Arabidopsis *RAP2.2*: An Ethylene Response Transcription Factor That Is Important for Hypoxia Survival[1[W][OA]]

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Arabidopsis (*Arabidopsis thaliana*) *RAP2.2* (At3g14230) is an APETALA2/ethylene response factor-type transcription factor that belongs to the same subfamily as the rice (*Oryza sativa*) submergence tolerance gene *SUB1A*. *RAP2.2* is expressed at constitutively high levels in the roots and at lower levels in the shoots, where it is induced by darkness. Effector studies and analysis of ethylene signal transduction mutants indicate that *RAP2.2* is induced in shoots by ethylene and functions in an ethylene-controlled signal transduction pathway. Overexpression of *RAP2.2* resulted in improved plant survival under hypoxia (low-oxygen) stress, whereas lines containing T-DNA knockouts of the gene had poorer survival rates than the wild type. This indicates that RAP2.2 is important in a plant's ability to resist hypoxia stress. Observation of the expression pattern of 32 low-oxygen and ethylene-associated genes showed that RAP2.2 affects only part of the low-oxygen response, particularly the induction of genes encoding sugar metabolism and fermentation pathway enzymes, as well as ethylene biosynthesis genes. Our results provide a new insight on the regulation of gene expression under low-oxygen conditions. Lighting plays an important regulatory role and is intertwined with hypoxia conditions; both stimuli may act collaboratively to regulate the hypoxic response.

Flooding is one of the most common stresses affecting plant growth and development. Many important crop plants are sensitive to flooding or waterlogging conditions caused by heavy rain or irrigation. Flooding and submergence conditions impose a variety of challenges on the plant. Most critical is maintaining an energy supply for continued metabolism and growth. To cope with the reduction in oxygen supply, plants have developed a number of metabolic and morphological adaptations that enable them to survive transient periods of complete or partial submergence (Kende et al., 1998; Drew et al., 2000; Bailey-Serres and Voesenek, 2008). Escape from hypoxia involves shoot elongation, development of aerenchyma, and adventitious root formation (Drew et al., 2000; Sauter, 2000; Voesenek et al., 2006; Perata and Voesenek, 2007). Nonetheless, oxygen may ultimately become limiting, necessitating a switch from aerobic respiration to anaerobic fermentation, with a variety of electron acceptors such as pyruvate and acetaldehyde replacing oxygen. This metabolic adaptation helps maintain ATP production and NAD^+ regeneration (Dennis et al., 2000).

Anaerobic fermentation is far less efficient in producing energy than aerobic respiration. Carbohydrate availability, either from photosynthesis or from stored reserves, becomes an important issue the longer the plant remains submerged. As a reaction to rising water levels, some plants extend their shoots and leaf blades to reestablish gas exchange (Kende et al., 1998; Voesenek et al., 2004; Pierik et al., 2009). However, this extension growth is at the expense of increased carbohydrate consumption and is detrimental when submergence is too deep or lasts for too long and carbohydrate reserves become depleted. Some plants, therefore, have adapted an alternative strategy that aims to conserve energy and carbohydrate consumption by limiting growth under low-oxygen conditions. Plant survival under flooding conditions is controlled by an intricate balance between escape and endurance (Fukao and Bailey-Serres, 2004).

The molecular basis of the morphological, anatomical, and metabolic adaptations to flooding and the signaling events leading to these responses remain to be elucidated. Recent research has focused on ethylene as a signal for the regulation of the early response to flooding. Expression levels of ethylene biosynthetic genes are up-regulated under hypoxic conditions (Van der Straeten et al., 1997; Peng et al., 2005). In sub-

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merged plants, ethylene levels increase rapidly due to physical entrapment of this volatile hormone (Métraux and Kende, 1983; Peng et al., 2001; Voesenek et al., 2004, 2006). The programmed cell death response that results in lysigenous aerenchyma formation is also controlled by ethylene (Drew et al., 2000), as is epidermal cell death above adventitious root primordia in rice (*Oryza sativa*; Mergemann and Sauter, 2000; Steffens and Sauter, 2009) and adventitious root growth in *Rumex palustris* and rice (Visser et al., 1996; Steffens and Sauter, 2005; Steffens et al., 2006). The internode elongation response, which serves as an important adaptation response in deepwater rice, is also controlled by ethylene (Hattori et al., 2009). In Arabidopsis (*Arabidopsis thaliana*), ethylene induces alcohol dehydrogenase gene expression (*ADH1*; Peng et al., 2001, 2005). Ethanolic fermentation through ADH1 activity contributes substantially to metabolic adaptation to low-oxygen stress; an *adh1* null mutant that showed lower survival rates when exposed to low oxygen (Ellis et al., 1999) and overexpression of pyruvate decarboxylase (*PDC1* and *PDC2*) in Arabidopsis results in improved survival under low-oxygen conditions (Ismond et al., 2003).

A major quantitative trait locus responsible for submergence tolerance, *Submergence1* (*SUB1*), was identified in lowland *indica* rice (Fukao et al., 2006; Xu et al., 2006). The locus consists of a cluster of three ethylene response factor (ERF) genes, *SUB1A*, *SUB1B*, and *SUB1C*. The *SUB1A* gene is present only in *indica* and not in *japonica* cultivars. Although *SUB1A* is present in several, but not all, *indica* cultivars, only the *SUB1A-1* allele from the submergence-tolerant *indica* cv FR13A was able to confer the flooding tolerance phenotype. Overexpression of *SUB1A-1* in a flooding-sensitive *japonica* cultivar resulted in increased *ADH1* expression and flooding tolerance (Fukao et al., 2006; Xu et al., 2006). The *SUB1A-1* allele appears to confer submergence tolerance via a complex regulatory pathway that reduces elongation growth and carbohydrate consumption (Fukao et al., 2006; Xu et al., 2006; Perata and Voesenek, 2007, Jung et al., 2010). A role of the *SUB1A-2* allele in submergence tolerance remains to be analyzed. However, more recently, it was shown that the opposite response in deepwater rice is also controlled by ethylene and ERF factors. The capacity of deepwater rice to elongate stem internodes and extend the hollow stem to the water surface requires the *SNORKEL1* (*SK1*) and *SK2* ERF transcription factors (Hattori et al., 2009). A detailed analysis of cell type-specific changes in translated mRNA in response to short-term hypoxic treatment of Arabidopsis seedlings revealed a ubiquitous increase in translated *ERF71* and *ERF73* mRNA (Mustroph et al., 2009). Increased expression of these two ERFs by low-oxygen conditions was recently confirmed, but induction of these factors by ethylene was not analyzed (Licausi et al., 2010).

ERF-type transcription factors belong to the plant-specific multigene family of APETALA2 (AP2)/ERF transcription factors. The AP2/ERF superfamily of proteins is characterized by having either one or two AP2 domains: the ERF or EREBP (for ethylene-responsive element-binding proteins) family has one AP2 domain, the RAV family has one AP2 domain and a B3 domain, and the AP2 family has two AP2 domains (Okamuro et al., 1997; Riechmann and Meyerowitz, 1998; Nakano et al., 2006). Some ERF factors can bind as activators or repressors to the GCC box (AGCCGCC) elements in the promoters of ethylene-responsive genes (Ohme-Takagi and Shinshi, 1995), while others mediate the response to cytokinin (Rashotte et al., 2006). ERF factors also play a role in a variety of developmental processes such as flower and seed development (Riechmann and Meyerowitz, 1998) and abiotic and biotic stress responses (Büttner and Singh, 1997; Stockinger et al., 1997; Fujimoto et al., 2000; Thomashow, 2001; Sakuma et al., 2002).

We studied the role of the ERF transcription factor, *RAP2.2* (At3g14230), in the Arabidopsis response to hypoxia. *RAP2.2* shows structural and phylogenetic relationships to the rice *SUB1A* gene (Nakano et al., 2006). Our results show that ethylene and *RAP2.2*, together with oxygen-dependent signal transduction, play an important role in the response to hypoxia.

RESULTS

Relationship between Arabidopsis RAP2.2 and the Rice SUB1 Proteins

BLASTP searches revealed that Arabidopsis RAP2.2 is related to the rice SUB1A protein. RAP2.2, however, is longer than SUB1 and shares only 37% amino acid identity over the aligned regions relative to SUB1A-1. The protein alignment of RAP2.2 with the rice SUB1 proteins (Fig. 1A) shows only short stretches of high homology, particularly the AP2 domain and two other domains, one with unknown function located between amino acids 49 and 70 (rich in Asp) and the other at the very N-terminal end (MCGG motif; Fig. 1A). The rice SK1 and SK2 proteins that are responsible for elongation growth have a different AP2 domain and therefore were not included in the alignment (Hattori et al., 2009). An extensive classification of the Arabidopsis and rice AP2/ERF transcription factors based on the AP2 domain structure grouped RAP2.2 and the rice SUB1B and SUB1C proteins together in subfamily VII (Nakano et al., 2006); rice SK1 and SK2 have a different AP2 domain structure and belong to subfamily XI. The group VII subfamily contains 15 members in *japonica* rice but only five in Arabidopsis. A phylogenetic analysis with the entire protein sequences of the Arabidopsis and rice group VII members and closely related AP2/ERF proteins from other plants was carried out (Fig. 1B; for the sequence alignment, see Supplemental Fig. S1). RAP2.2 was found to be most closely related to Arabidopsis RAP2.12 (At1g53910) and two other dicot ERF factors, tomato (*Solanum*

