

The Heat-Inducible Transcription Factor *HsfA2* Enhances Anoxia Tolerance in Arabidopsis^[W]

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Anoxia induces several heat shock proteins, and a mild heat pretreatment can acclimatize Arabidopsis (*Arabidopsis thaliana*) seedlings to subsequent anoxic treatment. In this study, we analyzed the response of Arabidopsis seedlings to anoxia, heat, and combined heat + anoxia stress. A significant overlap between the anoxic and the heat responses was observed by whole-genome microarray analysis. Among the transcription factors induced by both heat and anoxia, the heat shock factor A2 (*HsfA2*), known to be involved in Arabidopsis acclimation to heat and to other abiotic stresses, was strongly induced by anoxia. Heat-dependent acclimation to anoxia is lost in an *HsfA2* knockout mutant (*hsfa2*) as well as in a double mutant for the constitutively expressed *HsfA1a/HsfA1b* (*hsfA1a/1b*), indicating that these three heat shock factors cooperate to confer anoxia tolerance. Arabidopsis seedlings that overexpress *HsfA2* showed an increased expression of several known targets of this transcription factor and were markedly more tolerant to anoxia as well as to submergence. Anoxia failed to induce *HsfA2* target proteins in wild-type seedlings, while overexpression of *HsfA2* resulted in the production of *HsfA2* targets under anoxia, correlating well with the low anoxia tolerance experiments. These results indicate that there is a considerable overlap between the molecular mechanisms of heat and anoxia tolerance and that *HsfA2* is a player in these mechanisms.

Higher plants are aerobic organisms relying on oxygen for respiration (Bailey-Serres and Voesenek, 2008). Oxygen is required for oxygen-dependent enzymatic reactions and, most importantly, in mitochondrial respiration, where it acts as the terminal acceptor of electrons in oxidative phosphorylation that indirectly provides the plant with ATP (Perata and Alpi, 1993; Geigenberger, 2003; Voesenek et al., 2006). Shortage of oxygen (hypoxia) or a total absence of oxygen (anoxia) inhibits respiration and results in energy deficit.

Few plant species can tolerate flooding, the most common environmental cause of oxygen shortage in plants (Bailey-Serres and Voesenek, 2008). Plants species adopt different strategies to survive flooding. Some species display an "escape strategy" with rapid elongation of submerged organs in order to maintain contact with the atmosphere, allowing transport of oxygen to the submerged parts of the plant, while others tolerate low-oxygen conditions by modulating their metabolism and activating stress-tolerance pathways (Bailey-Serres and Voesenek, 2008). A breakthrough discovery in the field of plant adaptation to

submergence is the identification of the rice (*Oryza sativa*) *Submergence1A* gene as a key element for rice flooding tolerance (Fukao et al., 2006; Xu et al., 2006; Perata and Voesenek, 2007). Little is known, however, about the mechanisms of anoxia tolerance in other plant species. Even flood-intolerant species such as Arabidopsis (*Arabidopsis thaliana*) trigger many genes generally associated with flooding tolerance (Klok et al., 2002; Branco-Price et al., 2005, 2008; Loreti et al., 2005; van Dongen et al., 2009), suggesting that the regulation of flooding tolerance and the differences between species are far more complex than previously thought.

To date, genetic evidence supports the important role of the fermentative pathway in anoxia tolerance in Arabidopsis. Arabidopsis plants devoid of alcohol dehydrogenase (ADH) or Suc synthases are less tolerant to anoxia, although an increased expression of ADH cannot confer enhanced anoxia tolerance (Ismond et al., 2003; Bieniawska et al., 2007). Overexpression of pyruvate decarboxylase, catalyzing a rate-limiting step in ethanol fermentation, has been shown to increase Arabidopsis survival (Ismond et al., 2003). Recently, a role for the transcription factor ANAC102 has been demonstrated for the Arabidopsis response to low oxygen levels during germination (Christianson et al., 2009).

Transcript profiling revealed that, besides the genes involved in the fermentative pathway, anoxia also induces several heat-related genes, including various heat shock proteins (HSPs) and heat shock factors (Hsfs; Loreti et al., 2005). HSP production under stress

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conditions is the most important cellular response, preventing protein aggregation, denaturation, misfolding, and degradation (Hartl, 1996; Hofmann and Feder, 1999; Swindell et al., 2007; Liberek et al., 2008), and their importance in all living organisms, including plants, has been assessed under several different stress conditions potentially threatening cellular homeostasis and leading to protein dysfunction (Vierling, 1991; Hofmann and Feder, 1999; Koh, 2002; Swindell et al., 2007; Timperio et al., 2008). The heat shock response is primarily regulated at the transcriptional level by Hsfs. In *Arabidopsis*, 21 Hsfs have been identified (Nover et al., 2001). Interestingly, it seems that in *Arabidopsis*, different from tomato (*Solanum lycopersicum*; Mishra et al., 2002), there is not a unique master regulator for the Hsf-mediated heat shock response. Different Hsfs have been identified as potential crucial element for *Arabidopsis* response not only to heat stress (Busch et al., 2005; Schramm et al., 2006, 2008; Charng et al., 2007) but also to oxidative stress (Li et al., 2005; Nishizawa et al., 2008), hydrogen peroxide (H_2O_2), high light (Nishizawa et al., 2006), and salt stress as well (Ogawa et al., 2007).

The possible overlap between heat and anoxia responses is supported by evidence showing that mild heat pretreatment, in addition to hypoxic treatment (Ellis et al., 1999), can confer greater tolerance to anoxia in *Arabidopsis* (Loreti et al., 2005). Although the combination of heat and anoxia is not relevant in an ecophysiological context, the induction of Hsfs and HSPs under anoxia and hypoxia in both *Arabidopsis* roots and shoots demonstrates that the heat stress signaling pathway is activated under conditions of oxygen shortage at normal temperatures (Banti et al., 2008). A correlation between HSP mRNA induction by anoxia and low-oxygen tolerance has also been demonstrated (Banti et al., 2008). This evidence led to the speculation that HSPs, and possibly other heat shock-related genes, might be important in the physiological response to anoxia (Loreti et al., 2005; Banti et al., 2008).

In this study, we investigated the role of heat-induced genes in *Arabidopsis* tolerance to anoxia. We used a transcriptomic approach to identify the common elements in both the heat and anoxia responses, highlighting the repression of several anaerobic genes in the heat-anoxia combined treatment and the strong induction of several heat-related genes and transcription factors. We identified *HsfA2* as an important transcription factor that confers anoxia tolerance to *Arabidopsis* seedlings.

RESULTS

Transcriptomic Patterns of Anoxia and Heat Stress Are Very Similar

The induction of several heat-related genes under anoxia suggests that the anoxia response may partly

