The Low-Oxygen-Induced NAC Domain Transcription Factor ANAC102 Affects Viability of Arabidopsis Seeds following Low-Oxygen Treatment

Jed A. Christianson, Iain W. Wilson*, Danny J. Llewellyn, and Elizabeth S. Dennis
CSIRO Plant Industry, Canberra, Australian Capital Territory 2601, Australia

Low-oxygen stress imposed by field waterlogging is a serious impediment to plant germination and growth. Plants respond to waterlogging with a complex set of physiological responses regulated at the transcriptional, cellular, and tissue levels. The Arabidopsis (Arabidopsis thaliana) NAC domain-containing gene ANAC102 was shown to be induced under 0.1% oxygen within 30 min in both roots and shoots as well as in 0.1% oxygen-treated germinating seeds. Overexpression of ANAC102 altered the expression of a number of genes, including many previously identified as being low-oxygen responsive. Decreasing ANAC102 expression had no effect on global gene transcription in plants but did alter expression patterns in low-oxygen-stressed seeds. Increasing or decreasing the expression of ANAC102 did not affect adult plant survival of low-oxygen stress. Decreased ANAC102 expression significantly decreased germination efficiency following a 0.1% oxygen treatment, but increased expression had no effect on germination. This protective role during germination appeared to be specific to low-oxygen stress, implicating ANAC102 as an important regulator of seed germination under flooding.

Transient waterlogging, which can impose low-oxygen stress on established plants, has been shown to reduce yield in a number of crops, including cotton (Gossypium hirsutum; Hodgson and Chan, 1982), wheat (Triticum aestivum; Collaku and Harrison, 2002), barley (Hordeum vulgare; Setter and Waters, 2003), maize (Zea mays; Mason et al., 1987), and canola (Brassica napus; Cannell and Belford, 1980). Even rice (Oryza sativa), which is well adapted to growing partially underwater, is adversely affected when the entire plant is submerged (Singh et al., 2001). As well as in growing plants, waterlogging poses a significant problem to seeds prior to emergence. Germination rates decline dramatically as oxygen concentrations are reduced for 12 plant species, including Brassica vegetable species, soybean (Glycine max), pea (Pisum sativum), wheat, maize, and rice (Al-Ani et al., 1985). Soil-based experiments have also shown large impacts on seed viability following 4 d of flooding stress in oat (Avena sativa), triticale (x Triticosecale), wheat, and barley (Setter and Waters, 2003). Declines in seed germination in a number of low-oxygen environments (1%–15% oxygen) have also been observed in Brassica oleracea, a close relative of Arabidopsis (Arabidopsis thaliana; Finch-Savage et al., 2005).

The effects of low oxygen on seeds can vary between plant species. For many species, a lack of oxygen prevents germination. Some cereals, such as barley, rely on limiting oxygen availability to embryos as a mechanism for imposing and maintaining dormancy (Benech-Arnold et al., 2006). In contrast, rice seeds are able to germinate under full anoxia, and this capability is dependent on ethanolic fermentation pathways (Kato-Noguchi, 2001). However, very little is known about the effects of low oxygen on seed viability in Arabidopsis. Germination did not occur in wild-type Arabidopsis seeds in an anoxic environment and was severely reduced in those treated seeds following a recovery period (Mattana et al., 2007). This impairment of germination was partially ameliorated by overexpression of the rice transcription factor Mybleu (Mattana et al., 2007). Arabidopsis germination has also been found to be reduced significantly more in ALC-HOL DEHYDROGENASE1 (ADH1) mutants than in wild-type plants following anoxic treatments (Jacobs et al., 1988), which suggests a role for fermentative metabolism in sustaining seeds under low oxygen.

Following perception of a lack of oxygen, changes in gene transcripts, proteins, and metabolism rapidly result. A set of about 20 anaerobically induced peptides, the majority of which were involved in glycolysis and fermentation, have been identified as part of the low-oxygen response in a number of plant species (Sachs et al., 1980; Kelley and Freeling, 1984a, 1984b; Dennis et al., 2000).

Functional approaches to studying the effects of low oxygen have focused on adult plants and have involved altering the expression of these enzymes of fermenta-
tion and glycolysis. The genes SUCROSE SYNTHASE1 (SUS1) and SUS4, ADH1, and PYRUVATE DECARBOXYLASE1 (PDC1) have been shown to be vital in Arabidopsis for tolerance to low oxygen, as knocking out their function leads to a reduction in plant growth or survival (Ellis et al., 1999; Rahman et al., 2001; Ismond et al., 2003; Kursteiner et al., 2003; Bieniawska et al., 2007). Overexpression of PDC, considered to be rate limiting in ethanol fermentation, has been shown to increase Arabidopsis survival (Ellis et al., 2000; Ismond et al., 2003). Increased root survival was also observed in LACTATE DEHYDROGENASE (LDH)-overexpressing Arabidopsis lines (Dolferus et al., 2008). Altering the expression of ALANINE AMINOTRANSFERASE, which controls the Ala fermentation pathway, did not affect tolerance to low oxygen in Arabidopsis (Dolferus et al., 2003; Miyashita et al., 2007). While this candidate gene approach has yielded some promising results, overexpression of transcription factors that can alter the expression of stress response networks may have a greater impact. The SLUB1 ERF/AP2-type transcription factor in rice has proven capable of increasing survival in fully submerged conditions by regulating the transcription of a suite of genes associated with carbohydrate consumption, ethanolic fermentation, and cell expansion and by leading to decreased growth, chlorophyll degradation, and carbohydrate consumption (Fukao et al., 2006; Xu et al., 2006).

Global gene expression studies in Arabidopsis have revealed widespread and complex responses to low oxygen that have typically found significant changes in approximately 5% to 10% of all the genes assayed (Klok et al., 2002; Liu et al., 2005; Loreti et al., 2005). Early microarray experiments on low-oxygen responses in Arabidopsis hairy root cultures (Klok et al., 2002) identified known and putative transcription factors whose expression was induced early in low-oxygen stress, including the NAC domain-containing gene ANAC0102 (ANAC102; At5g63790).

NAC domain genes are a plant-specific class of transcription factors with functions in development and stress responses (for review, see Olsen et al., 2005). Arabidopsis contains 105 genes with NAC domains, which can be divided into two major groups and further partitioned into 18 subgroups (Ooka et al., 2003). ANAC transcription factors are characterized by an N-terminal DNA-binding NAC domain composed of five subelements and a variable C-terminal transcriptional activator region (Ooka et al., 2003). The NAC domain was first identified and characterized in the petunia (Petunia hybrida) NO APICAL MERISTEM, ATAF1, ATAF2, and CUP-SHAPED COTYLEDON genes (Souer et al., 1996; Aida et al., 1997). Numerous members of the ANAC gene family have been shown to be involved in response to stresses: ANAC019, ANAC055, and ANAC072 were all up-regulated in response to drought, high salinity, and abscisic acid (ABA), and overexpression of any one of ANAC019, ANAC055, and ANAC072 was shown to increase drought tolerance in Arabidopsis (Tran et al., 2004). In addition to modulating lateral root formation in Arabidopsis, AtNAC2 is also salt responsive (He et al., 2005). The NTL8 gene has been shown to influence flowering time under salt-stress conditions (Kim et al., 2007). ANAC102 belongs to the ATAF subfamily of NAC domain genes. Members of the ATAF subfamily, ATAF1 and ATAF2, negatively regulate responses to drought and wounding, respectively (Delessert et al., 2005; Lu et al., 2007).