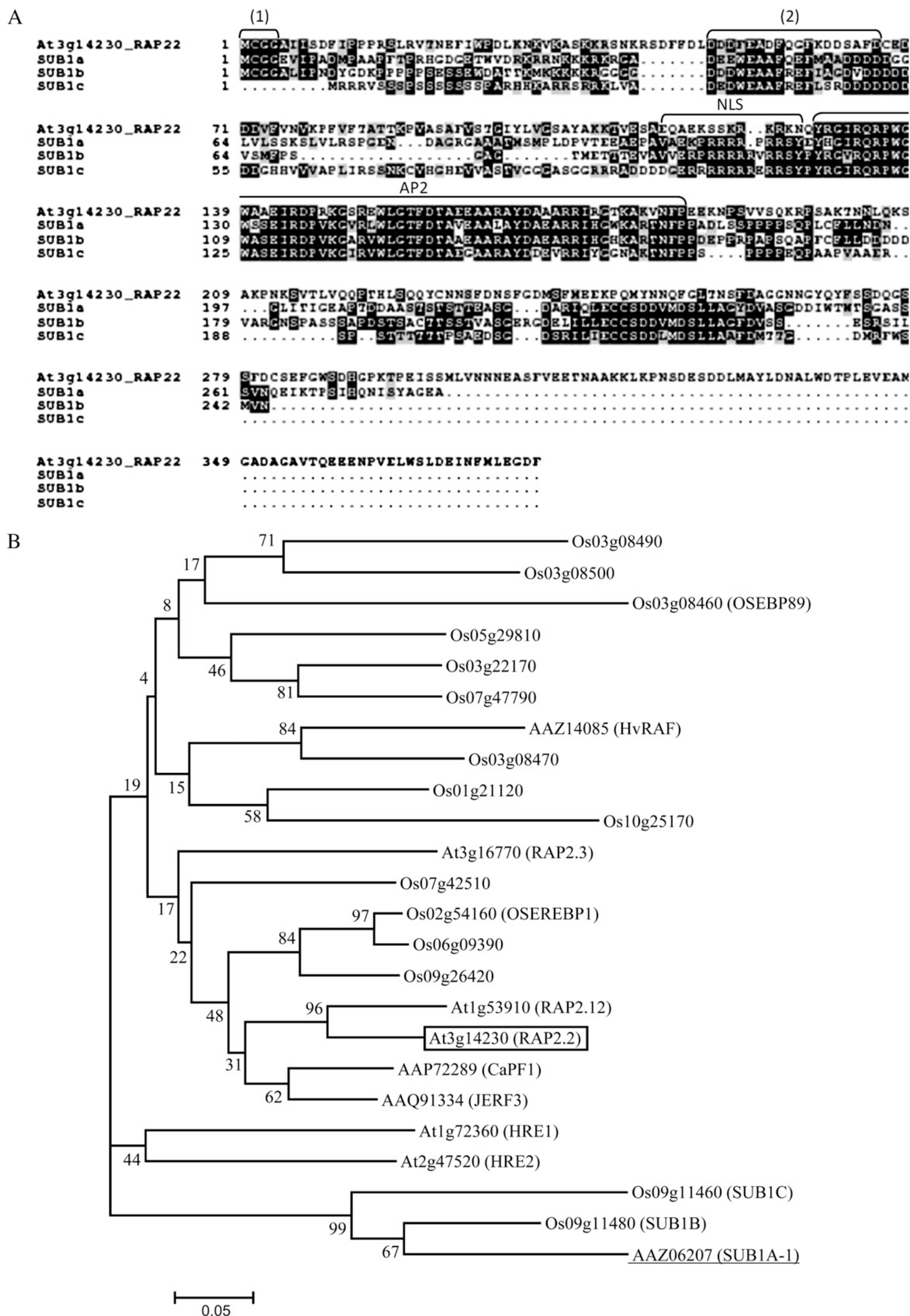

Figure 1. Phylogenetic tree of group VII AP2/ERF transcription factors from Arabidopsis and rice. A, Protein sequence alignment of RAP2-2 with the rice Sub1A, Sub1B, and Sub1C proteins. Alignment shows homology especially in the AP2 domain (amino acids 130–186). Another rather highly conserved region of unknown function is present between amino acids 49 and 70 (2). The

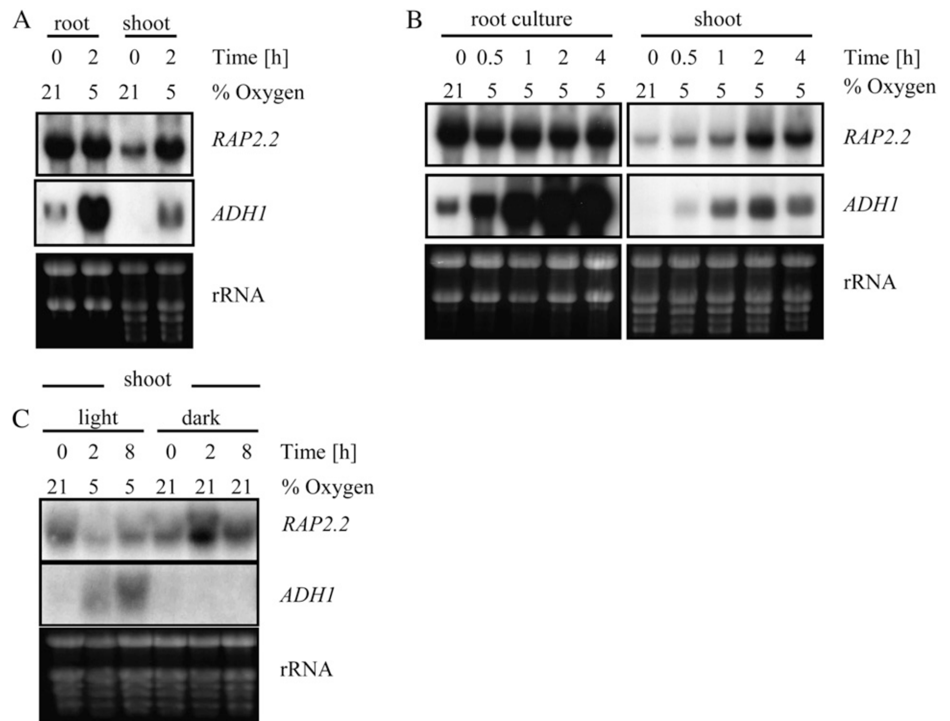

Figure 2. Expression analysis of *RAP2.2* and *ADH1* in roots and shoots during low-oxygen and dark treatments of Arabidopsis plants. A, RNA blots showing expression of *RAP2.2* and *ADH1* in roots and shoots of 17-d-old plants treated with 5% oxygen (hypoxia treatment) or 21% oxygen (aerobic control). B, RNA-blot analysis of *RAP2.2* and *ADH1* expression in hairy root cultures and shoots of plants treated with hypoxia or kept in aerobic conditions. C, RNA-blot analysis of *RAP2.2* and *ADH1* expression in the shoots treated with hypoxia (for 2 or 8 h) or kept in aerobic conditions (0 h) under normal lighting conditions and in the dark. Twenty micrograms of total RNA was used per lane, and ethidium bromide staining of rRNA was used as a loading control.

lycopersicum) JERF3 and pepper (*Capsicum annuum*) CaPF1, which play roles in salt and freezing tolerance, respectively (Wang et al., 2004; Yi et al., 2004; Fig. 1B). No rice group VII proteins clustered with the clade containing RAP2.2. The only rice proteins that cluster with the dicot proteins are Os02g54160, Os06g09390, and Os09g26420 (Fig. 1B). Os02g54160 (OSEREBP1) plays a role in pathogenesis-related gene expression (Cheong et al., 2003). This alignment of the amino acid sequences shows that the rice SUB1A, -B, and -C proteins cluster separately from the other rice group VII ERF factors and that their closest Arabidopsis relatives are At1g72360 (ERF73) and At2g47520 (ERF71; Fig. 1B) but not RAP2.2. But, as is the case for RAP2.2, ERF73, and ERF71, homology with SUB1A is mainly confined to the AP2 domain (overall amino acid identity of 26% and 33% for ERF73 and ERF71, respectively). These results indicate that Arabidopsis does not have a close relative of the rice SUB1 proteins and that the rice SUB1 genes may have evolved independently after the separation of monocots and dicots (Fukao et al., 2009).

RAP2.2 Is Induced in Dark-Grown Shoots

RAP2.2 was originally identified as a gene that was induced 2- to 3-fold on microarrays after 1 h of low-oxygen treatment (0.5% oxygen) of Arabidopsis hairy root cultures (Klok et al., 2002). Induction of *RAP2.2* by hypoxia was not observed on RNA blots of Arabidopsis root mRNA in our experimental system; expression of *RAP2.2* in roots was high in the dark under both aerobic and hypoxic conditions (Fig. 2A). In aerobic shoots, *RAP2.2* expression was much lower than in aerobic roots; expression was strongly induced when plants were transferred to hypoxic conditions in the dark, up to levels similar to those in roots (Fig. 2, A and B). *ADH1* expression was strongly induced by low-oxygen conditions in the roots and shoots, but overall expression levels were much lower in the shoots compared with the roots (Fig. 2, A and B). *RAP2.2* induction by low-oxygen stress was not observed in roots from dark-grown hairy root cultures, but overall expression levels were already very high in untreated root cultures (Fig. 2B). When low-oxygen stress was

Figure 1. (*Continued.*)
N-terminal MCGG motif (1) is strictly conserved between RAP2.2, Sub1A, Sub1B, and Sub1C and is also found in barley (*Hordeum vulgare*) HvRAF (Jung et al., 2007). A potential nuclear localization signal (NLS) is present between positions 113 and 128. B, A phylogenetic tree of group VII AP2/ERF transcription factors from Arabidopsis and rice was constructed using the entire protein sequence of only the group VII (Nakano et al., 2006) ERF transcription factors of *japonica* rice (prefix Os) and Arabidopsis (prefix At). Bootstrap figures are given at the branch points of the tree. For the SUB1 proteins from *indica* rice, the equivalent *japonica* locus is given for SUB1B and -C; the GenBank entry is given for the SUB1A locus. Subgroup VII corresponds to the B-2 subgroup according to the analysis of Sakuma et al. (2002). Known gene names are mentioned in parentheses. The phylogenetic tree also includes group VII AP2/ERF transcription factors from bell pepper (CaPF1) and tomato (JERF3; Nakano et al., 2006). Barley HvRAF is also included as a close relative of the group VII ERF subfamily. The bar indicates the number of amino acid substitutions per site.