overlap with the heat response (Loreti et al., 2005). In order to analyze the responses to heat, anoxia, and combined heat + anoxia, we performed a transcript profiling experiment using *Arabidopsis* seedlings. We identified 6,141 differentially regulated genes (genes showing a change of ≥ 2 -fold or ≤ -2 -fold in a hybridization signal, namely the ratio between the intensity in the treated spot versus the control, and a false discovery rate [FDR] of ≤ 0.05 ; two biological replicates for each treatment; Fig. 1, A and B). Among these, 2,210 were up-regulated following three different treatments (Fig. 1A; Supplemental Table S1) and 3,931 were down-regulated (Fig. 1B; Supplemental Table S2). Four hundred thirty-one genes were induced by anoxia, heat up-regulated 1,329 genes, and the combined heat + anoxia treatment resulted in 2,017 up-regulated genes. We observed a considerable overlap between the transcriptomic patterns of the three treatments. The heat + anoxia treatment up-regulated 702 genes that were not regulated by either heat or anoxia. Several of these genes were found to be heat regulated under various experimental conditions (35% when comparing our results with the data set of Larkindale and Vierling [2008]), while others were also actually modulated by either heat or anoxia in our experiments, although the statistical significance of their change in expression did not pass the threshold used to select differentially regulated genes. Analysis of data using MapMan (Thimm et al., 2004; Fig. 1C) revealed that some heat-induced genes ("heat genes" as identified by MapMan) were induced by anoxia, but both heat and the combined heat + anoxia treatments were much more effective, inducing a larger number of genes (Fig. 1C). A mild heat pretreatment (e.g. 38°C, 90 min) can induce acclimation to anoxia in *Arabidopsis* (Loreti et al., 2005; Banti et al., 2008). Therefore, we were interested in evaluating the effects of heat pretreatment on the expression of the genes induced by anoxia. Anoxia induced two groups of genes: those exclusively induced by anoxia ("anoxia genes") and genes induced by anoxia but also responsive to heat ("heat-induced anoxia genes"). While the expression of heat-induced anoxia genes was enhanced by the heat pretreatment, the induction of anoxia genes was reduced by the pretreatment (Fig. 1C). In order to expand this analysis to a larger number of genes, we identified a list of genes modulated by anoxia, and their expression was compared in plants only treated under anoxia and under heat + anoxia (Fig. 1, D and E). Statistical analysis showed that there was a significant general increase in the expression of the anaerobic gene cluster when seedlings had been previously acclimated by a heat pretreatment (Wilcoxon signed rank test, $P < 2.2e-16$). This result suggests that cross-acclimation may be due to an enhanced anaerobic response. However, the heat pretreatment inevitably induces genes related to the heat response. In fact, genes within the anaerobic cluster that were also induced by heat (red dots in Fig. 1D) showed enhanced mRNA levels in the combined treatment

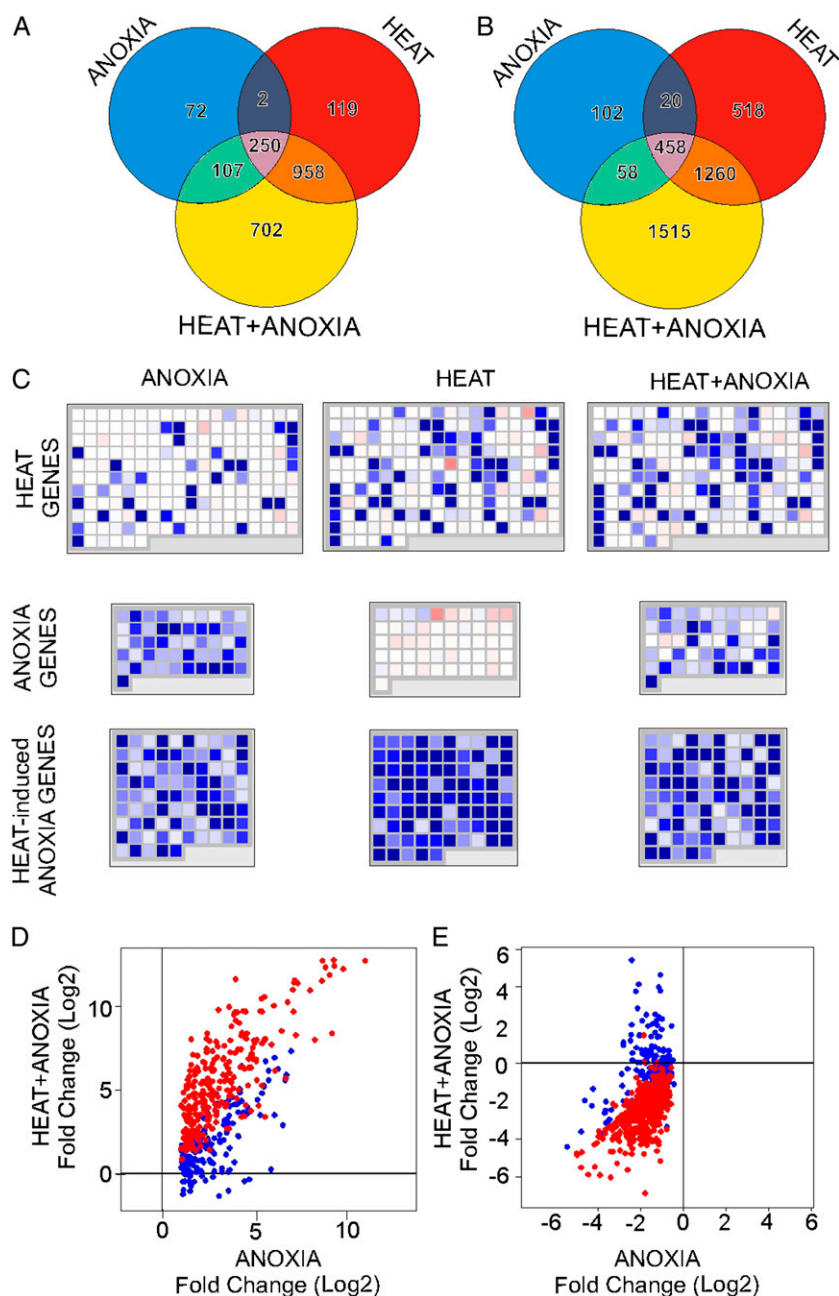


Figure 1. Transcriptome analysis of anoxia-, heat-, and heat + anoxia-treated Arabidopsis seedlings. A and B, Venn diagrams showing genes with differential expression (A, up-regulated; B, down-regulated). Genes were selected on the basis of their fold changes (≥ 2 -fold or ≤ -2 -fold) and their FDR (≤ 0.05). C, MapMan representation of the effects of anoxia, heat, and heat + anoxia on genes annotated as “heat responsive” or “anoxia responsive.” D and E, Effects of the heat pretreatment on the expression levels of anoxic genes. Genes induced (D) or repressed (E) by anoxia were plotted on the basis of their fold change following the anoxic treatment (horizontal axis) and the heat + anoxia treatment (vertical axis). Red dots represent genes induced or repressed by either anoxia or heat, while blue dots represent genes that were not found to be heat responsive. Relative expression levels are shown as fold change values (\log_2).

(Wilcoxon signed rank test, $P < 2.2e-16$). On the other hand, genes induced by anoxia but not by heat (“real” anoxia-dependent genes; blue dots in Fig. 1D) showed a significant reduction in gene expression (Wilcoxon signed rank test, $P = 2.280e-11$). A similar but opposite pattern was observed for down-regulated genes (Wilcoxon signed rank test, $P = 4.088e-07$; Fig. 1E). Genes repressed by both treatments (red dots) showed a stronger repression by the combined treatment (Wilcoxon signed rank test, $P < 2.2e-16$), whereas the opposite happened for genes exclusively repressed by anoxia (blue dots; Wilcoxon signed rank test, $P < 2.2e-16$).

Heat Pretreatment Dampens the Anaerobic Response

Heat pretreatment appears to dampen the induction of several anaerobic genes under anoxia (Fig. 2A). Examples of genes showing this pattern included *SUCROSE SYNTHASE1* (*SUS1*; *At5g20830*), *SUS4* (*At3g43190*), and *ADH* (*At1g77120*), as shown in Figure 2A, but not *PYRUVATE DECARBOXYLASE1* (*PDC1*; *At4g33070*; Supplemental Table S1). Real-time reverse transcription-PCR (qPCR) analysis confirmed the dampening effect of the heat pretreatment on the expression of anaerobic genes (Fig. 2B). Interestingly we also found reduced ADH activity and anaerobic