Here, we investigated the role of ANAC102 in plant responses to low oxygen and provide evidence for a role for this gene in transcriptional regulation during low-oxygen response and as an important positive regulator of seed viability under low-oxygen conditions.

RESULTS

ANAC102 Is Up-Regulated in Shoots, Roots, and Imbibed Seeds in Response to 0.1% Oxygen

ANAC102 is significantly up-regulated at early time points in hairy root cultures exposed to oxygen stress (Klok et al., 2002). To confirm that ANAC102 is induced in whole plants, 3-week-old Arabidopsis plants were subjected to 0.1% oxygen for 0, 0.5, 2, 4, 8, and 24 h (Fig. 1A). Both root and shoot tissues showed a similar response, with increased expression detected at 0.5 h and remaining high for at least 24 h (Fig. 1A). There was no substantial difference between root- and shoot-specific expression in untreated plants (1.2-fold). Low-oxygen induction of gene expression also occurred in the presence of 10 μM cycloheximide (17.0-fold; data not shown), demonstrating that no new protein synthesis is required for ANAC102 induction.

Several classical hypoxia-induced genes, such as ADH, LDH, and AlaAT, exhibit increased expression in germinating seeds following exposure to low oxygen levels (Ricoult et al., 2005). Imbibed and stratified seeds, both unsterred and subjected to 0.1% oxygen in the light for a period of 6 d (no germination occurs while the seeds are in 0.1% oxygen), were assayed for ANAC102 and ADH1 expression. Both ANAC102 and ADH1 expression in low-oxygen-stressed seeds was increased over unstressed seeds (2.5-fold and 11.9-fold, respectively), but the expression level of ANAC102 and ADH1 in unstressed seeds was lower than in unstressed adult tissue (3.2-fold and 2-fold lower expression in seeds than in roots; Fig. 1B).

ANAC102 Is Expressed in Both Roots and Early Rosettes, But Is Concentrated in the Root Cortex and Root Caps

To characterize the spatial and developmental patterns of ANAC102 expression in Arabidopsis, lines transformed with an ANAC102 promoter::GUS fusion were generated (Fig. 2). GUS staining was clearly visible in parts of the root system. In the aerial portion
of the plant, faint staining was detected in older sepals and leaves. The public Arabidopsis gene expression database Genevestigator (Zimmermann et al., 2004) largely corroborated the expression patterns observed in the ANAC102 promoter::GUS fusion lines, indicating high expression in the lateral root cap, stele, epidermal atrichoblasts, endodermis, and cortex. Public expression data also indicated that ANAC102 is relatively highly expressed in the stem and sepal. Sepal expression was observed in the ANAC102 promoter::GUS lines (Fig. 2D), but stem expression may have been too low or diffuse to be observed. GUS expression was not seen in imbibed seeds, but expression was visible in the radicle of seedlings at 2 d after emergence (Fig. 2E). No induction of GUS expression could be detected following treatment with 0.1% oxygen for 4 h, and the GUS signal appeared to be weaker in the treated plants (Fig. 2G and H). In contrast to the visible GUS staining, quantitative real-time (QRT)-PCR demonstrated that transcript levels of both the native ANAC102 gene and the ANAC102 promoter::GUS fusion gene were increased in these plants following a 4-h 0.1% oxygen treatment (Fig. 2I). It has been observed that maize ADH1 5′ and 3′ untranslated regions are important for maintaining transla-

tional efficiency under low-oxygen conditions (Bailey-Serres and Dawe, 1996). As the ANAC102 promoter::GUS construct used here does not contain the ANAC102 untranslated region sequences, it may be that the GUS mRNA produced under low oxygen is translated at decreased efficiency, resulting in lower amounts of protein formed despite having higher levels of transcript.

Arabidopsis Lines with Altered ANAC102 Levels Do Not Show Any Gross Phenotypic Differences from the Wild Type

Two independent Columbia (Col-0) lines carrying insertions in the second exon of the ANAC102 gene that would be predicted to eliminate protein function were obtained and designated KO-1 (SALK_030702) and KO-2 (SALK_094437). ANAC102 mRNA expression in these two lines was also found by both QRT-PCR and subsequent microarray experiments to be between 3.5- and 15-fold lower in KO-1 and approximately 15-fold lower in KO-2 than in the wild type (Supplemental Fig. S1). Also, two independent ANAC102-overexpressing lines (OX-1 and OX-2) were generated in the C24 ecotype, as this ecotype is more susceptible to low-oxygen stress than Col-0 but has similar ANAC102 expression profiles (data not shown), potentially making it easier to detect any increase in tolerance derived from ANAC102. Each OX line had 25- to 30-fold higher expression of ANAC102 than the wild type in whole 3-week-old plants (Supplemental Fig. S1). No gross phenotypic differences were observed between the knockout or overexpressing lines and their respective parental ecotypes grown under standard laboratory conditions, except for a slightly lighter green leaf color in the ANAC102-overexpressing lines. Lighter green coloration was also observed when the closely related ATAF2 gene (ANAC081; >92% amino acid similarity to ANAC102) was overexpressed in C24 (Delessert et al., 2005); however, none of the other ATAF2 overexpression phenotypes, such as increased leaf size, wrinkled leaves, or increased biomass, were observed in the ANAC102-overexpressing lines. Although ANAC102 is highly expressed in root tips, there were no obvious differences in root morphology or quantity (data not shown).