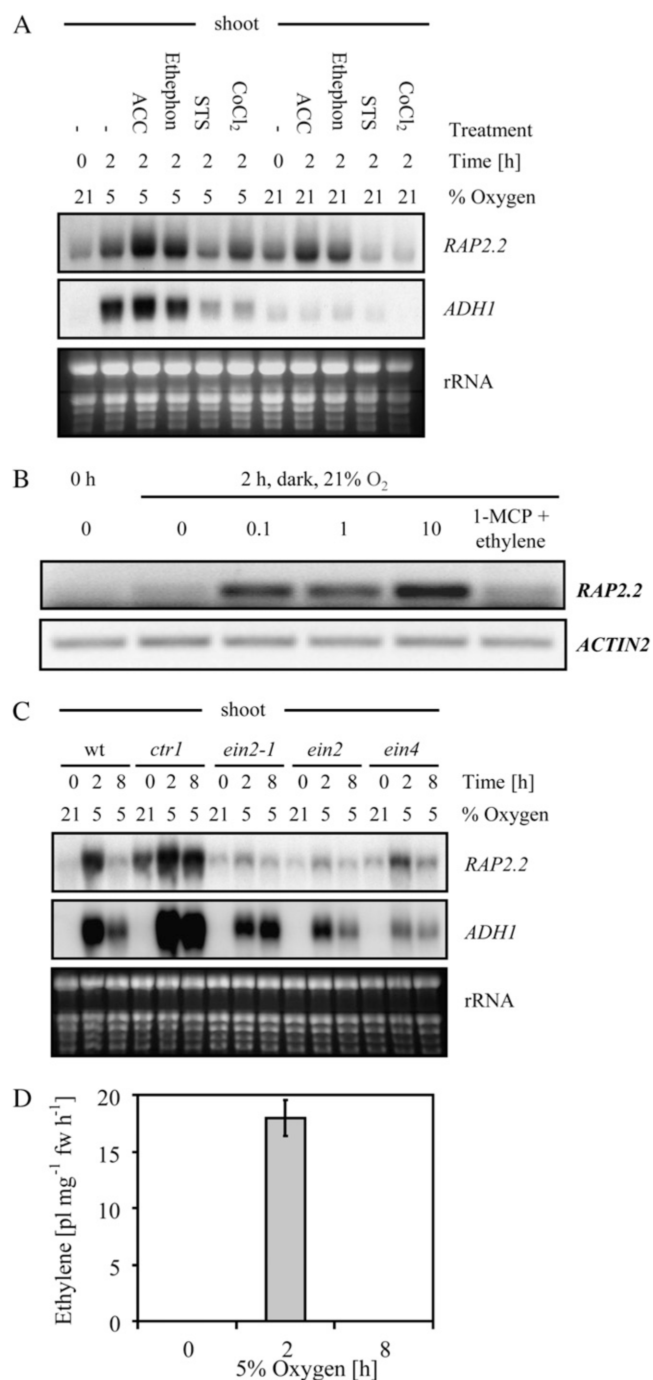

Figure 3. Effects of ethylene, ethylene-producing agents, inhibitors of ethylene synthesis, and ethylene signaling mutants on the expression of *RAP2.2* and *ADH1*. A, RNA-blot analysis of 17-d-old plants that were preincubated for 2 h with 50 μM ethephon, 50 μM ACC, 50 μM STS (an ethylene perception inhibitor), or 50 μM $CoCl_2$ (an ethylene synthesis inhibitor) and then subjected to hypoxic conditions in the dark. As a control, nontreated plants were left in the light. B, RT-PCR data showing the effects of ethylene treatment (0.1, 1, and 10 μL L^{-1}) and the combined treatment of ethylene (1 μL L^{-1}) and 1-MCP (10 μL L^{-1}) on *RAP2.2* gene expression. Amplification of *Actin1* cDNA was used as a control for RNA input. C, RNA-blot analysis of *RAP2.2* and *ADH1* in shoots of the wild type (wt) and the ethylene signaling mutants *ctr1*, *ein2-1*, *ein2*, and *ein4* under normoxic or hypoxic conditions in the dark. D, Ethylene production of 17-d-old plants kept at 21% oxygen (0 h) or at 5% oxygen for 2 or 8 h in the dark. Ethylene accumulation was determined by gas chromatography during the last hour of treatment. Average values ± SD were obtained from three independent biological experiments with three independent setups including 30 plants each. fw, Fresh weight.

carried out in the light, *RAP2.2* induction by 5% oxygen was not observed, but expression levels of *RAP2.2* increased in shoots in the dark, even under aerobic conditions; the induction is transient and peaks after 2 h of darkness (Fig. 2C). In contrast, expression of *ADH1*, a well-characterized low-oxygen-responsive gene, was strongly induced by hypoxia in roots, hairy root cultures, and shoots (Fig. 2, A and B) and responded specifically to low oxygen but not to darkness (Fig. 2C). These results indicate that *RAP2.2* responds only to darkness and not to low-oxygen conditions in shoots.

RAP2.2 Expression Is Regulated by Ethylene

Treatments were carried out in the dark on shoot tissues under normoxic (21% oxygen) or hypoxic (5% oxygen) conditions to determine whether RAP2.2 is regulated by ethylene. The ethylene-releasing chemical ethephon, the natural ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), ethylene itself, and the inhibitor of ethylene biosynthesis $CoCl_2$ were used to manipulate ethylene levels in the plant. In the presence of 50 μM ACC or 50 μM ethephon, *RAP2.2* expression was enhanced under both normoxic and hypoxic conditions in the dark. The 50 μM $CoCl_2$ treatment appeared to reduce expression only under normoxic conditions (Fig. 3A). We also applied the inhibitors of ethylene signaling silver thiosulfate (STS) and 1-methylcyclopropene (1-MCP). In the presence of 50 μM STS, *RAP2.2* transcript levels were reduced under both normoxic and hypoxic conditions (Fig. 3A). Treatment of Arabidopsis plantlets with ethylene (0.1, 1, and 10 μL L^{-1}) induced *RAP2.2* expression; this induction was blocked by the simultaneous treatment with 1-MCP (ethylene at 1 μL L^{-1} and 1-MCP at 10 μL L^{-1}; Fig. 3B). Treatments with the ethylene precursors ethephon and ACC increased *ADH1* transcript abundance in shoots, while the inhibitors of ethylene synthesis or signaling reduced *ADH1* expression (Fig. 3A).

To further study the role of ethylene signaling in *RAP2.2* regulation, the constitutive triple response *ctr1* mutant and three ethylene-insensitive mutants, *ein2*, *ein3*, and *ein4*, were employed (Olmedo et al., 2006). Expression of *RAP2.2* in shoots was increased in *ctr1* under both normoxic-light and hypoxic-dark conditions (Fig. 3C). In contrast, in *ein2*, *ein2-1*, *ein3-1*, and *ein4*, induction of *RAP2.2* expression under hypoxic-dark conditions in shoots was reduced (Fig. 3C). *RAP2.2* is induced weakly in *ein4* after 2 h of hypoxic treatment; this could be due to the fact that *ein4* is semidominant, as other ethylene receptors can partially

complement *ein4* (Hua and Meyerowitz, 1998). Similar to *RAP2.2*, *ADH1* mRNA levels in shoots were enhanced in *ctr1* but only under hypoxic-dark conditions (Fig. 3C), suggesting that *ADH1* is under dual control by low-oxygen signaling and ethylene signaling.

Direct measurement of ethylene production by Arabidopsis plantlets showed that ethylene levels increased strongly after 2 h of oxygen deprivation in the dark and returned to nondetectable levels after 8 h (Fig. 3D). The time of maximal ethylene synthesis coincided with the time of maximal expression of *RAP2.2* (Fig. 3B).

In conclusion, both ethylene and ethylene signaling affect *RAP2.2* expression under normoxic-dark and hypoxic-dark conditions, but *ADH1* expression is affected by ethylene signaling only when hypoxic conditions are present at the same time.

Spatial Expression Pattern of the *RAP2.2* Promoter

Seventeen-day-old Arabidopsis plants transformed with a *RAP2.2* promoter-driven GUS reporter gene showed GUS activity in both shoots and roots. Expression was weak in shoots but high in the roots (Fig. 4, A and B). When plants were transferred to the dark for 2 h, GUS staining increased in the shoot (Fig. 4C). In 4-d-old light-grown seedlings, GUS activity was high in the stele of the root and in the hypocotyl but weaker in the vasculature of the cotyledon (Fig. 4D). Expression was highest at the junction between the root and hypocotyl (Fig. 4, E and F) and the hypocotyl and cotyledons (Fig. 4, E and G). In light-grown seedling roots, GUS staining was present in the stele but not in the root tip (Fig. 4H). In 4-d-old dark-grown seedlings, GUS activity was high in the roots and the root-hypocotyl junction (Fig. 4, I–K), but in contrast to light-grown seedlings, expression in the root was also observed in the root tip (Fig. 4L). In the hypocotyl, overall expression appeared to be lower compared with light-grown seedlings of the same age and was mostly vascular (Fig. 4I). However, high levels of expression were seen in the subapical hook region (Fig. 4, I and M). The spatial distribution of *RAP2.2* promoter-driven GUS expression in whole plantlets showed quantitative differences in expression but no qualitative differences, nor did we observe any qualitative differences in the expression pattern following darkness or low-oxygen treatment (data not shown).

RAP2.2 Overexpression Increases *ADH1* and *PDC1* Expression under Hypoxic Conditions

RAP2.2 under the control of the 35S promoter was transformed into ecotype Columbia (Col-0), and the *ox1*, *ox2*, and *ox3* lines were recovered. Strong *RAP2.2* overexpression in shoots of light-grown aerobic plants was evident for *ox1*, *ox2*, and *ox3*, and mRNA levels were further increased in all lines following hypoxic treatment in the dark (Fig. 5).