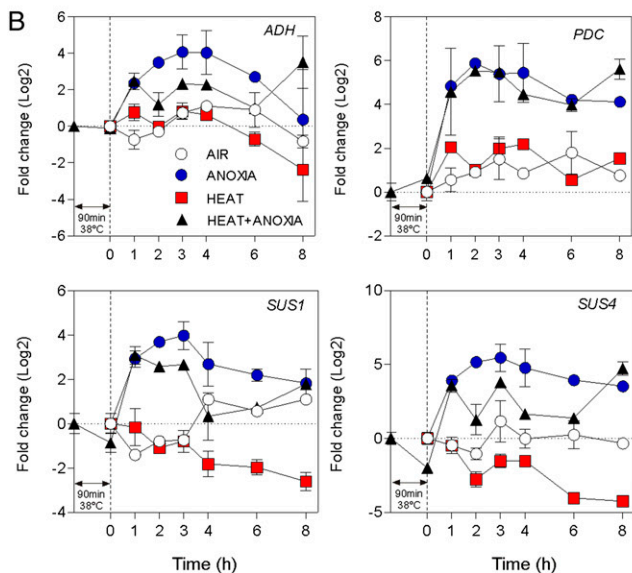
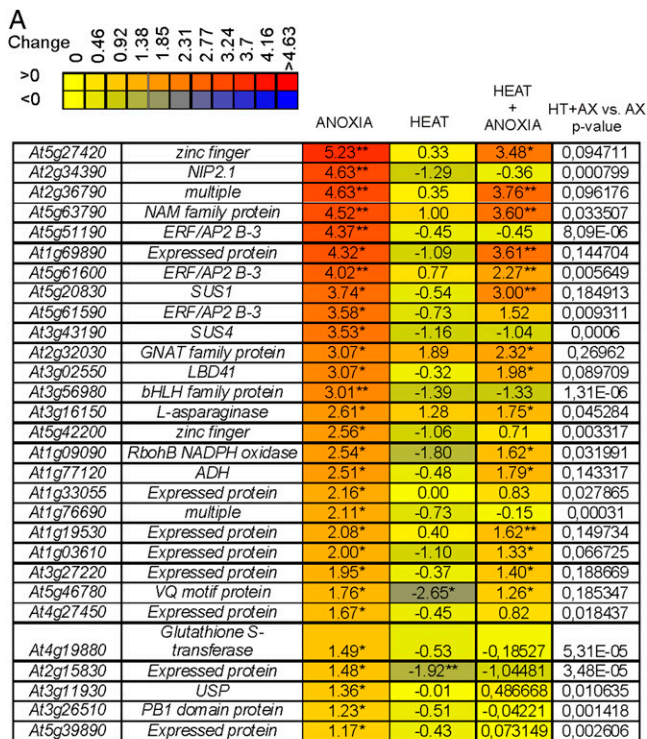


Figure 2. Effects of anoxia, heat, and combined heat + anoxia treatments on the expression of selected anaerobic genes. A, Heat map showing the expression of anaerobic genes showing a reduced expression in the combined heat + anoxia treatment. Expression data were visualized using Heatmapper Plus software (http://bbc.botany.utoronto.ca/ntools/cgibin/ntools_heatmapper_plus.cgi). The fold change is shown as \log_2 . Statistical significance for the comparison "treatment versus control (air, 23°C)" is reported: ** FDR \leq 0.01, * FDR \leq 0.05. The FDR P value for the comparison "combined heat + anoxia treatment versus anoxia" is shown in the table (HT + AX versus AX P value). B, Time courses of gene expression following anoxia, heat, and combined heat + anoxia treatments. The fold change (\log_2) was measured by qPCR using time = 0 (air) as a reference.

production of ethanol in the heat-pretreated samples (Supplemental Fig. S1). The increased expression of *ADH*, *SUS1*, *SUS4*, and *PDC* at the 8-h time point in acclimated plants (Fig. 2B, heat + anoxia), in comparison with nonacclimated plants, is probably due to the increased viability of the acclimated seedlings (Loreti et al., 2005; Banti et al., 2008).

HsfA2 Is Strongly Up-Regulated by Both Heat and Anoxia

To understand which transcriptional activator is involved in the induction of heat-related genes under anoxia, we searched our microarray data sets for abiotic stress-related transcription factors (Supplemental Fig. S2). We found several transcription factors strongly induced by both heat and anoxia, including *HsfA2*, the most highly induced transcription factor among the seven *Hsfs* up-regulated by anoxia (Fig. 3A). Interestingly, several putative *HsfA2* targets (Nishizawa et al., 2006; Schramm et al., 2006) are also induced by anoxia (Fig. 3B). Among them are several heat shock proteins and genes involved in oxidative stress responses, such as *Ascorbate Peroxidase2* (*APX2*) and *Galactinol Synthases* (*GolS*; Nishizawa et al., 2008). An analysis of the expression of *Hsp25.3-P*, *Hsp18.2-CI*, *APX2*, *GolS1*, and *Hsp101* over several hours of treatment under anoxia, heat, or heat + anoxia revealed that their expression pattern closely mirrored *HsfA2* (Fig. 3C).

HsfA2 Is Likely Up-Regulated by Heat- or Anoxia-Induced H_2O_2

HsfA2 and several *HSPs* are H_2O_2 -regulated genes in *Arabidopsis* (Vanderauwera et al., 2005). Both *HsfA2* and its target *Hsp25.3-P* are induced by an H_2O_2 treatment (Fig. 4A; Nishizawa et al., 2006). The production of H_2O_2 , therefore, may be the trigger for *HsfA2* and *HSP* expression following either heat or anoxic treatment (Blokhina et al., 2001; Volkov et al., 2006). H_2O_2 is quickly produced after the onset of both treatments (Fig. 4B), supporting the hypothesis of a mechanistic overlap in the physiological response to heat and anoxia. Interestingly, treating *Arabidopsis* seedlings with H_2O_2 results in enhanced anoxia tolerance (Fig. 4C).

HsfA2 Overexpression Confers Tolerance to Anoxia

HsfA2 is required for long-term acquired thermotolerance (Chang et al., 2007) and for salt and osmotic stress tolerance (Ogawa et al., 2007). To verify the role of *HsfA2* under anoxia, we isolated a knockout mutant for *HsfA2* (*hsfA2*) and produced an *HsfA2*-overexpressing line (*35S::HsfA2*). We tested the anoxia tolerance of *hsfA2*, *35S::HsfA2*, and the *hot1-3* mutant. *hot1-3* is deficient in *Hsp101* and negatively affected in both basal and acquired thermotolerance (Fig. 5A; Hong and Vierling, 2000; Queitsch et al., 2000). Interestingly,