Overexpression of ANAC102 Modifies Expression of Downstream Genes, But ANAC102 Knockout Lines Do Not Affect Global Gene Transcription in Growing Plants

As ANAC102 is a putative transcription factor, the global impact of overexpressing or knocking out ANAC102 on the Arabidopsis transcriptome was examined using the Affymetrix ATH1 Arabidopsis arrays. RNA extracted from both untreated and 4-h low-oxygen-treated 3-week-old seedlings of the wild type (Col-0), KO-1, and KO-2 lines was used for microarray analysis. Neither comparisons between

Figure 1. Expression of ANAC102 in response to low oxygen. A, Time course of ANAC102 expression subjected to 0.1% oxygen. Plants of ecotype Col-0 in liquid MS medium were placed in chambers containing 0.1% oxygen for 0.5, 2, 4, 8, or 24 h. Expression changes were monitored in both root (black triangles) and shoot (white triangles) tissues. Data are expression at different times relative to the 0-h time point (±SE; n = 9). B, Expression of ANAC102 and ADH1 in imbibed seeds under ambient and 0.1% oxygen (6 d of exposure) relative to 3-week-old root tissue in ambient oxygen (±SE; n = 9).
untreated KO lines and the wild type nor comparisons between low-oxygen-treated KO lines and the wild type showed any differences in gene expression at a fold change cutoff of 1.5 and an adjusted $P$ value of $<0.05$, save for $ANAC102$ itself, which was underexpressed in KO lines in both circumstances (17- to 50-fold; Table I). RNA was extracted from 3-week-old seedlings of the wild type (C24), OX-1, and OX-2 lines and used for microarray analysis. A total of 113 genes were up-regulated more than 1.5-fold at an adjusted $P$ value of $<0.05$, and 98 genes were found to be significantly down-regulated in the overexpressing line (Supplemental Table S1). Seventy-five of the up-regulated genes and 61 of the down-regulated genes have been identified as being differentially regulated in other low-oxygen microarray experiments (Klok et al., 2002; Branco-Price et al., 2005; Liu et al., 2005; Loreti et al., 2005). Notably, $ADH1$ and $SUS1$, which have long been recognized as key components of the low-oxygen response in plants, were up-regulated by $ANAC102$ overexpression (2.9-fold and 1.6-fold, respectively; Table I).

Relative expression levels in adult plants of 11 genes reported to be low-oxygen responsive and shown here to be significantly affected by $ANAC102$ overexpression were compared by QRT-PCR in KO-1, OX-1, and wild-type lines across six time points following exposure to 0.1% oxygen (Fig. 3; Supplemental Fig. S2). All of the genes examined changed in response to low oxygen levels, in agreement with previous reports. Of the 11 selected genes that were identified on the microarray as being up-regulated in OX-1, two ($At1g02850$ [glycosyl hydrolase] and $At2g43820$ [UDP-glucosyl transferase]) were shown by QRT-PCR to have higher expression in OX-1 over all time points. A further eight of these genes were more highly expressed in OX-1 during the initial stages of low-oxygen exposure, after which expression in the wild type increased to match that in OX-1 (Supplemental Fig. S2). The one selected gene that was identified on the microarray as being down-regulated in OX-1 was found by QRT-PCR to be down-regulated in comparison with the wild-type line at most time points. Taken together, overexpression of $ANAC102$ increased or at least preinduced expression of some low-oxygen-inducible genes and decreased or delayed the induction of others.
Table I. Microarray-derived expression ratios of selected genes with differential expression between ANAC102 mutant lines and the wild type

<table>
<thead>
<tr>
<th>Arabidopsis Genome Initiative No.</th>
<th>Gene Title/Description</th>
<th>Expression Ratio in OX-1</th>
<th>Expression Ratio in KO-1</th>
<th>Expression Ratio in KO-1 (0.1% Oxygen)</th>
<th>Expression Ratio in KO-1 Seeds (0.1% Oxygen)</th>
<th>NAC Strict</th>
<th>NAC Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT5G63790</td>
<td>ANAC102 (Arabidopsis NAC domain-containing protein 102), transcription factor; similar to ATAF2</td>
<td>18 &lt;0.01 0.06 0.4 0.02 0.04 0.03 &lt;0.01</td>
<td>1 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G02850</td>
<td>Glycosyl hydrolase family 1 protein</td>
<td>8.6 &lt;0.01 0.67 1 0.53 0.9 0.85 0.11 0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2G04040</td>
<td>MATE efflux family protein</td>
<td>5 &lt;0.01 0.86 1 0.48 0.9 0.63 0.11 0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G77120</td>
<td>Alcohol dehydrogenase (ADH)</td>
<td>2.9 &lt;0.01 1.2 1 0.99 1 1 0.9 0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2G43820</td>
<td>UDP-glucoronosyl/UDP-glucosyl transferase family protein</td>
<td>5.6 &lt;0.01 0.57 1 0.74 0.9 0.68 0.01</td>
<td>0 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G26770</td>
<td>Expansin, putative (EXP10)</td>
<td>5.1 &lt;0.01 0.94 1 1.1 0.96 0.96 0.76 1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G59140</td>
<td>ABC transporter family protein</td>
<td>3.4 &lt;0.01 0.81 1 0.89 0.93 0.93 0.6 1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT5G43580</td>
<td>Protease inhibitor, putative</td>
<td>2.6 0.01 0.58 1 1.2 0.9 1.1 0.45 1</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G72680</td>
<td>Cinnamyl-alcohol dehydrogenase, putative</td>
<td>2.7 0.01 0.66 1 0.71 0.9 0.77 0.09</td>
<td>0 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2G30140</td>
<td>UDP-glucoronosyl/UDP-glucosyl transferase family protein</td>
<td>2.4 &lt;0.01 0.68 1 0.66 0.9 0.98 0.94</td>
<td>0 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G68570</td>
<td>Proton-dependent oligopeptide transport (POT) family protein</td>
<td>2.5 &lt;0.01 0.86 1 1 1 0.9 0.41</td>
<td>1 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G56710</td>
<td>sigA-binding protein</td>
<td>0.4 &lt;0.01 1.2 1 0.86 0.9 1.1 0.38</td>
<td>1 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table I. Microarray-derived expression ratios of selected genes with differential expression between ANAC102 mutant lines and the wild type