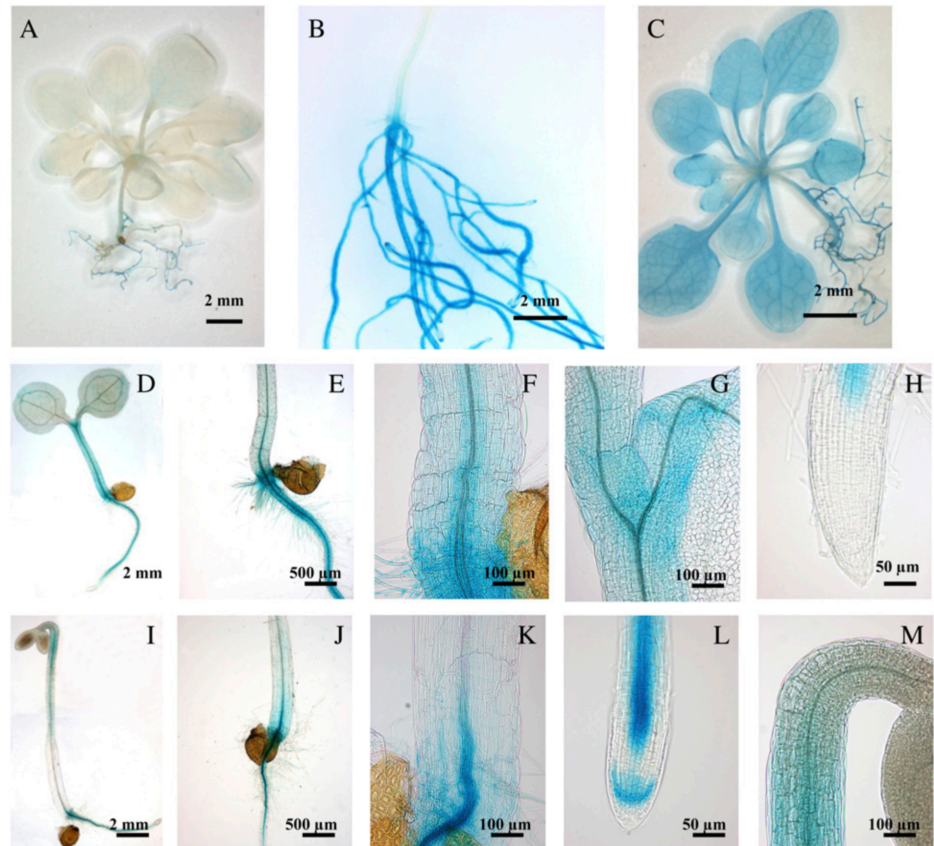

Figure 4. Localization of *RAP2.2* expression using promoter:GUS transgenic plants. A to C, Seventeen-day-old plants subjected to either normal lighting conditions (A and B) or dark conditions for 2 h (C). GUS staining for both light-grown and dark-treated plantlets was 30 min. D to H, Light-grown 4-d-old Arabidopsis seedlings. D, Complete seedling. E, Enlargement of the root-hypocotyl junction. F, Further enlargement of the root-hypocotyl junction. G, The vascular tissue leading from the hypocotyl to the cotyledons. H, Root tip. I to M, Four-day-old etiolated Arabidopsis seedlings. I, Intact seedling. J, Root-hypocotyl junction. K, Staining in the vascular tissue at the root-hypocotyl junction. L, Root tip. M, Staining in the apical hook region.

Overexpression of *RAP2.2* resulted in overexpression of *ADH1* and, to a lesser degree, of *PDC1* in shoots following hypoxic-dark treatment but not under normoxic-dark conditions (Fig. 5). This increase was reflected in increased ADH and PDC enzyme activities in shoots (Fig. 6). In shoots of Col-0 wild type, ADH activity increased after 8 and 24 h of hypoxic-dark treatment. The same time course experiment for the *RAP2.2*-overexpressing lines *ox1* and *ox2* showed significantly higher ADH and PDC activities than the wild type after 24 h of hypoxia treatment (Fig. 6; $P < 0.01$, $n = 6$). In the *ox2* line but not the *ox1* line, ADH and PDC activities were already increased significantly compared with the wild type after 8 h of hypoxia treatment (Fig. 6). Similar results were obtained for a third *RAP2.2*-overexpressing line, *ox3* (Supplemental Fig. S2). The increased ADH activity of the *RAP2.2*-overexpressing lines was significant under hypoxic-dark conditions and mirrors the observations from the RNA blots (Fig. 5). These results clearly indicate that overexpression of *RAP2.2* leads to increased levels of gene expression and enzyme activity levels of the two ethanol fermentation pathway enzymes, ADH and PDC. This overexpression is mainly evident under a hypoxic environment in the dark. Similarly, expression of *ERF71* (*HRE2*) and *ERF73* (*HRE1*) in roots of Arabidopsis seedlings is induced by treatment with 1% oxygen in the dark. Overexpression of *ERF73* improved survival of Arabidopsis seedlings exposed to anaerobic treatment in the dark and resulted in up-regulation of *ADH1* expression (Licausi et al., 2010). Since anaerobic treatments were carried out in the dark, it is possible that darkness or hypoxia or both treatments is required as signals.

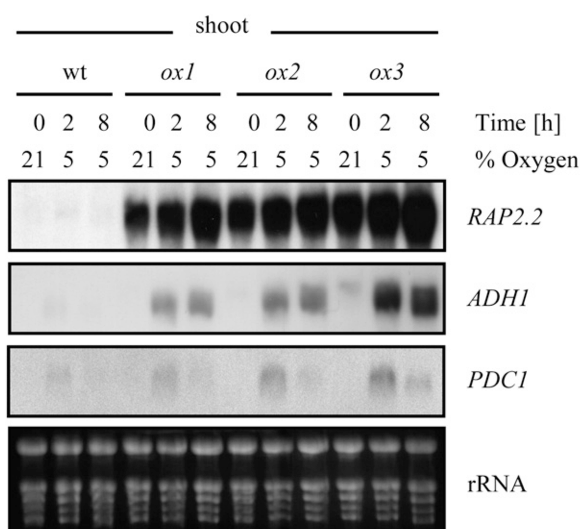

Figure 5. *RAP2.2*, *ADH1*, and *PDC1* expression in the wild type (wt) and *RAP2.2*-overexpressing lines. Seventeen-day-old wild-type plants and the 35S::*RAP2.2 ox* transgenic lines *ox1*, *ox2*, and *ox3* were treated with 21% or 5% oxygen in the dark, and relative transcript levels in shoots of *RAP2.2*, *ADH1*, and *PDC1* were determined by RNA-blot analysis.

T-DNA Insertions in *RAP2.2* Reduce *ADH1* and *PDC1* Hypoxic Induction in Shoots

Two independent T-DNA insertion lines of *RAP2.2*, *rap2.2-1* (CS871911) and *rap2.2-2* (CS876942), segregated as single locus insertions. The *rap2.2-1* and *rap2.2-2* T-DNA insertions are located in the first and second exons of *RAP2.2*, respectively, and are expected to eliminate protein function. Expression levels were reduced more strongly in the *rap2.2-2* insertion line compared with *rap2.2-1*, but both lines still showed induction of mRNA levels following hypoxia in the shoots (Fig. 7). T-DNA insertions in *RAP2.2* resulted in slightly lower shoot *ADH1* and *PDC1* mRNA induction levels under hypoxia in the dark. The fact that *RAP2.2* knockdown lines only partially reduced *ADH1* and *PDC1* expression may be an indication that other redundant ERF factors can compensate for lower *RAP2.2* expression levels.

RAP2.2 Is Important for Survival of Hypoxia

To determine whether *RAP2.2* is important for plant survival under low oxygen, the two highest overexpressing lines (*ox2* and *ox3*) and the two insertion knockout lines (*rap2.2-1* and *rap2.2-2*) were subjected to low-oxygen survival assays as described by Christianson et al. (2009). Different survival assay conditions had to be established for overexpressing and knockout lines of *RAP2.2* because of their different behavior in terms of low-oxygen survival relative to the wild type. The insertion line treatment consisted of 24 h of 5% oxygen pretreatment followed by 40 h of 0.1% oxygen treatment, both in the dark. The overexpressing lines were subjected to 24 h of 5% oxygen pretreatment followed by 48 h of 0.1% oxygen treatment in the dark. Control aerobic plants were also kept in the dark. Each assay consisted of three replicates of 12 plants, using Col-0 as the wild-type control, and results were averaged from at least three independent experiments. Plant survival in the light was scored 1 week after the stress treatment. The results show that *rap2.2-1* and *rap2.2-2* had significantly lower survival than Col-0 ($P < 0.01$; Figs. 8A and 9A), whereas the *ox2* and *ox3* overexpressing lines possessed significantly higher survival levels than the wild type ($P < 0.01$; Figs. 8B and 9B). These results indicate that there is a correlation between *RAP2.2* expression and survival under the low-oxygen conditions imposed in this assay. Root and shoot fresh weights were determined from *rap2.2-2* and *ox3* plants recovered from the survival assays shown in Figure 9, A and B, and expressed relative to the fresh weights of their respective untreated controls (Fig. 9C). Results indicate that knockout plants have in general a lower fresh weight than the wild type, while plants of the *ox3* line tend to have higher shoot and root fresh weights.

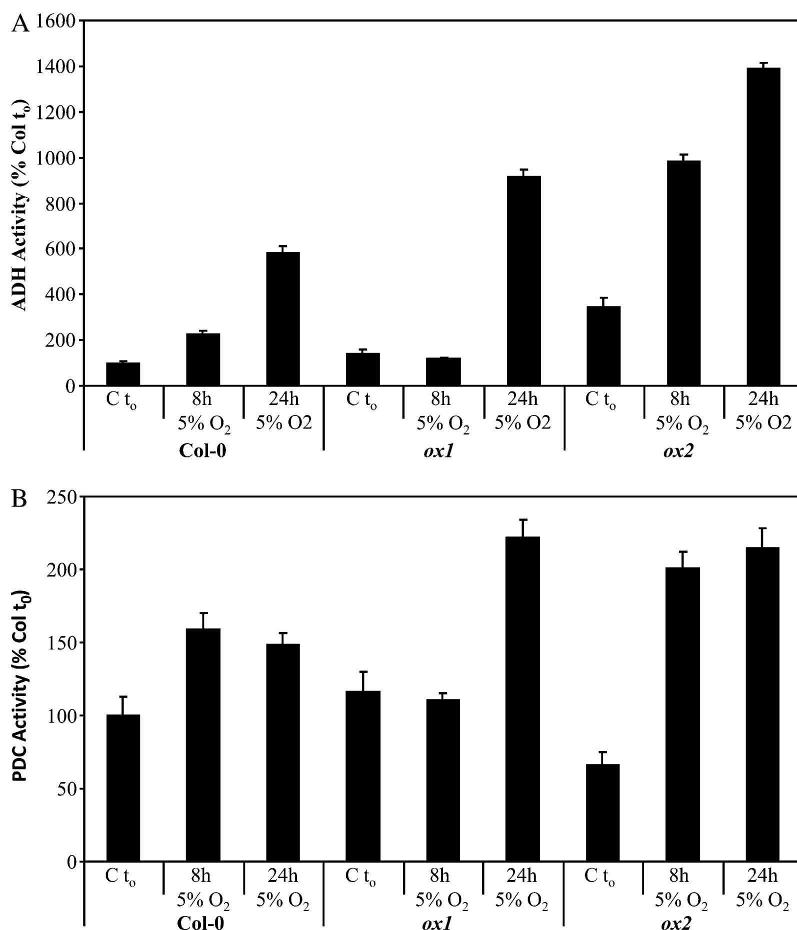

Figure 6. ADH and PDC enzyme activity measurements in shoots of wild-type Col-0 and the *RAP2.2*-overexpressing lines *ox1* and *ox2*. A, ADH enzyme activity measurements following 0 (t_0), 8, and 24 h of hypoxia (5% oxygen) treatment, showing significantly increased levels of ADH activity in the overexpressing lines. B, PDC enzyme activity measurements following 0 (t_0), 8, and 24 h of hypoxia (5% oxygen) treatment, showing overexpression of PDC activity in the two overexpressing lines. The data represent averages ± SE of three biological repeats with three measurements per sample.