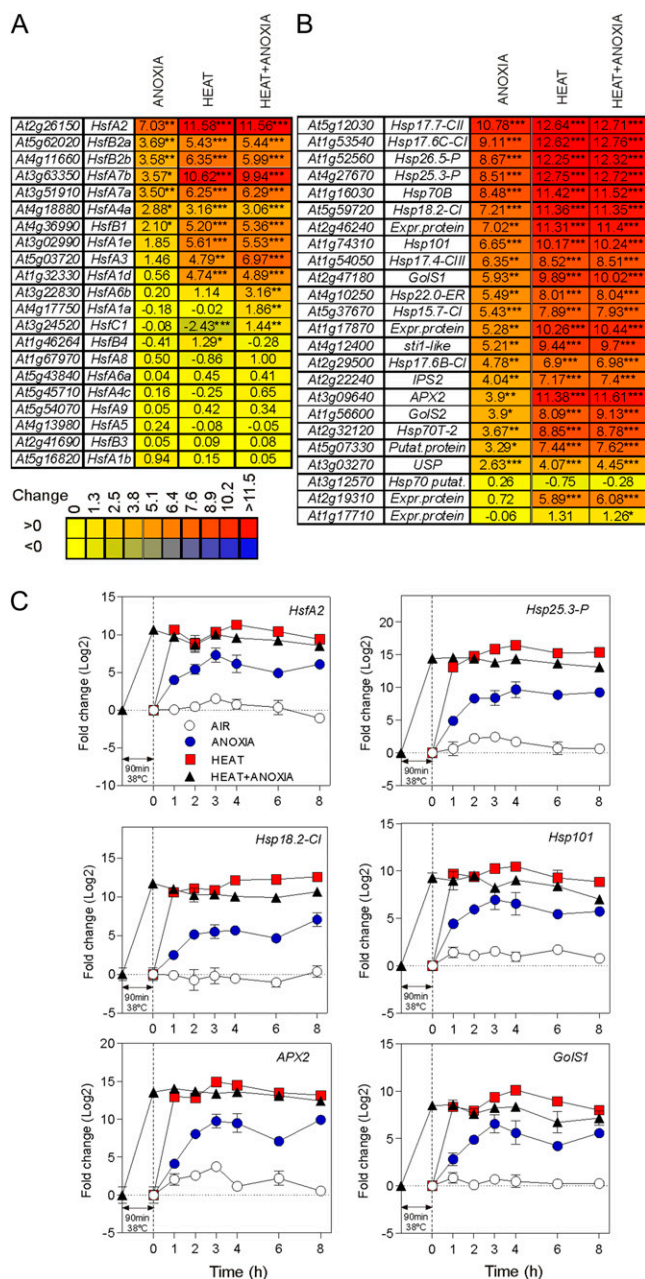


Figure 3. Effects of anoxia, heat, and combined heat + anoxia treatments on the expression of *Hsf*-related genes. **A**, Heat map showing the effects of anoxia, heat, and combined heat + anoxia treatments on the expression of *Hsf* genes. Expression data were visualized using Heatmapper Plus software (http://bbc.botany.utoronto.ca/ntools/cgibin/ntools_heatmapper_plus.cgi). The output of the software is shown. The fold change is shown as \log_2 . Statistical significance is reported: *** FDR \leq 0.001, ** FDR \leq 0.01, * FDR \leq 0.05. **B**, Heat map showing the effects of anoxia, heat, and combined heat + anoxia treatments on the expression of putative targets of *HsfA2*. Details are as in **A**. **C**, Time courses of gene expression following anoxia, heat, and combined heat + anoxia treatments. The fold change (\log_2) was measured by real-time qPCR using time = 0 (air) as a reference.

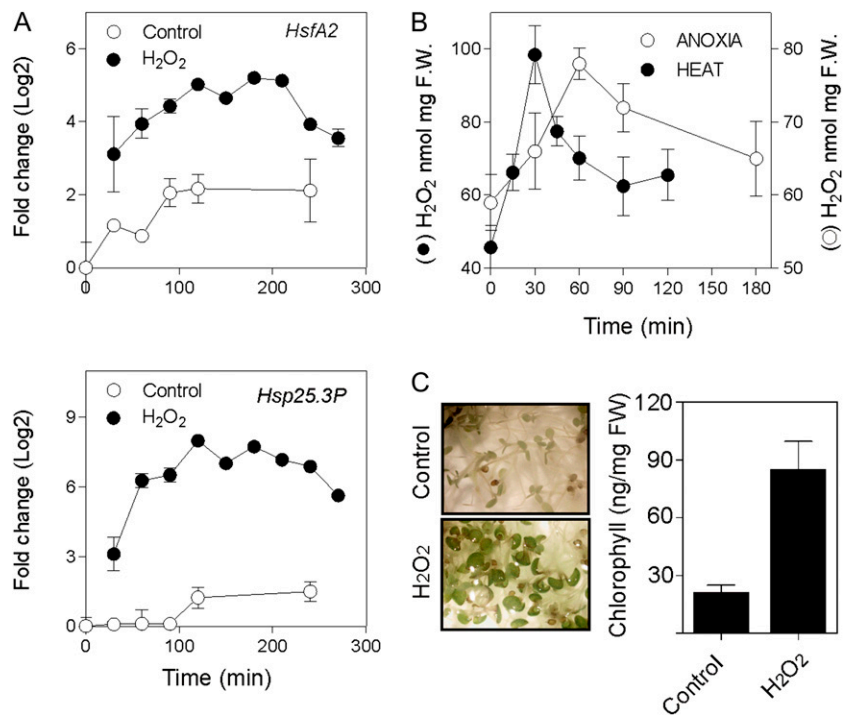
35S::HsfA2-overexpressing lines survived a 13-h-long anoxic treatment, while the wild-type (Columbia ecotype [Col-0]), *hsfA2*, and *hot1-3* seedlings died (Fig. 5A). *hsfA2* and *hot1-3* seedlings, however, were as tolerant as the wild type even when anoxia treatments were sublethal for the wild type (data not shown). *hsfA2* seedlings showed lower anoxia tolerance than the wild type when heat pretreated, but only after a long-term (2 d) recovery from the heat treatment followed by anoxia (Fig. 5A). This indicates that while both HsfA2 and Hot1 are required for acquired thermotolerance (Hong and Vierling, 2000; Queitsch et al., 2000), only HsfA2 is required for heat-induced anoxia tolerance. The tolerance of *35S::HsfA2* to anoxia is particularly evident when seedlings were grown on vertical plates. *35S::HsfA2* seedlings cannot be distinguished from the wild type before the treatment and grow normally in air (Fig. 5B, air before treatment). After anoxia only, *35S::HsfA2* seedlings recover and grow at a rate comparable to the aerobic seedlings, while the Col-0 seedlings display stunted growth (Fig. 5B, anoxia). Anoxia-treated *35S::HsfA2* seedlings produce more ethanol than Col-0 seedlings (Supplemental Fig. S3), a likely consequence of the ability of *35S::HsfA2* seedlings to better tolerate anoxia. Interestingly, 3-week-old *35S::HsfA2* pot-grown plants also showed better tolerance (survival, 100%; chlorophyll content, 220 ± 22 ng mg^{-1} fresh weight) to submergence when compared with Col-0 (survival, 50%; chlorophyll content, 150 ± 21 ng mg^{-1} fresh weight), *hsfA2* (survival, 40%; chlorophyll content, 145 ± 23 ng mg^{-1} fresh weight), and *hot1-3* (survival, 40%; chlorophyll content, 144 ± 24 ng mg^{-1} fresh weight; Fig. 5C).

The expression of most HsfA2-regulated genes appears also to be dependent on HsfA1a/HsfA1b (Busch et al., 2005). Although *HsfA1a/HsfA1b* are not induced by either heat or anoxia (Fig. 3A), these two HSFs appear to cooperate with HsfA2 in the heat response (Schramm et al., 2006). Therefore, we checked whether HsfA1a/HsfA1b are required for the heat-induced anoxia tolerance and found that the double mutant *hsfA1a/1b* displayed the inability to tolerate anoxia when heat pretreated (Fig. 5D).

HsfA2 Overexpression Up-Regulates Several Heat-Related Genes

We analyzed the expression of anaerobic and heat-related genes in the *35S::HsfA2* and *hsfA2* plants (Fig. 6A). As expected, *hsfA2* and *35S::HsfA2* lines showed lower and higher *HsfA2* mRNA levels, respectively. Interestingly, *35S::HsfA2* seedlings showed increased amounts of *HsfA2* mRNA following exposure to anoxia. This may be due to the endogenous transcripts, whose level increased following the onset of the stress. *ADH* and *SUS4* are induced by anoxia, irrespective of the genotype. A higher expression of both *ADH* and *SUS4* in the *35S::HsfA2* seedlings is likely due to the higher anoxia tolerance of this genotype, preventing the decline in expression of these genes toward the end

Figure 4. Effects of H_2O_2 on the expression of *HsfA2* and *Hsp25.3P*. A, Expression of *HsfA2* and *Hsp25.3P* following treatment with 5 mM H_2O_2 for the times indicated. Seedlings were grown in liquid medium for 4 d in the dark. H_2O_2 (5 mM) was added for the time shown. The fold change (\log_2) was measured by qPCR using time = 0 as a reference. B, H_2O_2 production under anoxia and heat (38°C). C, Effects of 5 mM H_2O_2 on anoxia tolerance. Seedlings were grown in liquid medium for 4 d in the dark. H_2O_2 (5 mM) was added (2-h treatment) to the wells containing the seedlings followed by rinsing in fresh medium and transfer to anoxia (26 h). The photographs show the seedlings recovering (5 d) after the anoxic treatment. The chlorophyll content in replicated ($n = 3$) samples is shown in the histogram. FW, Fresh weight.



of the anoxic treatment. *Hsp25.3-P*, *Hsp18.2-CI*, *APX2*, and *GolS1* were clearly up-regulated in *35S::HsfA2* seedlings, both in air and, mirroring *HsfA2* patterns, also 6 h after the onset of anoxia (Fig. 6A). *Hsp101* did not appear to be up-regulated in the *35S::HsfA2* line. The absence of a significant reduction in the anoxic expression of *HsfA2* targets in the knockout line suggests that other *Hsfs*, besides *HsfA2*, might be involved in the regulation of these genes under anoxia.