<table>
<thead>
<tr>
<th>Arabidopsis Genome Initiative No.</th>
<th>Gene Title/Description</th>
<th>Expression Ratio in OX-1</th>
<th>Expression Ratio in KO-1</th>
<th>Expression Ratio in KO-1 (0.1% Oxygen)</th>
<th>Expression Ratio in KO-1 Seeds (0.1% Oxygen)</th>
<th>NAC Strict</th>
<th>NAC Consensus</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT3G22370</td>
<td>Alternative oxidase 1a, mitochondrial (AOX1A)</td>
<td>0.94 0.81 0.86 1 0.95 0.97 1.8 &lt;0.01</td>
<td>0 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT3G23150</td>
<td>Ethylene receptor, putative (ETR2)</td>
<td>1 0.91 1.1 1 1.2 0.9 0.51 &lt;0.01</td>
<td>0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G11580</td>
<td>Pectin methylesterase, putative</td>
<td>0.91 0.68 1.2 1 1.1 0.95 0.48 &lt;0.01</td>
<td>0 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT1G03090</td>
<td>3-Methylcrotonyl-CoA carboxylase 1 (MCCA)</td>
<td>1.2 0.28 0.97 1 0.83 0.9 0.47 &lt;0.05</td>
<td>1 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT2G40220</td>
<td>Abscisic acid-insensitive 4 (ABI4)</td>
<td>0.95 0.85 1.2 1 1 1 0.6 &lt;0.01</td>
<td>0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The promoter regions of all of the genes showing differential expression in OX-1 were analyzed for evidence of conserved motifs. Analysis with the Athenā visualization tool (O’Connor et al., 2005), which can identify transcription factor-binding motifs in promoter sequences, showed some enrichment for the ABA-responsive element (ABRE)-like binding site motif (Shinozaki and Yamaguchi-Shinozaki, 2000) in the up-regulated genes, with 34 of the 113 genes containing at least one copy of this element. The genes found to be down-regulated in OX-1 were found to be enriched for an AGC box enhancer element identified from tobacco (*Nicotiana tabacum*) class I β-1,3-glucanase GLB gene (Hart et al., 1993), the LS7 promoter element required for salicylic acid induction of Arabidopsis PR-1 (Despres et al., 2000), and the JASE1 motif required for leaf senescence and jasmonic acid induction of Arabidopsis OPRI (He and Gan, 2001). As Athenā
does not identify NAC domain transcription factor binding sites, the Toucan analysis tool, which can perform searches for specified motifs, was used to search for these sequences. Both the set of genes up-regulated in OX-1 and the set of genes down-regulated in OX-1 had higher than expected incidences of the NAC-binding site motif: 37.8% to 48.7% of the promoters in each data set contained one or more instances of the strict NAC core-binding site (TTNCGTA), and 90.9% to 96.5% of the promoters in each data set contained one or more instances of the general consensus NAC-binding site ([TA][GT][TACG]CGT[GA]; Olsen et al., 2005; Table II; Supplemental Table S1). The Web-based tool POBO, which compares the frequency of a given motif in a set of promoter sequences with a random set of Arabidopsis promoter sequences, confirmed that both the strict and general consensus NAC-binding sites were statistically overrepresented in promoters of genes affected by ANAC102 overexpression ($P < 0.0001$; Kankainen and Holm, 2004).

Overexpression or Underexpression of ANAC102 Has No Significant Effect on Adult Plant Tolerance to Low Oxygen

To determine whether ANAC102 is important for plant survival under low oxygen, both KO and OX lines were subjected to severe low-oxygen stress. In five separate experiments, plants were subjected to 0.1% oxygen, with or without a 24-h 5% oxygen pretreat-

Table II. Promoter region analysis for NAC-binding sites in genes affected by ANAC102 expression

<table>
<thead>
<tr>
<th>Gene Set</th>
<th>No. of Genes</th>
<th>NAC Motif Strict</th>
<th>POBO T Statistic</th>
<th>POBO P</th>
<th>NAC Consensus</th>
<th>POBO T Statistic</th>
<th>POBO P</th>
</tr>
</thead>
<tbody>
<tr>
<td>OX-1 up-regulated</td>
<td>113</td>
<td>48.7%</td>
<td>46.5</td>
<td>&lt;0.0001</td>
<td>96.5%</td>
<td>83.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OX-1 down-regulated</td>
<td>98</td>
<td>37.8%</td>
<td>49.7</td>
<td>&lt;0.0001</td>
<td>90.9%</td>
<td>34.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO-1 up-regulated</td>
<td>94</td>
<td>38.3%</td>
<td>41.7</td>
<td>&lt;0.0001</td>
<td>89.4%</td>
<td>88.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>KO-1 down-regulated</td>
<td>113</td>
<td>38.9%</td>
<td>43.6</td>
<td>&lt;0.0001</td>
<td>82.3%</td>
<td>36.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

The gene sets used can be found in Supplemental Tables S1 and S4. The strict NAC-binding site motif is TTNCGTA, as defined by Olsen et al. (2005). The T statistic was calculated by the promoter analysis program POBO (Kankainen and Holm, 2004) and is a measure of the difference between the frequency of the searched-for motif in a given sequence set as compared with a randomly generated background set of sequences. The consensus NAC-binding site motif is [TA][GT][TACG]CGT[GA], as defined by Olsen et al. (2005).
ment, in the dark for 3 d and then scored for survival, shoot weight, and root weight 1 week after removal from the stress condition. Survival counts were variable (Supplemental Fig. S3), and no significant differences in survival or weight measures between either KO lines or OX lines and their respective wild types were found in experiments with or without a 5% oxygen pretreatment. As expected, however, there was a significant difference in survival between the Col-0 ecotype lines and the C24 ecotype lines in experiments in which no 5% oxygen pretreatment was used (Fig. 4; accumulated analysis of deviance, 5 degrees of freedom [df]; deviance ratio $= 6.77$; approximate $F$ probability, $P < 0.05$). B, Plants were treated for 1 d with 5% oxygen, followed by 3 d with 0.1% oxygen. Survival percentages are averages over three experiments ($\pm s e; n = 9$).

### Seed Viability Is Impaired in ANAC102 Knockout Lines following a Low-Oxygen Treatment

Six months after harvest, seeds from each line either overexpressing or knocked out for ANAC102 and their parental wild types, collected from plants grown at the same time in the same growth cabinet, were subjected to 6 d of exposure to 0.1% oxygen in the light. During the exposure to 0.1% oxygen, no germination occurred, in contrast to seeds exposed to the ambient atmosphere, which reached maximum germination within 2 to 4 d. Germination percentages in the normal atmosphere were lower for seeds from C24 background plants (63%–80%; Fig. 5A) than for seeds from the Col-0 background plants (92%–97%; Fig. 5A), which appears to be a feature of C24 germination on unsupplemented agarose, as these seeds will all

**Figure 4.** Survival after exposure to 0.1% oxygen. Plants with T-DNA insertions within the ANAC102 gene (KO-1, KO-2, and Col-0 background) and plants overexpressing the ANAC102 gene (OX-1, OX-2, and C24 background) and their respective parental ecotypes were subjected to low-oxygen treatments. A, Plants were treated with 0.1% oxygen in the dark for 3 d. Survival percentages are averages over five experiments ($\pm s e; n = 15$). Data points with different lowercase letters denote lines with survival percentages significantly different from each other as determined by ANOVA and Tukey's honestly significant difference test ($P < 0.05$). B, Plants were treated for 1 d with 5% oxygen, followed by 3 d with 0.1% oxygen. Survival percentages are averages over three experiments ($\pm s e; n = 9$).