RAP2.2 Expression Affects Only a Subset of Low-Oxygen-Responsive Pathways

To understand the role of *RAP2.2* under hypoxia conditions, we investigated what genes (apart from *ADH1* and *PDC1*) are affected by *RAP2.2* in darkness, hypoxia (5% oxygen), and the combination of darkness and hypoxia. We studied the effect of these three treatments in wild-type plants as well as in *RAP2.2*-overexpressing and knockout lines (*ox3* and *rap2.2-2*) and investigated the effect of altering *RAP2.2* expression levels on the expression of 32 genes that have previously been shown to be affected by hypoxia treatment in plants (Supplemental Table S1). The genes were chosen from two low-oxygen stress microarray experiments that were carried out in the dark (Klok et al., 2002; Loreti et al., 2005) and one carried out under light conditions (Liu et al., 2005; Supplemental Table S1). The choice was influenced by function under low-oxygen concentrations (e.g. sugar metabolism, fermentation pathway genes) or a potential role in senescence or ethylene-mediated responses. Also included were ethylene biosynthesis genes and other ERF genes, particularly members of the group VII ERF factors (Fig. 1B) and those ERF factors that were differentially expressed in the microarray experiments. Since *RAP2.2* expression levels were shown to reach a maximum around 2 h after induction in the shoots, a treatment length of 5 h was chosen for the darkness and hypoxia treatments to analyze the effect of changing *RAP2.2* expression on downstream genes. Quantitative reverse transcription (QRT)-PCR expression profiling was carried out for three individual repeat samples; all expression data are provided in Supplemental Figure S3, and a heat map summarizing the expression data is provided in Supplemental Figure S4.

Hierarchical clustering of the expression profiles of the 32 genes and treatments/genotypes revealed that the combined hypoxia-darkness treatment responses were closer to the darkness-only treatment under normoxic conditions than they were to hypoxia treatment under light conditions (Fig. 10). Of the 21 genes that were induced by at least 2-fold under hypoxia in the dark in wild-type plants, only eight were induced by hypoxia in the light, whereas 11 were induced by darkness alone (Fig. 10; Supplemental Figs. S3 and S4). Those genes that were induced by hypoxia in the light had a much stronger induction by hypoxia in darkness (Supplemental Figs. S3 and S4). Only three genes appeared to have a weak induction by hypoxia in the light and did not further increase their expression under hypoxia in darkness (*RopGAP4*, *RAP2.12*, and *ACO*; Supplemental Fig. S3). Many genes thought to be "low-oxygen-induced" genes are in fact induced by

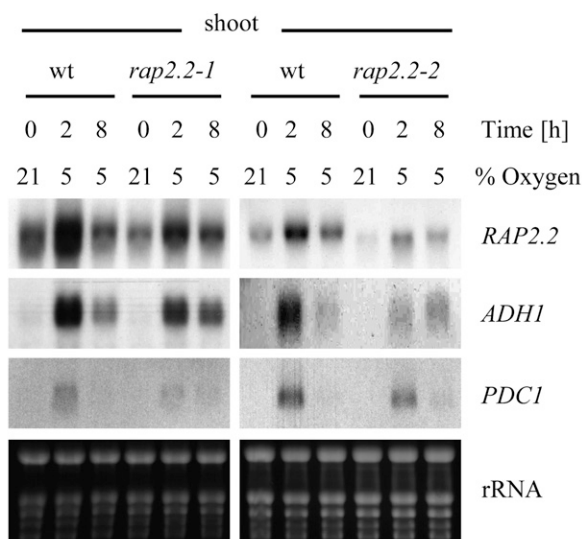

Figure 7. RNA-blot analysis of *RAP2.2*, *ADH1*, and *PDC1* expression in the wild type (wt) and the *RAP2.2* T-DNA insertion lines *rap2.2-1* and *rap2.2-2*. Seventeen-day-old wild-type plants and plants of the T-DNA insertion lines *rap2.2-1* and *rap2.2-2* were treated with 21% oxygen or 5% oxygen in the dark for 2 or 8 h before isolation of RNA from the shoot.

darkness during the assay. Changing *RAP2.2* expression caused changes to specific genes, but overall expression profiles were similar to wild-type responses and clustered with them. Overexpression of *RAP2.2* resulted in up-regulation of *ADH1* and down-regulation of *ACS9* under normoxia in the light. When hypoxia-darkness was imposed on *ox3*, increases in expression of *AlaAT1* and *RAP2.3* and decreases in *AtMYB2* and *ACS2* were observed (Fig. 10). Loss of a functional RAP2.2 resulted in up-regulation of a number of genes under normoxia-light, including the ethylene biosynthesis genes *ASC2*, *ACS7*, *ASC9*, and *AOX1* as well as *NIP2;1* and *AtMYB2*. Loss of hypoxia-darkness induction in *AtMYB2*, *ACS2*, and *ETR2* was also observed in *rap2.2-2* (Fig. 10).

Overall, there were three major clusters in expression profiles (Fig. 10). Group I consisted of eight genes that were mainly darkness and hypoxia-darkness induced but repressed under hypoxia-light conditions. This group contained the senescence-associated genes *SEN1* and *SEN5* as well as a number of other ethylene-associated genes, such as *ACS2* and *AOX1*. Group II is the largest cluster (16 members) and contains predominantly genes induced by darkness, hypoxia-darkness, and hypoxia-light treatments. All of the known low-oxygen-induced genes involved in fermentation and sugar metabolism (*ADH1*, *PDC1*, *LDH1*, *AtSUS1*, and *AtSUS4*) as well as four transcription factors (*ANR1*, *AtERF4*, *ERF71*, and *ERF73*), the ethylene biosynthesis genes *ASC7* and *ACO1*, and the ethylene receptor *ETR2* were present in this group. Group III was made up of eight genes that were normally not induced by any of the three treatments in wild-type plants but whose expression was altered by changing *RAP2.2* expression levels. This group contains *RAP2.12*, a close relative of *RAP2.2*.

The expression data demonstrate that *RAP2.2* affects only part of the response to low-oxygen stress. Specific genes important for metabolic adaptations such as fermentation and sugar metabolism, as well as some ethylene biosynthetic genes, are affected by *RAP2.2*.

DISCUSSION

Ethylene plays a major role in hypoxia-induced gene expression in plants: ethylene biosynthesis genes are up-regulated under low-oxygen conditions, ethylene induces *ADH1* in Arabidopsis, and, more recently, quantitative trait locus mapping revealed that both submergence tolerance and elongation growth in deepwater rice are controlled by transcription factors of the ERF family (Xu et al., 2006; Hattori et al., 2009). Ethylene regulates several morphological adaptation responses to flooding and submergence, such as shoot elongation, aerenchyma formation, and adventitious root growth (for review, see Bailey-Serres and Voesenek, 2008). Both tolerance to submergence (*SUB1A-1*) and the escape reaction of elongation growth in deepwater rice (*SK1* and *SK2*) are controlled by particular alleles of ERF genes.

ERFs that play a role in low-oxygen stress conditions have recently also been identified in dicots. The Arabidopsis *ERF71* and *ERF73* genes (*HRE2* and *HRE1*, respectively) are induced by low-oxygen stress in the dark, and overexpression results in improved hypoxia survival (Licausi et al., 2010). We studied Arabidopsis *RAP2.2* (At3g14230), originally thought to be responsive to hypoxia in roots (Klok et al., 2002) but shown here to be constitutively expressed under hypoxia in that tissue. *RAP2.2* belongs to the same group VII ERF family as *SUB1A* (Fig. 1; Nakano et al., 2006) and was recently shown to activate two Arabidopsis genes encoding the carotenoid biosynthesis pathway enzymes phytoene synthase and phytoene desaturase (Welsch et al., 2007). These genes are also differentially expressed under hypoxic conditions (Loreti et al., 2005). Our phylogenetic analysis of the group VII ERF factors from rice and Arabidopsis shows that the dicot ERF transcription factors have evolved independently. This is reflected by the relatively high degree of sequence divergence outside the highly conserved AP2 domain. This complicates finding a true SUB1 homolog in Arabidopsis based on sequence similarity only. But *RAP2.2*, according to AP2 domain structure, is certainly more related to *SUB1* from rice than to the rice *SK1* and *SK2* genes, which belong to a different ERF subfamily (Hattori et al., 2009). However, two other Arabidopsis group VII proteins, ERF73 and ERF71, are even more closely related to SUB1A than RAP2.2 (Fig. 1B). These transcription factors also play a role in low-oxygen stress in the dark (Licausi et al., 2010).

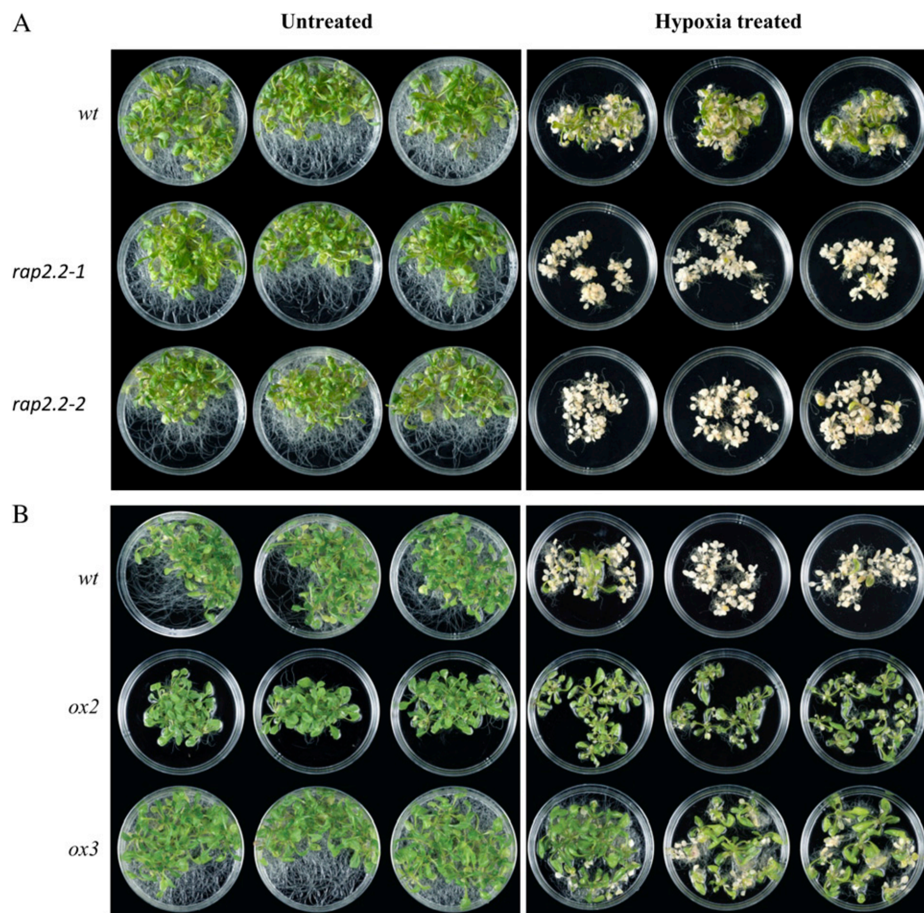

Figure 8. Survival of plants with elevated and reduced expression of *RAP2.2* as compared with the wild type (wt). A, Images of wild-type seedlings and the T-DNA insertion lines *rap2.2-1* and *rap2.2-2* at 1 week after recovery from 24 h of 5% oxygen and 40 h of 0.1% oxygen in the dark. B, Images of wild-type seedlings and the overexpression lines *ox2* and *ox3* at 1 week after recovery from 24 h of 5% oxygen and 48 h of 0.1% oxygen in the dark. The control plants in this experiment were also kept in the dark.