HSPs Are Present in the Anoxic *35S::HsfA2* Seedlings But Not in the Wild Type

Translational efficiency under anoxia can be greatly impaired, a likely consequence of a low ATP content and posttranscriptional regulation mechanisms (Branco-Price et al., 2005, 2008). This may result in a lack of correlation between the mRNA and corresponding protein levels. To verify whether the increased *HSP* mRNA levels resulted in correspondingly increased protein levels, we checked three anoxia-induced HSPs (small HSP class I [sHSP-CI], *Hsp25.3-P*, and *Hsp101*) by immunoblotting. The results showed that heat and heat + anoxia strongly increase these HSP protein levels (Fig. 6B). Under anoxia, the wild-type seedlings do not appear to accumulate HSPs. We only managed to detect faint sHSP-CI and *Hsp25.3-P* signals in Col-0 after 4 h under anoxia (Fig. 6B). Despite the increased HSP mRNA steady state in *35S::HsfA2* (Fig. 6A), these plants did not accumulate the corresponding proteins under aerobic conditions (Fig. 6B). However, when transferred to anoxia, *35S::HsfA2* seedlings showed a clear increase in the levels

of sHSP-CI and *Hsp25.3-P* (Fig. 6B), while only a slight increase in *Hsp101* was observed. Therefore, HSPs do not accumulate under anoxia but are produced in the anoxic *35S::HsfA2* plants.

The level of sHSP-CI and *Hsp25.3-P* proteins is high in heat-pretreated seedlings transferred to anoxia, while it is low in anoxia-treated seedlings (Fig. 6B). This suggests that under anoxia, HSPs fail to accumulate despite the presence of high levels of the corresponding transcripts. We tested whether anoxia negatively affects HSPs transcripts and protein stabilities using actinomycin D and cycloheximide (Supplemental Fig. S4, A and B). The results showed that the *Hsp18.2-CI* and *Hsp25.3-P* mRNAs induced by a heat treatment are degraded at a slower rate under anoxia when compared with the effects of an aerobic incubation following the heat treatment (Supplemental Fig. S4C). Anoxia, however, enhances HSP degradation through a mechanism requiring de novo protein synthesis, since cycloheximide stabilizes HSPs under anoxia (Supplemental Fig. S4D). We then compared the effects of a heat treatment applied before anoxia or “during anoxia” to evaluate their consequences of HSP protein accumulation (Fig. 7A). Seedlings treated at 38°C before a 4.5-h anoxia treatment (heat + anoxia) accumulate HSP mRNAs at a level comparable to that of the heat-only treatment (Fig. 7B). The level of HSP proteins is only slightly lower in the heat + anoxia samples when compared with the heat-only treatment (Fig. 7C). When the heat treatment is applied during anoxia (anoxia + heat), the level of HSP mRNAs is higher than under anoxia only, showing the additive effect of the two stress treatments and indicating that

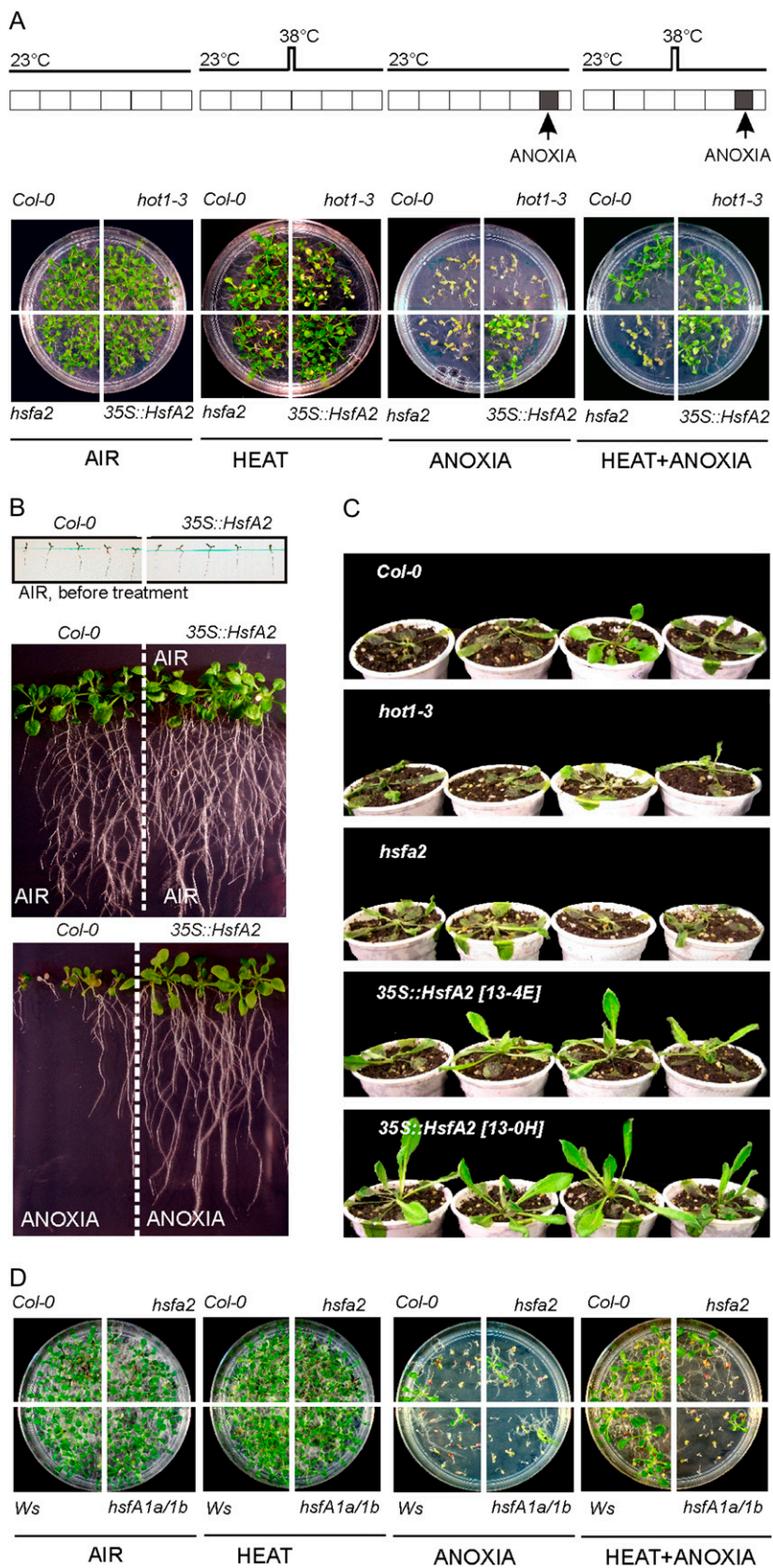


Figure 5. Role of HsfA2 in anoxia tolerance. **A**, Tolerance assay in Col-0, *hot1-3*, *hsfA2*, and *35S::HsfA2*. A schematic representation of treatments is shown. Each box represents 1 d. The gray box represents the anoxic treatment (13 h). Photographs were taken 1 week after the end of the treatments. The results shown refer to *35S::HsfA2* line 13-OH. Comparable results were obtained using the independent *35S::HsfA2* line 13-4E. **B**, Tolerance assay in Col-0 and *35S::HsfA2* seedlings grown on vertical plates. The anoxic treatment was 13 h long. Photographs were taken before treatment and 2 weeks after the end of the anoxic treatment. The results shown refer to *35S::HsfA2* line 13-OH. Comparable results were obtained using the independent *35S::HsfA2* line 13-4E. **C**, Tolerance assay in Col-0, *hot1-3*, *hsfA2*, and *35S::HsfA2* plants grown in pots and subjected to 24 h of complete submergence in the dark. The results shown refer to both *35S::HsfA2* lines 13-OH and 13-4E. **D**, Tolerance assay in *hsfA2*, its wild type (Col-0), the double mutant *hsfA1a/1b*, and its wild type (Wassilewskija [Ws]). A schematic representation of the treatments is shown in A. Photographs were taken 1 week after the end of the treatments.