**Figure 5.** Effects on germination of various abiotic stresses. Seeds were scored for germination at intervals up to 1 week. Data represent average numbers of germinated seeds from three replicates containing 20 to 50 seeds each ($\pm s e; n = 3$). Black symbols represent data for untreated seeds, and white symbols represent treated seeds. Data points with different lowercase letters denote lines with germination percentages significantly different from each other (only differences between lines within a treatment are shown) as determined by ANOVA and Tukey’s honestly significant difference test ($P < 0.05$). A, Seeds treated with 0.1% oxygen for 6 d. Germination was scored after removal to ambient oxygen levels. B, Seeds sown on medium containing 200 mM NaCl. C, Seeds sown on medium containing 5% (w/v) mannitol. D, Seeds sown on medium containing 15 mM ABA.
germinate on other media. Modification of ANAC102 expression appeared to have no effect on seed germination in the absence of stress, as there was no significant difference in germination between ANAC102 KO or OX seeds and their respective wild types under normal conditions. Following removal from 0.1% oxygen, OX-1, OX-2, and the wild type all showed a reduction in germination percentage, with no significant differences in response between the lines (Fig. 5), but there was a marked difference between the ANAC102 knockout lines and the wild type. Following the low-oxygen treatment, wild-type germination percentages were reduced to between 65.4% and 80.0% of the untreated control, while the ANAC102 knockout lines KO-1 and KO-2 had germination percentages reduced to between 26% and 38% of their untreated controls (Fig. 5A). Germination percentages of KO-1, KO-2, and the wild type untreated and 0.1% oxygen-treated seeds at 7 d after stress were found by ANOVA to have highly significant treatment and line differences (treatment, df = 4; line, df = 1, F = 140.0662, P = 9 × 10⁻¹¹; line, df = 2, F = 13.4125, P = 2 × 10⁻⁴; treatment × line, df = 2, F = 9.25, P = 0.001; Fig. 5A).

To determine whether the 0.1% oxygen treatment had killed or only imposed a secondary dormancy (where seeds that were viable and nondormant remain viable but refuse to germinate) in those seeds that had not germinated, all of the ungerminated seeds from the assay were placed back at 4°C for 1 week (486 seeds total). Only a small proportion (2%) of the ungerminated seeds germinated following the cold treatment, indicating that a secondary dormancy had not been induced in these seeds (Supplemental Table S3). Another set of ungerminated seeds from a separate experiment were stained with tetrazolium salts (510 seeds total); 95% of these seeds turned pink, indicating that respiration was occurring (Supplemental Table S3). Vital staining with propidium iodide and fluorescein diacetate was performed on another set of low-oxygen-treated, ungerminated seeds, consisting of five ungerminated seeds from each of KO-1, KO-2, and the wild type. Endosperm tissues were all alive, but in all cases save one, seeds from KO-1, a mixture of live and dead cells were seen in the embryo (Fig. 2F). The remaining KO-1 embryo appeared to be completely viable.

We further examined whether germination signals may be blocked in these seeds using ATGA3OX2 (At1g80340) expression as a marker for germination. In Arabidopsis, the biosynthesis of gibberellins is a crucial step toward initiating germination (Koornneef and Vanderveen, 1980; Nambara et al., 1991). One of the final steps in the production of active gibberellin is catalyzed by ATGA3OX2, expression of which increases in germinating seeds (Yamaguchi et al., 1998). QRT-PCR assays of ATGA3OX2 expression between wild-type and KO-1 lines treated with 0.1% oxygen showed no significant reduction in ATGA3OX2 expression in the KO-1 lines (1.2-fold induction in KO-1 line; t test; two-tailed P = 0.17).

To determine whether the decreased germination observed under low oxygen in the KO lines was specific to low-oxygen treatments or was a generalized stress response, we subjected Arabidopsis wild-type and mutant lines to other stresses during germination. Salt and osmotic stresses were chosen for these experiments, as public data available at the Genevestigator Web site indicated that ANAC102 expression is also inducible by salt and osmotic stresses (Zimmermann et al., 2004). Due to its role in inhibiting germination in Arabidopsis, we also tested the ANAC102 mutant lines for differences in ABA sensitivity during germination. Although germination in KO lines was transiently lower than in the wild type when subjected to 200 mM NaCl, neither 5% mannitol nor 15 μM ABA appeared to have a stronger effect on KO lines compared with the wild type (Fig. 5, B–D). These results suggest that the decreases in germination observed in KO lines are likely to be specific to low-oxygen stress.

**ANAC102 Knockout Lines Show Changes in Global Gene Expression in 0.1% Oxygen-Treated Seeds**

Affymetrix ATH1 microarrays were used to interrogate RNA samples derived from wild-type (Col-0) and KO-1 lines to assess the impact on global gene expression of loss of ANAC102 function. In comparisons made using 3-week-old plants under both ambient atmosphere and 0.1% oxygen for 4 h, no genes other than ANAC102 itself (which decreased 17- to 50-fold; Table I) were found to have significantly different expression (greater than 1.5-fold change between KO-1 and the wild type, with an adjusted P value of ≤0.05). In contrast, when KO-1 and wild-type imbibed seeds were subjected to 6 d of 0.1% oxygen and assayed for differences in gene expression, 94 genes were found to be more highly expressed and 113 were more lowly expressed in KO-1 than in the wild type (greater than 1.5-fold change at an adjusted P value of <0.05; Supplemental Table S4). As was the case in the ANAC102 OX lines, a large proportion of the genes with altered expression in the ANAC102 KO seeds have previously been reported to be responsive to low oxygen; 29 of the 94 up-regulated genes and 58 of the 113 down-regulated genes have been listed as having significant expression changes following low oxygen in one or more microarray studies (Klok et al., 2002; Branco-Price et al., 2005; Liu et al., 2005; Loreti et al., 2005).

Promoters of genes with altered expression in KO-1 seeds under low oxygen were analyzed using the Athena visualization tool (O'Connor et al., 2005). This analysis identified at least one of the following related motifs (all share the core sequence ACGTG): the ABRE-like-binding site motif (Shinozaki and Yamaguchi-Shinozaki, 2000), the ABRE-binding site motif (Choi et al., 2005).
et al., 2000), the ABF-binding site motif, the G-box motif CACGTG (Menkens et al., 1995), the Z-box motif (Ha and An, 1988), or a GA down-regulation motif (Ogawa et al., 2003) in 60 promoters of the 94 ANAC102 KO-1 up-regulated genes. Analysis of the promoters of the 113 genes down-regulated in ANAC102 KO-1 seeds identified 39 with the DREB1A/CBF3 or DRE core motif found in many stress-inducible genes (Maruyama et al., 2004). Searching promoter sequences with Toucan (Aerts et al., 2003, 2005) revealed that 38.3% and 38.9% of the ANAC102 KO-1 up-regulated and down-regulated genes, respectively, contain the strict NAC-binding domain in their promoters and 89.4% and 82.3% of the ANAC102 KO-1 up-regulated and down-regulated genes, respectively, contain the general NAC-binding domain in their promoters (Table II; Supplemental Table S4). The cis-element analysis tool POBO confirmed that both the strict and general NAC domain binding sequences are overrepresented in the promoter sequences at P < 0.0001.