We show that *RAP2.2* does play a significant role in metabolic adaptation to flooding stress in Arabidopsis, suggesting that the group VII ERF factors are specialized in abiotic stress responses. RAP2.12, the closest relative of RAP2.2 (Fig. 1), was recently shown to regulate *ADH1* expression (Papdi et al., 2008). Other related dicot ERFs, tomato JERF3 and pepper CaPF1, play a role in salt and freezing tolerance, respectively (Wang et al., 2004; Yi et al., 2004). *RAP2.2* was expressed at high levels in roots and at low levels in the shoots, a distribution similar to fermentation pathway genes like *ADH1* (Dolferus et al., 1994), *PDC1* (R. Dolferus, unpublished data), *LDH1* (Dolferus et al., 2008), and *AlaAT1* (Miyashita et al., 2007). While *RAP2.2* expression in roots is constitutively high and not affected by hypoxia, in shoots its expression appeared to be induced by darkness but not by hypoxia. The gene is also regulated by ethylene and ethylene-generating compounds, and the fact that mutations in ethylene signal transduction components affect its expression clearly indicates that *RAP2.2* functions in an ethylene-responsive signaling pathway. Low-light conditions trigger leaf senescence, which involves ethylene accumulation and the ethylene signaling pathway (for review, see Gan and Amasino, 1997; Quirino et al., 2000; Wingler and Roitsch, 2008; Lin et al., 2009). Darkness may trigger ethylene biosynthesis, as Arabidopsis ethylene biosynthesis genes *ACS4* and *ACS7* are induced by darkness (Wang et al., 2005). It is not clear why *RAP2.2* expression in roots is so high and no superinduction can be seen by hypoxia or darkness. It is possible that roots grown in soil (darkness) or in liquid medium (entrapment) naturally accumulate higher ethylene levels.

A variety of experimental setups have been used to study low-oxygen responses in plants. Some of the more recent studies have used anaerobiosis (0% oxygen; Mustroph et al., 2006, 2009), while others used hypoxic conditions (0.1%–5% oxygen; Van Dongen et al., 2009; Licausi et al., 2010). Most of these experiments use darkness or low-light conditions, but the effect of light has so far not been investigated. We have routinely carried out our low-oxygen treatments on Arabidopsis plantlets in sealed tanks with a defined gas composition and in the dark to stop complications arising from altered oxygen concentrations due to photosynthesis or the release of trapped oxygen from plant tissues. These treatments resemble complete submergence, which in nature also leads to reduced photosynthetic activity due to a combination of restricted light and CO_2 availability. It is conceivable that low light and CO_2 availability, induction of ethylene synthesis, and induction and promotion of senescence

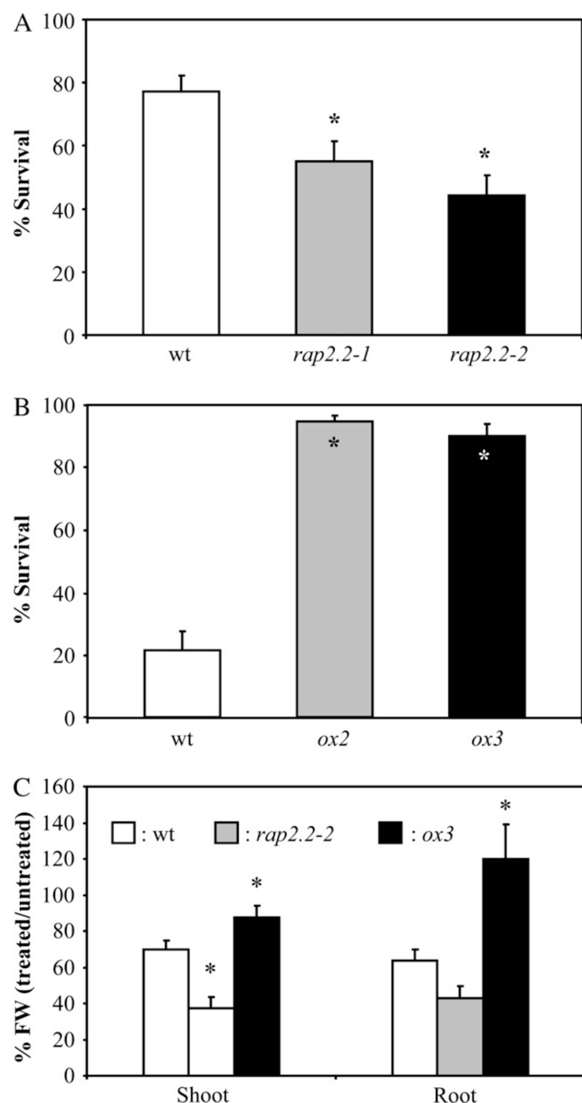

Figure 9. Effects of low-oxygen treatment on the percentage of plant survival. A, Survival percentage of the wild type (wt) and the T-DNA insertion lines *rap2.2-1* and *rap2.2-2* measured 1 week after recovery from 24 h of 5% oxygen and 40 h of 0.1% oxygen (±SE; *n* = 5). Asterisks denote line survival percentages significantly different from each other as determined by *t* test ($P < 0.01$). B, Survival percentage of the wild type and the overexpression lines o*x2* and *ox3* measured 1 week after recovery from 24 h of 5% oxygen and 48 h of 0.1% oxygen (±SE; *n* = 3). Asterisks denote line survival percentages significantly different from each other as determined by *t* test ($P < 0.01$). C, Fresh weight (FW) of roots and shoots of 4-week-old light-grown plants of the wild type, the T-DNA knockout line *rap2.2-2*, and the *RAP2.2*-overexpressing line *ox3* measured after the recovery period of the survival assays. *rap2.2-2* has consistently lower fresh weight. The opposite was observed for the *ox3* overexpression line. Data points with asterisks denote fresh weights significantly different from the wild type as determined by *t* test ($P < 0.01$).

are naturally intertwined and therefore inherent to natural submergence conditions. *ADH1* expression in roots is low and is strongly induced by hypoxic conditions (Dolferus et al., 1994). Previous work on Arabidopsis seedlings indicated that *ADH1* is induced within 12 h by ethylene, but this induction is only strong under hypoxic conditions (Peng et al., 2001). These studies were carried out on intact seedlings, likely in the light, but nonetheless support the conclusion that ethylene is not the only trigger required for inducing *ADH1* expression. Hypoxic conditions are essential to fully induce *ADH1* expression. The rice *SUB1A* gene is induced by submergence conditions, but it remains to be shown whether *SUB1A* also responds to darkness.

Overexpressing *RAP2.2* using a strong constitutive promoter did not cause any noticeable phenotypic changes under normal growth conditions, but both *ADH1* and *PDC1* expression in shoots was increased following hypoxic-dark treatment. The overexpressing lines also had higher ADH and PDC enzyme activity in shoots, suggesting that RAP2.2 is a regulator of the alcohol fermentation pathway genes. The overexpression of *ADH1* and *PDC1* in the *RAP2.2*-overexpressing lines was only evident under hypoxic conditions in the dark but not under normoxic-dark conditions. This suggests that ethylene accumulation and *RAP2.2* induction are not sufficient for *ADH1* and *PDC1* activation and that hypoxic conditions are also required.

The *RAP2.2*-overexpressing lines showed significantly higher hypoxia plant survival rates in the dark, while the knockout lines showed the opposite phenotype. This demonstrates that RAP2.2 can act as a regulator of the plant's response to hypoxia in the dark and that either expression of *RAP2.2* is rate limiting for regulating genes that lead to hypoxia tolerance or that higher preexisting levels of RAP2.2 enable a quicker response to hypoxia, resulting in enhanced hypoxia tolerance. Submergence-tolerant rice plantlets produced highly elevated levels of ethanol during anaerobiosis in the dark with sufficient ATP synthesis to ensure plantlet survival, whereas flooding-sensitive wheat (*Triticum aestivum*) seedlings were much less efficient in fermentative ATP production. Fermentation occurred mainly in the shoot, indicating a vital role of anaerobic shoot metabolism for plantlet survival in the dark (Mustroph et al., 2006).