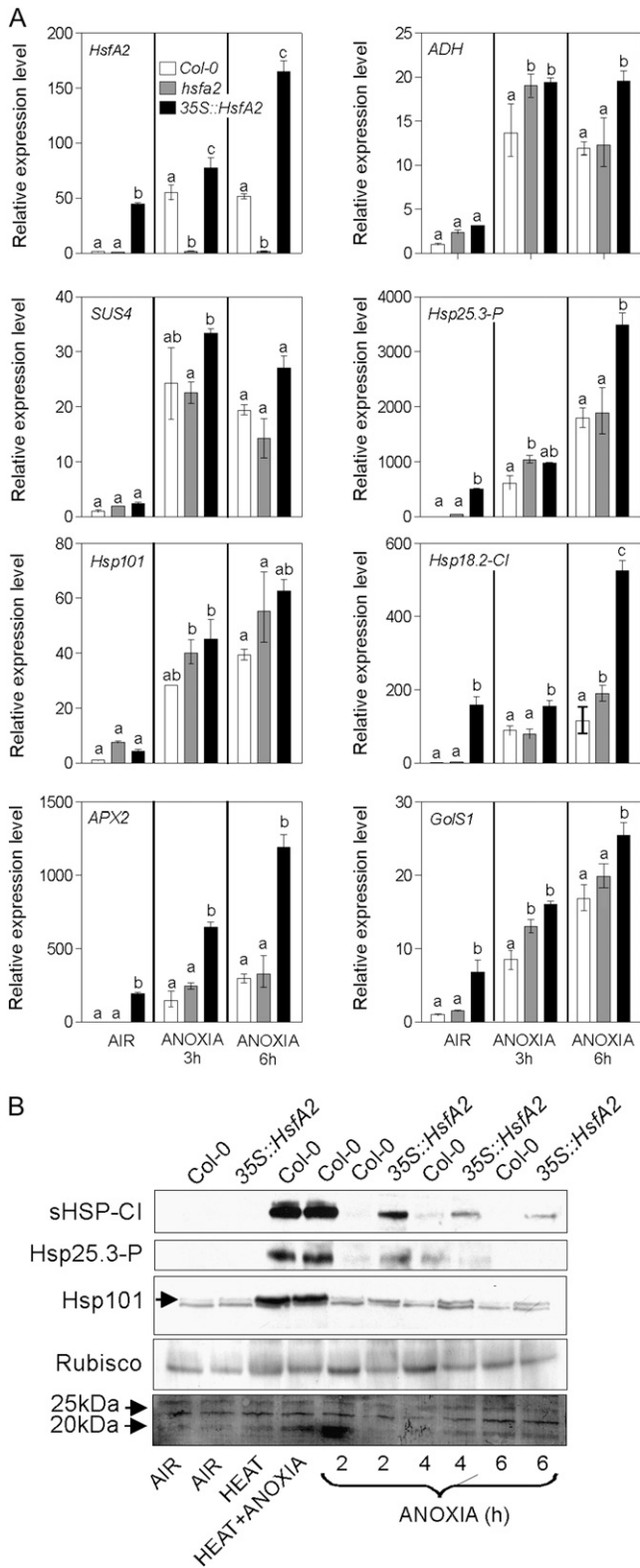


Figure 6. Effects of HsfA2 on the expression of anaerobic and heat-related genes and proteins. A, Expression of HsfA2, ADH, SUS4, HSP25.3-P, HSP101, HSP18.2-Cl, APX2, and GolS1 in Col-0, *hsfA2*, and *35S::HsfA2* seedlings. Relative expression levels are shown as fold change values (1 = Col-0, air). Differences among genotypes within

anoxia does not interfere with the triggering effect of heat at the mRNA level (Fig. 7B). However, HSP proteins fail to accumulate at significant levels (anoxia + heat; Fig. 7C), indicating that, despite a mRNA level comparable to that of the heat treatment (compare heat with anoxia + heat; Fig. 7A), the HSP proteins fail to accumulate (Fig. 7B). The level of HSP proteins in the heat + anoxia treatment (heat treatment before anoxia) is only slightly lower than in the heat-only sample, suggesting that 4.5 h under anoxia had a moderate effect on HSP protein stability. On the other hand, a heat treatment on seedlings that already experienced 4.5 h under anoxia (heat treatment during anoxia) was only effective at the mRNA level (Fig. 7B) but produced a modest HSP protein accumulation (Fig. 7C).

DISCUSSION

Mild heat treatment results in acquired anoxia tolerance (Loreti et al., 2005). The molecular basis of this phenomenon is unknown, and there are several possible hypotheses. First, heat may induce several stress-related transcription factors, thus activating a large number of signaling pathways conferring tolerance to anoxia. A second possibility is that heat pretreatment enhances tolerance by dampening the anoxic responses of the seedlings, allowing them to enter into a quiescent state preserving cellular viability. Third, the increased tolerance could be due to the activation of a functional overlap between the heat and the anoxia acclimation mechanisms.

The first hypothesis is reasonable, since we observed a strong induction of the genes belonging, for example, to the ERF, MYB, WRKY, NAC, and HSF families (Supplemental Fig. S2). However, none of the transcription factors listed in Supplemental Table S1 is known to positively affect anoxia tolerance. The dampening of the anoxic response as part of a “low oxygen quiescence syndrome” (Bailey-Serres and Voesenek, 2008) is also feasible, since heat pretreatment reduces the expression of several anaerobic genes (Fig. 2) and also slows down the ethanol production (Supplemental Fig. S1). However, the higher production of ethanol observed in the *35S::HsfA2* seedlings (Supplemental Fig. S3), which were also more tolerant to anoxia (Fig. 5A), suggests that the dampening effect of heat over the anaerobic response may simply reflect the hierarchies of stress response regulatory systems.

each treatment (air, 3 h of anoxia, and 6 h of anoxia) were evaluated by two-way ANOVA (Bonferroni posttest $P < 0.05$). Bars represent means of at least three measurements \pm SD. B, Immunoblotting of proteins extracted from 4-d-old dark-grown seedlings treated under anoxia. The antibodies used recognized HSP25.3-P, sHSP-Cl, and HSP101 (the specific bands are indicated by the arrows). Blots were stained with Ponceau-S and probed with an antibody recognizing the large subunit of Rubisco to confirm even loading and transfer. Treatments were as follows: air, heat (90 min at 38°C), heat + anoxia (90 min at 38°C followed by 6 h under anoxia), and anoxia (2–6 h).

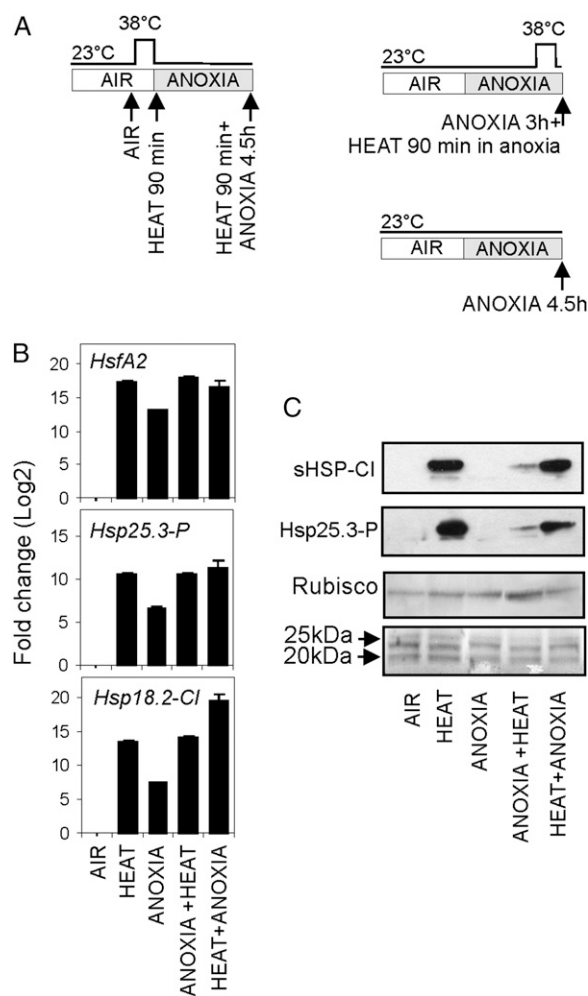


Figure 7. Effects of anoxia on HSP mRNA and protein accumulation. A, Schematic representation of treatments. B, Expression of *HsfA2*, *Hsp25.3-P*, and *Hsp18.2-CI* in Col-0 seedlings. Relative expression levels are shown as fold change values (\log_2 ; using "air" as a reference). C, Immunoblotting of proteins extracted from 4-d-old dark-grown seedlings treated under anoxia. The antibodies used recognized *Hsp25.3-P* and sHSP-Cl. Blots were stained with Ponceau-S and reprobed with an antibody recognizing the large subunit of Rubisco to confirm even loading and transfer.