Ten genes, including ANAC102, identified as differentially regulated between wild-type and KO-1 seeds following 6 d of exposure to 0.1% oxygen, were assayed via QRT-PCR to determine their relative expression levels in wild-type, KO-1, and OX-1 lines at 4 d under 0.1% oxygen as well as 6 d (Fig. 6). All genes tested

Figure 6. Relative expression of selected genes in seeds exposed to 0.1% oxygen for 4 or 6 d. Seeds of each line were imbibed, cold stratified, and then placed in a 0.1% oxygen atmosphere for 4 or 6 d. Expression ratios for selected genes are given relative to expression levels in untreated wild-type (Col-0 for Col-0 and KO-1; C24 for C24 and OX-1) imbibed seeds. Bars represent expression ratios for Col-0 (black columns), KO-1 (dotted columns), C24 (shaded columns), and OX-1 (diagonal lined columns; ± SE; n = 9). Data points with different letters denote lines with relative expression ratios significantly different from each other as determined by two-way ANOVA (line × time) and Tukey’s honestly significant difference test (P < 0.05). Uppercase letters differentiate between Col-0 and KO-1 at the different times, while lowercase letters differentiate between C24 and OX-1 at the different times. No comparisons were made between lines in the Col-0 background and lines in the C24 background.
showed a greater response to low oxygen after 6 d at 0.1% oxygen as compared with 4 d. SUS1, a key low-oxygen-responsive gene, showed induction in low-oxygen conditions at both time points tested, but this induction was significantly lessened in the KO-1 seeds. For only one gene (AOX; At3g22370) was there a significant difference in expression between KO-1 and Col-0 after 4 d in 0.1% oxygen (Fig. 6). Differences in expression between KO-1 and Col-0 were much more apparent in most genes after 6 d at 0.1% oxygen (Fig. 6). Overexpression of ANAC102 also had a significant impact on gene expression under 0.1% oxygen (Fig. 6). Surprisingly, overexpression of ANAC102 had a similar impact on gene expression as reduced expression of ANAC102; that is, where gene expression was decreased in KO-1 as compared with Col-0, it was also decreased in OX-1 as compared with C24.

DISCUSSION

ANAC102 is not required for normal growth and development, and its primary role in Arabidopsis is very likely to respond to stress. Neither of the two independent ANAC102 knockout lines showed any apparent phenotype under nonstressed conditions, including no global changes in gene expression. Knockouts of the stress-inducible NAC genes ATAF2 (Delessert et al., 2005), ATAF1 (Lu et al., 2007), and AtNAC2 (He et al., 2005) similarly showed no phenotype under normal growth conditions. ANAC102 expression increases very early following the imposition of low oxygen levels. The rapid response of ANAC102 to low oxygen coupled with the ability to respond to low oxygen in the presence of cycloheximide, which blocks protein synthesis, suggest that ANAC102 responds directly to low-oxygen stress and does not require for induction any upstream low-oxygen-responsive transcription factors. ANAC102 has been classed as an unstable transcript with a half-life of less than 60 min under normal conditions (Gutierrez et al., 2002). The increase in ANAC102 transcript levels under low oxygen may be partially due to an increase in transcript stability rather than an increase in transcription, although the ANAC102 promoter is capable of driving an increase in GUS transcript in response to low oxygen (Fig. 2).

In contrast to lines overexpressing ATAF2 (Delessert et al., 2005), AtNAC2 (He et al., 2005), or ANAC055 (Tran et al., 2004), overexpression of ANAC102 had little effect on plant phenotype other than a mild yellowing of the leaves. Overexpression of ANAC102 resulted in altered expression of 211 genes under normal conditions. Most (96.5%) of the genes with altered expression in OX-1 contained the general consensus NAC-binding site, indicating that these genes may be targets for ANAC102 binding. Both genes up-regulated and down-regulated in ANAC102-overexpressing lines had high frequencies of the NAC domain consensus-binding sequence, suggesting the possibility that ANAC102 can act bifunctionally as both an activator and a repressor of transcription. This type of bifunctionality has been observed in the NFYA5 transcription factor, overexpression of which affected the abundance of mRNA from a number of genes containing the NFYA5 recognition site CCAAT, inducing some and repressing others (Li et al., 2008). Overrepresentation of the WRKY-binding motif has also been observed in promoters of genes both induced and repressed by overexpression of WRKY70 (Kankainen and Holm, 2004). Two-thirds of the genes induced or repressed in ANAC102-overexpressing lines have been previously identified as being low-oxygen responsive (Supplemental Table S1), including ADH1 and SUS1, which are known to be vital for adult plant tolerance of low oxygen (Ellis et al., 1999; Bieniawska et al., 2007). In contrast, ANAC102 KO lines showed no transcriptional differences from the wild type in normal and low-oxygen conditions. Neither ANAC102 OX nor KO lines showed any differences in growth or survival when adult plants were subjected to low-oxygen stress. The ANAC102 OX microarray results under normal conditions, and QRT-PCR results under low-oxygen conditions suggest that ANAC102 has a role in modifying transcriptional responses to low oxygen, but the survival assays show that ANAC102-mediated responses are not sufficient to protect the plants or, as is the case with ADH1 overexpression, were not rate limiting for survival under low oxygen (Baxter-Burrell et al., 2002; Ismond et al., 2003).

The ANAC102 KO microarray and survival assay results demonstrate that ANAC102 function is not required for transcriptional or phenotypic response to low oxygen in adult plants. However, ANAC102 KO lines had impaired germination after 0.1% oxygen treatment, and comparisons between wild-type and KO-1 seeds showed expression differences in 207 genes. Loss of ANAC102 did not alter germination in unstressed seeds, nor was any effect on germination observed under salt, osmotic, or ABA stress. This indicates that although ANAC102 is not essential for adult tolerance to low oxygen, it is important for tolerance to low-oxygen levels during germination. A possible explanation for the difference between adult and seed low-oxygen phenotypes is redundancy of ANAC102 gene function. A total of 23 other NAC domain transcription factors also possess significantly altered expression under low oxygen, and many have high sequence similarity to ANAC102, particularly ANAC002 (ATAF1) and ANAC032 (Okka et al., 2003). In adult plants, ANAC102 is highly expressed in the lateral root cap (Fig. 2; Supplemental Fig. S4), as are ANAC002 and ANAC032 (Supplemental Fig. S4). ANAC102 is also expressed at relatively high levels in the radicle; however, ANAC002 and ANAC032 are not strongly expressed in this tissue (Supplemental Fig. S4). Differences in tissue-specific expression between these highly similar genes may account for the difference in adult survival/germination rates under...
low-oxygen conditions and the lack of transcriptome results observed in the ANAC102 mutant lines in adult tissue.

Very little is known about how Arabidopsis seeds respond to low oxygen, and it is unclear how disruption of ANAC102 function compromises seed tolerance to low oxygen. Two-thirds of the genes up-regulated in stressed ANAC102 KO seeds have a core motif of ABA-responsive elements in their promoters, and the ABA response gene ABI4 shows lower than wild-type expression in KO lines following exposure to low oxygen (Table I; Fig. 6), implying a role for ABA in the ANAC102-mediated response to 0.1% oxygen in seeds. ABA and oxygen levels interact to regulate dormancy in barley, where the glumella limits oxygen diffusion through the barley embryo, resulting in increased sensitivity to ABA (Benech-Arnold et al., 2006). However, in germination experiments on media supplemented with ABA, neither KO nor OX lines showed any change from the wild type in ABA sensitivity. Furthermore, the Arabidopsis seeds used in this work were nondormant, and low oxygen did not induce a secondary dormancy, as seeds that failed to germinate after low-oxygen treatment did not germinate after a dormancy-breaking cold treatment.