We investigated the overlap between hypoxic treatment under light and darkness as well as the effect of RAP2.2 overexpression and knockout lines on these responses using 32 candidate genes that play a role in hypoxia and ethylene responses. The clustering of the treatments indicates that hypoxia-darkness treatment responses were closer to the darkness-only treatment under normoxic conditions than they were to hypoxia treatment under light conditions. Darkness alone generally causes a lower induction than that observed when both darkness and hypoxia are combined. This suggests that there is a clear link between hypoxia and darkness responses in shoots. Observing the effect of altering levels of RAP2.2 showed that it affects only a subset of these pathways. The major effect observed was on fermentation genes such as *ADH1* and *PDC1* and ethylene biosynthesis and receptor genes such as *ACS2*, *ASC7*, *ASC9*, and *ETR2*. It is possible that

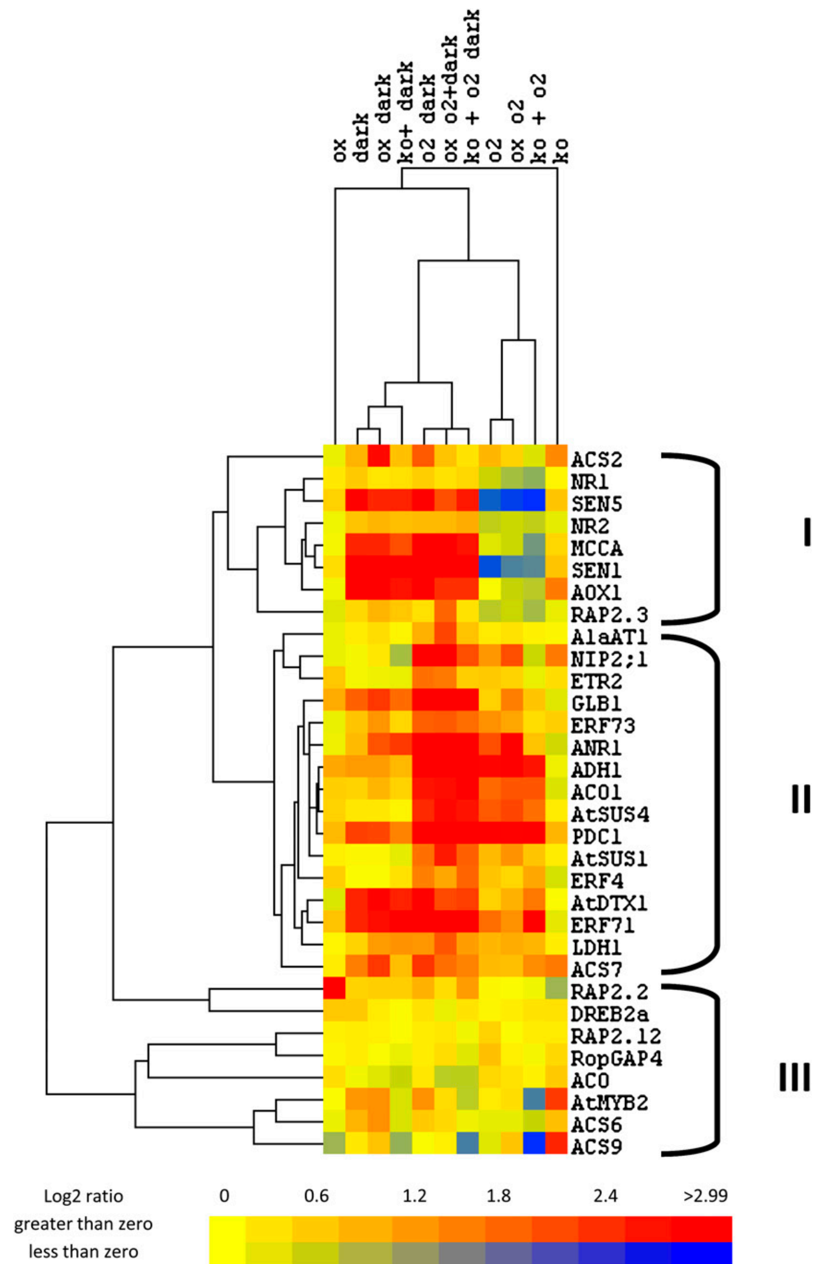

Figure 10. Hierarchical clustering of expression data for 32 candidate genes. Expression analysis was carried out on RNA extracted from whole seedlings of the wild type, *RAP2.2*-overexpressing line *ox3* (ox), and *RAP2.2* knockout line *rap2.2-2* (ko). Plant treatments consisted of 5 h in the dark under normoxia (dark), 5 h with 5% oxygen in the light (o_2), or a combination of 5 h in the dark with 5% oxygen (o_2 + dark). Expression ratios for wild-type plants are treatment divided by the value obtained under normoxia-light conditions. ox and ko values are *ox3* and *rap2.2-2* expression under normoxia-light compared with the wild type under normoxia-light. Treatment values for ox and ko are treatments divided by ox and ko values obtained under normoxia-light. The full description of the genes used (locus, gene name, and function) is listed in Supplemental Table S1. Hierarchical clustering was used to divide the gene set in three clusters: group I consists of genes induced by darkness and hypoxia-darkness but repressed under hypoxia-light; group II consists of predominantly genes induced by darkness, hypoxia-darkness, and hypoxia-light treatments and contains the best known low-oxygen-induced genes that are involved in fermentation and sugar metabolism as well as ethylene biosynthesis genes and the ethylene receptor *ETR2*; group III consists of genes that were normally not induced by any of the three treatments in wild-type plants but whose expression was altered by changing *RAP2.2* expression levels.

RAP2.2 may affect other genes; however, they would have to be involved in pathways not yet associated with hypoxia tolerance.

The importance of fermentation and glycolysis for surviving low oxygen has been well established. *ADH1*, *PDC1*, and the Suc synthase genes *AtSUS1* and *AtSUS4* are vital in Arabidopsis for tolerance to low oxygen (Ellis et al., 1999; Rahman et al., 2001; Ismond et al., 2003; Kürsteiner et al., 2003; Bieniawska et al., 2007). Increased root survival was also observed in *LDH1*-overexpressing Arabidopsis lines (Dolferus et al., 2008). Expression of *PDC1* is considered to be the rate-limiting step in ethanol fermentation, and overexpression has been shown to increase Arabidopsis survival (Ellis et al., 1999; Ismond et al., 2003). The observation that in the shoots, overexpression of *RAP2.2* resulted in higher ADH and PDC activity indicates that the improved hypoxia survival of plants overexpressing *RAP2.2* is in part via enhancement of fermentation. The poor hypoxia tolerance of the RAP2.2 knockouts may only be partly related or even unrelated to deficiencies in fermentation, as *ADH1* and *PDC1* expression is only partly reduced. Knocking out *RAP2.2* does result in changes to *ACS2*, *ACS7*, and *ACS9* that indicate negative feedback regulation of ethylene biosynthesis by RAP2.2. It may be hypothesized that altered ethylene signaling in *rap2-2* plants contributes to poor hypoxia tolerance.

Although ERF factors were shown to interact with the GCC box or AP2/ERF-binding motif (Ohme-Takagi and Shinshi, 1995; Lin et al., 2008), RAP2.2 was shown to bind to an ATCTA promoter element (Welsch et al., 2007). The *ADH1* promoter contains an ATCTA element proximal to the TATA box (positions −57 to −53; data not shown). A GCC box-like motif, the GC motif, is together with the GT motif part of the anaerobic response element (Olive et al., 1991; Dolferus et al., 1994). Transactivation assays with wild-type and deleted *ADH1* promoter constructs are required to investigate whether RAP2.2 interacts directly with the GC motif or the ATCTA element. *RAP2.2* may also act indirectly on *ADH1* expression via other transcription factors. ERF transcription factors are known to interact with each other in complicated networks (Oñate-Sánchez et al., 2007). *ERF71*, *ERF73*, and *ERF4* are all expressed in the same group of genes as *ADH1*; it is possible that these ERF factors function downstream of RAP2.2 to activate group II genes. A hypoxic signal is required to activate *ADH1* expression, suggesting that there are two regulatory pathways that act on the *ADH1* promoter. The ethylene-dependent pathway that involves RAP2.2 alone is not able to induce *ADH1*, but hypoxic conditions are also essential. The fact that hypoxia is able to induce ethylene biosynthesis suggests that the ethylene pathway is not completely independent of hypoxic conditions. On the other hand, AtMYB2, which binds to the GT motif (Hoeren et al., 1998), appears to be affected by darkness and ethylene (Fig. 10); this suggests that there is cross talk between the two regulatory pathways. Oxygen-dependent regulatory mechanisms that trigger anaerobic gene expression have been identified in bacteria, yeast, and mammals but remain so far elusive in plants (Poyton, 1999; Gilles-Gonsalez, 2001; Schofield and Ratcliffe, 2004; Bailey-Serres and Chang, 2005). To further unravel how gene regulation works under hypoxic conditions and to identify the components of the ethylene pathway and especially the hypoxic pathway will require more carefully designed experiments that take into account lighting conditions during hypoxic treatment. It will also be important to further establish the importance of lighting conditions and the difference between real submergence conditions and artificial assays that use gas mixtures in combination with light or dark conditions.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Low-Oxygen Treatments

Experiments were carried out on Arabidopsis (*Arabidopsis thaliana* ecotype Col-0). Col-0 wild-type seeds were originally obtained through GABI-Kat (Max-Planck-Institut), the T-DNA insertion lines SAIL_184_G12 (CS871911) and SAIL_799_D10 (CS876942) were from the SALK Institute, and the ethylene signaling mutants *ctr1*, *ein2*, and *ein3* were from the Arabidopsis Biological Resource Center. Seeds were surface sterilized for 20 min in 1 mL of 0.5% (w/v) sodium hypochlorite and washed five times with autoclaved water. Five or 10 seeds were sown per plate (60 mm × 15 mm or 120 mm × 120 mm; height, 17 mm) on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), sealed with 3M micropore tape (Eydam), and kept at 4°C in the dark for 2 d. Plates were then transferred to a growth chamber, and plants were grown at 22°C in a photoperiod of 16 h of light/8 h of dark for the times indicated. Arabidopsis root cultures were generated by incubating leaf discs on callus induction medium (Valvekens et al., 1988) for 3 to 4 d in the dark. Leaf discs were then infected with *Agrobacterium rhizogenes* strain A4RS (Vilaine et al., 1987). When hairy roots appeared, the leaf discs were transferred to liquid MS medium and incubated at 50 rpm on a rotary shaker platform in the dark.

For RNA isolation for northern-blot analysis, promoter:GUS, and enzyme activity assays, plants were grown on half-strength MS medium containing 1.5% (w/v) Suc and 0.8% (w/v) agar at pH 5.7 (70 μE m^{-2} s^{-1}). Hypoxic treatment of 17-d-old plants was carried out in an atmosphere containing 5% (v/v) oxygen, 0.04% (v/v) CO_2, and 94.96% (v/v) N_2 in the dark for the duration indicated. For ethylene measurements, plantlets were transferred after 1 week to MS medium containing 2% (w/v) agarose to prevent root growth into the agarose and subsequent wounding during the transfer of plants to the vials that were used for ethylene measurement, as described previously (Peng et al., 2001). For treatment with the effectors ACC, ethephon, STS (freshly prepared from 100 mM sodium thiosulfate and 100 mM silver nitrate at a ratio of 1:1), and $CoCl_2$, plants were first grown on solid medium and transferred 2 d before treatment to liquid MS medium to improve uptake of the effectors. After preincubation for 2 h with ethephon, ACC, STS, or $CoCl_2$ at the concentrations indicated, the plants were exposed to hypoxic treatment as described above.