However, the activation of a heat-dependent anoxia acclimation mechanism(s) that shares similarities with acquired thermotolerance pathways would seem to be possible, so we turned our attention to *HsfA2* (Fig. 3). *HsfA2* plays an important role in acquired thermotolerance and also appears to play a role in abiotic stress tolerance. Indeed, some studies have reported the role of *HsfA2* in oxidative stress (Li et al., 2005), high light conditions and chilling (Nishizawa et al., 2006, 2008), and salt/osmotic stress tolerance, suggesting a role in multiple stress tolerance (Ogawa et al., 2007). The results obtained through a genetic approach demonstrate that the *hsfA2* mutant cannot cross-acclimate to anoxia through mild heat pretreatment, in contrast to the wild type (Fig. 5). Assuming that *HsfA2* is impor-

tant for anoxia tolerance, we would also have expected a lower anoxia tolerance in the *hsfA2* plants when a sublethal anoxic treatment was applied, but this was not the case (data not shown). However, expression of *HsfA2* targets in *hsfA2* explains this result and is most likely due to possible redundancy among the 21 Arabidopsis HSFs (Lohmann et al., 2004). Seven *Hsfs* (Fig. 3A) were induced under anoxia and may have compensated for the absence of *HsfA2* in the *hsfA2* mutant. Differences between the wild type and *hsfA2* in heat-dependent acquired anoxia tolerance were observed only after a long (2-d) recovery from the heat treatment, in line with the role of *HsfA2* in acquired thermotolerance (Charng et al., 2007). It has been demonstrated that only after a long-term recovery from heat pretreatment is it possible to observe differences both in heat tolerance and expression of *HsfA2*-dependent HSPs, such as *Hsp25.3-P* (Charng et al., 2007). Additionally, as previously suggested for heat stress (Schramm et al., 2006), *HsfA2* appears to cooperate with *HsfA1a/1b* in the mechanism of heat-induced anoxia tolerance (Fig. 5D). Both *HsfA2* and *HsfA1a/1b* are indeed involved in the mechanism of acquired thermotolerance (Wunderlich et al., 2003; Charng et al., 2007).

35S::HsfA2 seedlings and plants showed enhanced tolerance to anoxia and to submergence, respectively (Fig. 5). The enhanced anoxia tolerance could be explained either as a real tolerance to the lack of oxygen or a better ability to recover during the post-anoxic phase. The results obtained with the submergence experiments, which showed healthier *35S::HsfA2* plants during and immediately after the end of the submergence (Fig. 5C), suggest that *HsfA2* overexpression protects the plants during the stress treatment rather than from the consequences of reaeration. These results demonstrate that *HsfA2* is not only responsible for the heat-dependent acclimation to anoxia but also plays a role in the low oxygen-tolerance response in Arabidopsis.

HsfA2 is the most H_2O_2 -responsive *Hsf* (Miller and Mittler, 2006). It is likely that H_2O_2 is the trigger that induces *HsfA2* under anoxia, since H_2O_2 is produced at the onset of anoxia (Fig. 4B). Exogenous H_2O_2 treatment induces *HsfA2* and confers anoxia tolerance (Fig. 4, A and C). Signaling by reactive oxygen species under oxygen deprivation (Bailey-Serres and Chang, 2005) and protection from oxidative stress are important under anoxia (Blokhina et al., 2002). HSPs themselves may be involved in protecting the cell from oxidative stress (Timperio et al., 2008). *GolS1* is induced by *HsfA2* and is important for plant acclimation to oxidative stress (Nishizawa et al., 2008). Galactinol, produced by *GolS*, may well play a role under anoxia, as de novo galactinol biosynthesis from Suc would be required, and Suc is known to enhance Arabidopsis tolerance to low oxygen (Banti et al., 2008). Genes regulated by *HsfA2* include several sHSPs (Nishizawa et al., 2006; Schramm et al., 2006). sHSPs are overrepresented under several stress conditions, underlining

the putative importance of their ATP-independent role in stabilizing proteins and membranes undergoing conformational disruption (Swindell et al., 2007). This ATP-independent function may be particularly important under anoxic conditions, since it reduces the energy-dependent de novo protein synthesis.

Despite the anoxic induction of *HsfA2* at levels close to those observed after heat treatment (Fig. 3), Arabidopsis seedlings are highly susceptible to anoxia. Heat-pretreated or *35S::HsfA2* plants, on the other hand, are highly tolerant to anoxia. This can be explained by the failure of wild-type plants to induce the *HsfA2* targets at the protein level (Fig. 6B). Indeed, while *HsfA2* target mRNAs are induced under either heat or anoxia, we only detected a faint protein band corresponding to sHSP-CI and Hsp25.3-P in the 4-h anoxia-treated Col-0 plants (Fig. 6B). Plants able to withstand anoxic treatment (heat pretreated or overexpressing *HsfA2*), on the other hand, showed HSP accumulation under anoxia (Fig. 6B). The lack of expression of HSPs at the protein level in the anoxic samples is somewhat surprising, since HSP mRNAs are loaded onto polyribosomes under low oxygen (Branco-Price et al., 2008). This suggests that the translation of HSP mRNA is controlled by a complex regulatory mechanism. This hypothesis is reinforced by the evidence of a lack of HSP protein accumulation even in the *35S::HsfA2* aerobic seedlings, despite the strong expression of the respective HSP mRNAs. The HSP proteins only accumulated when the *35S::HsfA2* plants were exposed to anoxia, indicating that a "stress" signal is required for the translation of the HSP mRNAs. The failure to induce HSP accumulation under anoxia in wild-type plants is possibly due to the slower timing of the *HsfA2* expression and its targets under anoxia compared with the expression following heat and heat + anoxia (Fig. 3C). In the *35S::HsfA2* plants, *HsfA2* and its target mRNAs are already present in air, which is likely to lead to their rapid translation as soon as anoxia is established, before the ability to produce and accumulate HSP proteins declines (Fig. 6B).