Vital staining of seeds that failed to germinate indicated that portions of the embryo may have been killed by the low-oxygen stress. This damage may have been sufficient to prevent the radicle from being able to break through the seed coat. An alternative hypothesis is that mobilization of energy reserves is required to break through the seed coat. An alternative is that mobilization of energy reserves is required to break through the seed coat. This damage may result in a weaker ethylene-mediated low-oxygen response in these seeds.

A set of genes identified as differentially expressed between KO and wild-type seeds at a single low-oxygen time point via microarray were assayed in both OX and KO lines using QRT-PCR at two time points under low-oxygen stress. For many of these genes, lower levels of expression were observed in both the OX and KO lines than in their wild-type counterparts after 6 d of exposure to low oxygen, raising the possibility that excess ANAC102 expression may also be somewhat detrimental to seed responses to low oxygen. Although the differences were found to be not statistically significant, both OX lines did show lower germination rates than the wild type following low-oxygen stress (Fig. 5A).

The increased expression of putative low-oxygen-responsive genes in ANAC102 overexpressors and the reduced germination rates of ANAC102 knockout lines in response to low-oxygen treatment suggest that ANAC102 positively regulates the response to low oxygen, while the lack of global gene expression change at the adult plant stage in ANAC102 knockouts and the lack of significant adult plant survival differences between ANAC102 OX and KO lines and the wild type suggest that the role of ANAC102 may be functionally redundant at the adult plant stage. Both of these features are in direct contrast with the mode of action of both ATAF1 and ATAF2, which negatively regulate responses to drought and wounding, respectively, and for which a loss of function of either of these genes results in a readily observable phenotype under stress conditions (Delessert et al., 2005; Lu et al., 2007).

ANAC102 is important for the Arabidopsis response to low-oxygen levels during germination. This work illustrates that responses to environmental stresses, in this case low oxygen, are dependent on developmental stage and that mechanisms for tolerance can be different at different stages. ANAC102 is required for tolerance to low oxygen at the seed stage but not at the early adult plant stage. The transcriptional responses to low oxygen also differ between developmental stages, since in KO lines no genes show differences in transcript levels in adult plant microarrays but 207 genes show significant expression differences in seeds. At the other end of the developmental scale, it has recently been reported that 12-week-old Arabidopsis plants form aerenchyma in response to waterlogging stress (Muehlenbock et al., 2007). This response to
waterlogging was not previously thought to occur in Arabidopsis, as most prior research had been conducted on younger, rosette stage plants that do not form aerenchyma. Taken together, these findings highlight the different responses to stress at different developmental growth stages.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) lines carrying T-DNA insertions in ANAC102 (AT5g63730; SALK_030702 and SALK_094437) were identified using the SIGnAL Web site (http://signal.salk.edu). The two insertion lines were obtained from the Arabidopsis Biological Resource Center (Alonso, 2003). Insertions were verified and homozygote lines selected using PCR according to the protocols provided by the SIGnAL Web site. KO-1 is a derivative of SALK_030702 and KO-2 is a derivative of SALK_094437. Both insertions are within the second exon of ANAC102, and both segregated as single-locus insertions. Ecotype Col-0 was used as the wild type in all comparisons with the insertion mutant lines. Since C24 is more susceptible to low-oxygen stress than Col-0, the ANAC102 coding sequence under the control of the cauliflower mosaic virus 35S promoter was transformed into ecotype C24 to make it easier to detect any increase in low-oxygen tolerance, highly expressing lines (as assayed by QRT-PCR), designated OX-1 and OX-2, were chosen for use in these experiments. Ecotype C24 was used as the wild type in all comparisons with the ANAC102 overexpression lines. A promoter::GUS fusion line was created by amplifying 1,611 bp upstream of the ANAC102 transcription start site and cloning the amplicon into a binary plant transformation vector, and transformed into C24 by the floral dip method.

All plants were grown on Murashige and Skoog (MS) medium with 3% Suc and 0.8% agar. For seed germination experiments, seeds were plated on either 0.6% agarose in water or half-strength MS medium. Plants or seeds for all experiments were kept in a growth room at 22 °C with a day/night period of 16/8 h and fluorescent lighting levels of approximately 75 μE. For gene expression and low-oxygen assays, plants were transferred to liquid MS with 3% Suc. To obtain seeds for germination experiments, plants were grown in soil at 22°C under a 16/8-h day/night cycle, and all lines tested were grown at the same time and in the same location to minimize environmental influences on seed development and subsequent germination.

Low-Oxygen Survival Assay

Low-oxygen survival assays were performed as outlined previously (Ellis et al., 1999), except that argon was not used to flush the anaerobic chambers and plants were not subjected to aoxia but rather to mild hypoxia (5% oxygen, balance nitrogen) and/or severe hypoxia (0.1% oxygen, balance nitrogen). Briefly, plants were grown to the four- to six-leaf stage on solid medium and then transferred to liquid medium for 1 d prior to treatment. Immediately prior to treatment, plants were transferred to liquid medium that had been sparged with either 5% oxygen, for pretreated plants, or 0.1% oxygen, for nonpretreated plants. Plants were then placed in 3.5-L anaerobic chambers (Oxoid) and purged with either 5% or 0.1% oxygen at a flow rate of approximately 10 L min⁻¹ for 20 min. Pretreated plants were left in 5% oxygen, in the dark with gentle shaking, for 24 h and then purged with 0.1% oxygen and left for a further 3 d. Nonpretreated plants were purged with 0.1% oxygen and left in the dark with gentle shaking for 3 d. Nonpretreated plants were placed in anaerobic chambers but left exposed to the ambient atmosphere and left in the dark with gentle shaking for 3 d. 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 and weight measurements.

Survival data from five independent experiments were pooled and analyzed for differences between lines in their response to low oxygen. The low-oxygen survival data were not well approximated by the assumption of normality. Consequently, these data were analyzed with a binomial generalized linear model with a logit link. The dispersion factor was initially fixed at 1, but the analysis then flagged a large number of large standardized residuals, so the data were reanalyzed, allowing the dispersion to be estimated from the data. The model assessed variation due to Arabidopsis line differences, blocked for variation between experiments. Predicted probabilities of survival were compared between each pair of lines using the LSD of each comparison at a significance level of 0.033% (5% after a Bonferroni correction).

QRT-PCR

All QRT-PCRs were performed in triplicate on either a Corbett Rotorn Genez (Corbett Life Sciences) or an Applied Biosystems 7900HT (Applied Biosystems) system. Product was detected by fluorescence of incorporated SYBR Green. All data were normalized to the expression of Act2 (Corbett Life Sciences) and has been found not to change expression in response to low oxygen in microarray experiments we have conducted. Primer sequences are listed in Supplemental Table S5. In all cases, relative expression levels were calculated using the ∆ΔCt method of Pfaffl et al. (2002). Expression levels in various experiments were analyzed using a two-tailed t test assuming equal variances to identify statistically significant changes in expression levels.