For RNA isolation for QRT-PCR, plants were grown on half-strength MS medium containing 1.5% (w/v) Suc and 0.8% (w/v) agar at pH 5.7 (70 μE m^{-2} s^{-1}). Plants were divided into one of four treatments (five plants per treatment): normoxia in the light (70 μE m^{-2} s^{-1}) for 5 h, normoxia in the dark for 5 h, hypoxia (5% oxygen) in the light for 5 h, or hypoxia (5% oxygen) in the dark for 5 h.

Hypoxic Assays

Low-oxygen survival assays were performed as outlined by Christianson et al. (2009). Briefly, 2.5-week-old plants were grown under a 16-h light cycle (100 μE m^{-2} s^{-1}) on MS medium containing 3% (w/v) Suc and 0.8% (w/v) agar at pH 5.7 and then transferred to MS liquid medium containing 3% (w/v) Suc for 1 d prior to treatment. Immediately prior to treatment, plants were transferred to liquid medium that had been sparged with 5% (v/v) oxygen and then placed in 3.5-L anaerobic chambers (Oxoid) and purged with 5% (v/v) oxygen at a flow rate of approximately 10 L min^{-1} for 20 min. The plants were left in 5% (v/v) oxygen in the dark with gentle shaking for 24 h, then purged with 0.1% (v/v) oxygen and left for 40 to 48 h. Following treatment, plants were given fresh liquid medium and allowed to recover on an orbital shaker in normal growth cabinet conditions for 1 week before survival scoring. Survival scores were averaged from at least three independent experiments. Each experiment contained three technical replicates, with each replicate consisting of 12 plants.

Generation and Characterization of Transgenic Plants

To constitutively overexpress *RAP2.2*, a 1.9-kb genomic fragment including the open reading frame, 5′ and 3′ untranslated regions, and two introns was amplified from Col-0 using the gene specific primers 5′-**GAATTC**GAGTAGAGCTTTCGTGAAGCCACCAT-3′ (forward) and 5′-**GGATCC**AGAAGATTCATTGAACAGATA-3′ (reverse). The fragment was restricted with *Eco*RI and *Bam*HI and cloned into the *Eco*RI and *Bam*HI sites of pART7 (Gleave, 1992). The resulting 35S-*RAP2.2*-OCS fragment was then subcloned as a *Not*I fragment into the binary vector pART27 and transformed into the Arabidopsis ecotype Col-0 using the floral dip method (Clough and Bent, 1998). The presence and integrity of the *RAP2.2* genomic DNA sequence was verified by sequencing the insertion in pART27.

To generate a *RAP2.2* promoter construct, a 1,112-bp *RAP2.2* promoter region was amplified from genomic DNA of ecotype Col-0 using the gene-specific primers 5′-**GAATTC**CAATATTGCCACTCCATGATAA-3′ (forward) and 5′-ATAGCTCCTCCAC**CCATGG**TGGCTTCACGA-3′ (reverse). The resultant PCR fragment, which contained an *Eco*RI site and an *Nco*I site, was ligated in front of the GUS-NOS cassette of pHW9 (Dolferus et al., 1994) and subsequently cloned into the binary vector pBIN19 using the restriction sites *Eco*RI and *Xba*I (Bevan, 1984). Arabidopsis ecotype Col-0 plants were transformed, and GUS-expressing lines were analyzed.

Phylogenetic Analysis of ERF Sequences

Full-length ERF protein sequences were retrieved from the GenBank database and analyzed using the MEGA 4.0 software (Tamura et al., 2007). Sequences were aligned using the ClustalW progressive alignment algorithm (Higgins et al., 1994) and the neighbor-joining method (Saitou and Nei, 1987). Bootstrap figures are indicated in the phylogenetic tree.

RNA Extraction, Northern Blotting, and QRT-PCR

Total RNA was extracted by grinding the frozen tissue in liquid nitrogen in a mortar with a pestle. The root and shoot sections of the rosette-stage plants were separated by butting the base of the rosette with a scalpel. Tri-Reagent (Sigma-Aldrich) was added to the ground tissue, and RNA was isolated according to the manufacturer's instructions. For all gels, 20 μg of RNA each per sample was separated on a denaturing agarose gel (1% [w/v] agarose, 40 mM MOPS buffer, and 6% [v/v] formaldehyde). The ribosomal RNA staining pattern (ethidium bromide) was used as a loading control. RNA was transferred to a nylon membrane (Hybond N$^+$; GE Healthcare) and hybridized to ^{32}P-labeled probes obtained using a Ready-To-Go dCTP labeling kit (GE Healthcare). Hybridization was performed overnight at 68°C in hybridization solution (5% [w/v] dextran sulfate, 1 M NaCl, 1% [w/v] SDS, and 0.1 mg mL^{-1} heat-denatured salmon sperm DNA). Blots were washed two times in 2× SSC/1% (w/v) SDS for 1 and 10 min, once in 1× SSC/1% (w/v) SDS for 10 min, and once in 0.5× SSC/1% (w/v) SDS for 10 min. The gene-specific primer pairs used to generate the PCR probes from genomic DNA were 5′-CCTAGCGTCGTATCC-CAGAA-3′ and 5′-CAAGGCGTTGTCAAGGTATGC-3′ for *RAP2.2*, 5′-CGA-GACACCTTACAACAACAC-3′ and 5′-CAGATGAGAGGTTCAAACACAT-3′ for *RAP2.12*, 5′-GATTGTTGAGAGTGTTGGAG-3′ and 5′-CTTGGTCGAATC-TTTTAGAGT-3′ for *ADH1*, and 5′-TCTCTCACACACATACACAAAC-3′ and 5′-CAAGCAAAGTGAGGTTGAAATC-3′ for *PDC1*. For *RAP2.2* and *ADH1*, probes were generated from the coding region. For *RAP2.12*, a probe including the coding region and part of the 3′ untranslated region was generated. For *PDC1*, a probe including part of the 5′ untranslated region and the coding region was used. The probes for *RAP2.2* and *RAP2.12* shared only 23% sequence identity.

QRT-PCR was performed as described previously (Wilson et al., 2005). The RT product was amplified using gene-specific primers (Supplemental Table S2). Reactions were performed in triplicate on a Rotor-Gene 6000 (Qiagen). Data were normalized to At5g08290 (Czechowski et al., 2005) and analyzed using a comparative quantification procedure (Wilson et al., 2005). Hierarchical clustering of log-transformed expression data was carried out using the Cluster and Treeview (Eisen et al., 1998) programs. Heat maps were constructed using the University of Toronto BAR Heatmapper tool (http://www.bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi).

Expression Analysis of *RAP2.2*:GUS Plants

For *RAP2.2*:GUS studies on etiolated seedlings, seeds were exposed to light for 4 h to synchronize germination and then incubated in the dark for 4 d. In addition, *RAP2.2*:GUS seedlings were grown for 4 d and plants were grown for 17 d on half-strength MS medium containing 1.5% (w/v) Suc and 0.8% (w/v) agar at pH 5.7 and 70 μE m^{-2} s^{-1} in a light/dark cycle as described above. To analyze *RAP2.2* promoter activity under 5% (v/v) oxygen and in the dark, 17-d-old plants were grown under a 16-h light cycle at 100 μE m^{-2} s^{-1} on MS medium containing 3% (w/v) Suc and 0.8% (w/v) agar at pH 5.7 and then transferred to MS liquid medium containing 3% (w/v) Suc for 1 d prior to treatment. Immediately prior to treatment, plants were transferred to liquid medium. For the low-oxygen-treated plants, the medium was sparged with 5% (v/v) oxygen whereas the control was not sparged. Plants were then placed in 3.5-L anaerobic chambers. The low-oxygen-treated plants were purged with 5% (v/v) oxygen at a flow rate of approximately 10 L min^{-1} for 20 min. The plants were left in the dark with gentle shaking before harvest. Two independent transgenic *RAP2.2*:GUS transgenic lines were analyzed for GUS activity at the seedling and vegetative stages (Blázquez et al., 1997; Bomblies, 2000).

ADH and PDC Activity Assays

For ADH enzyme activity measurements, soluble protein was extracted in cold extraction buffer (100 mM Tris-HCl, pH 8.0, 25% [v/v] glycerol, 0.8% [v/v] β-mercaptoethanol, 2% [w/v] polyvinylpyrrolidone, and 5 mM dithiothreitol) and centrifuged at 15,000*g* for 15 min at 4°C. PDC enzyme activity was measured using a modified protocol by Rivoal et al. (1997). Soluble protein was extracted in cold extraction buffer (50 mM MES-NaOH, pH 6.2, 5 mM dithiothreitol, 1 mM $MgCl_2$, and 1 mM thiamine pyrophosphate) and centrifuged at 15,000*g* for 15 min at 4°C. Protein concentration was determined with the Bradford assay (Roti-Quant; Roth). The enzymatic reaction was started by the addition of 800 μL of activation buffer (0.15 M Tris-HCl, pH 8.0, 0.3 mM NAD$^+$, and 0.175% [v/v] ethanol for ADH activity measurements or 50 mM MES-NaOH, pH 6.2, 0.1 mM thiamine pyrophosphate, 0.5 mM $MgCl_2$, 0.15 mM NADH, 3.3 mM sodium pyruvate, and 40 units mL^{-1} yeast ADH for PDC enzyme activity measurements). Activities were calculated either from the increase or decrease in A_{340} over time.

Ethylene Measurement

Thirty plants each were treated with 21% (v/v) or 5% (v/v) oxygen as described above, transferred to a 22-mL vial, and capped gas tight. Ethylene was measured after 1 h in the head space using a gas chromatograph (GC-14B; Shimadzu). Plant fresh weights were determined, and ethylene production per mg fresh weight was calculated.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_180251.

Supplemental Data

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

Supplemental Figure S1. Amino acid sequence alignment of RAP2.2-related ERF transcription factors.

Supplemental Figure S2. ADH and PDC activity measurements in shoots of wild-type Col-0 and the *RAP2.2*-overexpressing line *ox3*.

Supplemental Figure S3. QRT-PCR expression data for 32 candidate genes.

Supplemental Figure S4. Detailed heat map of QRT-PCR gene expression data for 32 candidate genes.

Supplemental Table S1. List of 32 candidate genes used for gene expression profiling studies by QRT-PCR.

Supplemental Table S2. List of PCR primers used in QRT-PCR experiments.

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