Low-Oxygen Treatments of Arabidopsis Seedlings and Seeds

Low-oxygen treatments for microarray and QRT-PCR experiments were carried out in the same manner as for the low-oxygen survival assays. Plants were grown on solid MS medium for 3 weeks, to around the four- to six-leaf stage, then transferred to liquid medium 1 d prior to stress. For the low-oxygen treatments, plants were placed in 3.5-L anaerobic chambers (Oxoid) and purged with a 0.1% oxygen/balance nitrogen gas mixture for 20 min, after which plants were left in the 0.1% oxygen atmosphere in the dark. For cycloheximide treatment, 3-week-old plants were moved to liquid medium containing 10 μm cycloheximide for 1 h, medium was refreshed with new medium plus 10 μM cycloheximide, and plants were low-oxygen treated for 4 h. After treatment, plants were flash frozen and ground in liquid nitrogen. RNA was extracted using a Trizol buffer (Invitrogen) following the manufacturer’s instructions. For seed microarray and real-time PCR experiments, seeds were plated on 0.6% agarose in water, allowed to imbibe for approximately 4 h, and then cold stratified at 4°C overnight. Following stratification, plated seeds were allowed to equilibrate to room temperature, then placed in the anaerobic chambers and purged with the 0.1% oxygen gas mixture for 20 min. Seeds were then left in the 0.1% oxygen atmosphere for 6 d. Following treatment, seeds were frozen and ground in liquid nitrogen. RNA was extracted using a hot borate method (Cadman et al., 2006).

Microarray Analysis of Gene Expression

Whole plant or seed RNA was sent to the Australian Genome Research Facility for labeling and hybridization to Affymetrix Arabidopsis ATH1 genome arrays (22,500 probes). In unstressed microarray experiments, two biological replicates were used for the wild type, KO-1, and KO-1 and one replicate was used for KO-2 and OX-2. As no differences were found between KO-1 and KO-2 or between OX-1 and OX-2, data presented here are from KO-1 and OX-1 comparisons only. At 3 weeks old, five plants of each line from each replicate plate were bulked, flash frozen in liquid nitrogen, and ground. RNA was extracted using a Trizol buffer (Invitrogen) following the manufacturer’s instructions. For low-oxygen stress microarrays at both plant and seed stages, at least two biological replicates of the wild type and KO-1 were used. For experiments on seeds, seeds were plated on 0.6% agarose in water and low-oxygen treated as described above. Following treatment, seeds were frozen and ground in liquid nitrogen. RNA was extracted using a hot borate method (Cadman et al., 2006).

Resulting signal data were analyzed using the limma Bioconductor package in R. Array data were normalized using the EXPRESSO function (Gautier et al., 2005). A robust multichip average was calculated using quantile normalization, background correction, and the median polish method as recommended by Bolstad et al. (2003) and Irizarry et al. (2003).

Promoter Analysis

Promoter regions of selected genes were screened for common motifs. Three different Web-based analysis tools were used: Athena (O’Connor et al.,
Germination Assays

Seeds of each line to be tested that had been harvested at the same time from plants grown in the same environmentally controlled growth room were imbibed in 1.5-mL microfuge tubes for approximately 2 h at room temperature. Twenty to 50 seeds of each line were then plated out approximately 0.5 cm apart on 0.6% agarose and then placed at 4°C for 1 week. For low-oxygen treatments, seeds were placed in 3.5-L anaerobic chambers and either purged with 0.1% oxygen at a flow rate of 10 L min⁻¹ for 20 min or exposed to the ambient atmosphere. Seeds left exposed to ambient air were scored for germination daily. Seeds treated with 0.1% oxygen remained in sealed anaerobic chambers at 0.1% oxygen for 6 d, during which time no seed germination occurred. Following removal from 0.1% oxygen, seed germination was scored daily. For the other stress treatments, seeds were plated on half-strength MS medium containing 200 mM NaCl, 5% (w/v) mannitol, or 15 µM ABA. In all cases, germination was scored at intervals for 1 week. In all cases, seeds were counted as germinated when the radicle penetrated the seed coat. Data on the proportion of seeds germinated at 7 d from three replicate experiments were analyzed with a one-way ANOVA blocked for replicate to detect significant differences between the lines. Arabidopsis lines were grouped on the basis of germination percentage using Tukey’s honestly significant difference.

Seed Staining

For GUS staining, plant material to be stained was immersed in a solution composed of 3 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid, 10 mM EDTA, 100 mM NaPO₄ buffer, pH 7.2, 0.3% Triton X, 500 µM ferrocyanide, 500 µM ferricyanide, and 10% methanol, vacuum infiltrated, and incubated at 37°C until color developed. Following staining, plant material was washed in distilled water and cleared with two washes of 70% ethanol. Ungerminated seeds of each line to be tested that had been harvested at the same time from plants grown in the same environmentally controlled growth room were collected and seeds to be tested were scored with a 21-gauge needle to break the seed coat. Data on the proportion of seeds germinated at 7 d from three replicate experiments were analyzed with a one-way ANOVA blocked for replicate experiments to detect significant differences between the lines. Arabidopsis lines were grouped on the basis of germination percentage using Tukey’s honestly significant difference.

Supplemental Data

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

Supplemental Figure S1. Expression levels of ANAC102 in knockout and overexpressing lines.

Supplemental Figure S2. Expanded version of Figure 3 showing expression of selected genes over a time course in each of Col-0, C24, KO-1, and OX-1.

Supplemental Figure S3. Survival after exposure to 0.1% oxygen for five independent experiments.

Supplemental Figure S4. Heat map of gene expression for 23 low-oxygen-induced NAC genes.

Supplemental Table S1. Genes identified with significant changes in expression in OX-1 adult plants.

Supplemental Table S2. Average root and shoot weights for Arabidopsis lines following a 2-week recovery from low-oxygen stress treatments.

Supplemental Table S3. Germination of low-oxygen-treated seeds following cold treatment and seed mortality as determined by tetrazolium staining.

Supplemental Table S4. Genes identified with significant changes in expression in low-oxygen-treated KO-1 seeds.

Supplemental Table S5. Sequences of primers used.

ACKNOWLEDGMENTS

We thank Jun Yang for technical assistance and Donna Bond for providing cDNA samples.

Received October 30, 2008; accepted January 23, 2009; published January 28, 2009.

LITERATURE CITED


Cottrell HJ (1947) Tetrazolium salt as a seed germination indicator. Nature 159: 748


Jones KH, Senft JA (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate propidium iodide. J Histochem Cytochem 33: 77–79


Plant Physiol. Vol. 149, 2009 1737
genes encoding 1-aminocyclopropane-1-carboxylate synthase in Arabidopsis during hypoxia. Plant Mol Biol 58: 15–25