In this study, we demonstrated the importance of *HsfA2* in protecting Arabidopsis plants under low-oxygen conditions. The overexpression of *HsfA2* transactivates its target genes early enough to enable them to be translated even under anoxic conditions. It is likely that both HSPs and genes involved in the protection against oxidative stress are responsible for the acquisition of anoxia tolerance. These results indicate that there is a considerable overlap between the molecular mechanisms of heat and anoxia tolerance and that *HsfA2* is a major player in these mechanisms.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0, its *hot1-3* mutant (kindly provided by Dr. Elizabeth Vierling), *HsfA2* (*At2g26150*) T-DNA insertion line

SALK_008978 (obtained from the Nottingham Arabidopsis Stock Centre; code N508978), the double mutant *hsfA1a/HsfA1b* (abbreviated *hsfA1a1b*; kindly provided by Dr. Friedrich Schöffl), and the *hsfA1a1b* wild type (Wassilewskija) were used in this study. Transgenic plants overexpressing *HsfA2* (*35S::HsfA2*) were obtained by infiltration with the floral dip method (Clough and Bent, 1998). Cloning and construction of the expression cassette for *HsfA2* were performed using Gateway technology (Karimi et al., 2002; Invitrogen). For gene expression experiments, seeds were sterilized with diluted bleach (10 min of incubation in 1.7% sodium hypochlorite, rinsed, and washed seven times in sterile water) and incubated in 2.5 mL of liquid growing medium (Murashige and Skoog half-strength solution on six-well plates) containing 90 mM Suc. The plates were incubated in the dark at 4°C for 2 d (vernalization, to promote homogeneous germination) and then transferred to 23°C for 4 d in the dark before the treatment. Anoxic treatments were carried out in the dark. An enclosed anaerobic workstation (Anaerobic System model 1025; Forma Scientific) was used to provide an oxygen-free environment for seedling incubation. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis to less than 10 $\mu\text{g mL}^{-1}$ oxygen (according to the manufacturer's specifications). High-purity N_2 was used to initially purge the chamber, and the working anaerobic gas mixture was $\text{N}_2\text{:H}_2$ with a ratio of 90:10. Three independent, replicated experiments were performed for each experimental condition. Each independent experiment consisted of four replicated seedling cultures pooled before RNA extraction. Agar plates were used to evaluate anoxia tolerance (Fig. 5). They were prepared using a solid growing medium (Murashige and Skoog half-strength solution containing 1% agar and 90 mM Suc). The plates were then transferred to the light for the postanoxic recovery (14-h/10-h photoperiod at 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Treatments were performed by transferring the plates containing the 7-d-old (vertical plates) or 5-d-old (horizontal plates) Arabidopsis seedlings to 38°C for 90 min immediately before the anoxic treatments. The submergence treatments shown in Figure 5C were performed using 14-d-old Arabidopsis plants grown in pots and subjected to 24-h complete submergence in the dark. The tolerance experiment reported in Figure 4 (effect of H_2O_2 treatment of tolerance) was performed using Arabidopsis seedlings (dark germinated, 4 d old) grown in liquid growing medium (Murashige and Skoog half-strength solution on six-well plates) containing 90 mM Suc. H_2O_2 (5 mM) was added 2 h before the anoxia treatment. Seedlings were rinsed in fresh Murashige and Skoog half-strength solution to remove H_2O_2 immediately before the anoxia treatment. All the treatments started at 9 AM.

qPCR

RNA was extracted from the whole seedlings. The total RNA was extracted with an RNAqueous kit (Ambion) according to the manufacturer's instructions and then subjected to a DNase treatment using a TURBO DNA-free kit (Ambion). Two micrograms of each sample were reverse transcribed into cDNA with a high-capacity cDNA archive kit (Applied Biosystems). qPCR amplification was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems) using *Ubiquitin10* (*At4g05320*) as an endogenous control. SYBR Green probes for each gene were used. The primers used are reported in Supplemental Table S3. PCR was carried out using 50 ng of cDNA and SYBR PCR master mix (Applied Biosystems) following the manufacturer's protocol. Relative quantitation of each single gene expression was performed using the comparative threshold cycle method, as described in the ABI PRISM 7700 Sequence Detection System User Bulletin No. 2 (Applied Biosystems).

RNA Isolation, Copy RNA Synthesis, and Hybridization to Affymetrix GeneChips

Total RNA was extracted from seedling samples using an Ambion RNAqueous kit (Ambion). Two biological replicates for each experimental condition were used. RNA quality was assessed by agarose gel electrophoresis and spectrophotometry. RNA was processed for use on Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays as described previously (Loreti et al., 2005). Hybridization, washing, staining, and scanning procedures were performed by Genopolis (University of Milano-Bicocca), as described in the Affymetrix technical manual. Microarray analysis was performed using R/Bioconductor (Gentleman et al., 2004). Expression measures were obtained using GeneChip Robust Multi-Array (Wu and Irizarry, 2005), a multiarray

analysis method that estimates probe set signals taking into account the physical affinities between probes and targets. Normalization was carried out using a quantile method (Bolstad et al., 2003). To reduce the number of noninformative genes, two different filters were applied: the first removes the probe sets presenting an Affymetrix absent call for both conditions; the second eliminates the probe sets showing, as the maximum signal in the two conditions, a value of less than or equal to the 95th percentile of the overall absent call signal distribution. To identify a statistically reliable number of differentially expressed genes between the two conditions, a linear model was used (Wettenhall and Smyth, 2004). To assess the differential expression, an empirical Bayesian method (Smyth, 2004) was used to moderate the SE of the estimated log-fold changes. To control *P* values in a multiple testing problem context, a Benjamini-Hochberg correction of the FDR (Reiner et al., 2003) was applied (adjusted $P \leq 0.05$), leading to a list of differentially expressed probe sets. Microarray data sets were deposited in a public repository with open access (accession no. GSE16222; <http://www.ncbi.nlm.nih.gov/projects/geo>). The differentially expressed genes were selected by applying a filter that considers both the fold change (≥ 2 or ≤ -2) in a hybridization signal (namely the ratio between the intensity in the treated spot versus the control) and the FDR (≤ 0.05). Scatterplots and the statistical analyses were performed using R and BRB array software for a multidimensional scaling comparison. In order to build gene expression maps, MapMan software (Thimm et al., 2004) from <http://gabi.rzpd.de/projects/MapMan/> was used. Heat shock-related genes were used as defined by MapManx BINs. For anoxia custom maps, a consensus of up-regulated genes (≥ 2 -fold) from Loreti et al. (2005), Branco-Price et al. (2005, 2008), and this study (Supplemental Table S4) was used.

H₂O₂ Quantitation

Liquid-grown whole seedlings were sampled immediately after treatment and homogenized in a HEPES buffer, pH 7.5, 1 mM EDTA. The resulting extract was then centrifuged for 10 min at 14,000g at 4°C. A 2:1 volume of chloroform:methanol was added to the supernatant. The mixture was vortexed and subsequently centrifuged for 3 min at 10,000g at 4°C. The aqueous phase was then used for the analyses. H₂O₂ abundance was quantified according to Guilbault et al. (1967) and the subsequent modification by Muhlenbock et al. (2007), in a reaction volume of 3 mL containing 50 mM HEPES, pH 7.5, 50 mM homovanilic acid, 4 μ M peroxidase, and an aliquot of extract. Fluorescence was then measured at 425 nm after excitation at 315 nm, 30 min after the beginning of the reaction. Samples were kept strictly in the dark. H₂O₂ was determined as a limiting factor in the oxidation mediated by homovanilic acid, calculated on the basis of the standard curve obtained with different amounts of H₂O₂.

ADH Assay

ADH activity was assayed after anoxia (6 h), heat (90 min), and heat (90 min) followed by anoxia (6 h) in 4-d-old dark-grown seedlings. Soluble proteins were extracted using 100 mM HEPES buffer, pH 7.0, and 5 mM dithiothreitol. Protein amount was estimated using the Bio-Rad Protein Assay, based on the method of Bradford (1976). ADH activity was monitored using the spectrophotometric assay (A₃₄₀) as described by Russell et al. (1990).

Ethanol Assay

The seedlings used for the ethanol production experiment were grown in liquid medium contained in petri dishes sealed with Parafilm. Ethanol evaporation under these conditions was negligible. Samples were assayed through coupled enzymatic assay methods, measuring the increase in A₃₄₀ as described previously (Banti et al., 2008).

Chlorophyll Assay

Chlorophyll quantitation was performed on plant material that was frozen immediately after the treatments. The plant material was weighed, ground in liquid nitrogen, and extracted with 80% (v/v) acetone. Chlorophyll was quantified photometrically as described by Lichtenthaler (1987) and Porra (2002).

Immunoblotting

Samples (about 500 mg) were ground in liquid nitrogen. An extraction buffer, described by Siddique et al. (2008), was added in a 1:2 ratio (plant tissue:buffer). Total protein content was quantified with a BCA Protein Assay (Pierce). SDS-PAGE was performed on a 10% Criterion polyacrylamide gel (Bio-Rad Laboratories). Blotting on an Amersham Hybond-P polyvinylidene difluoride membrane was performed with a Novablot electrophoretic transfer system (Amersham Pharmacia Biotech). Immunoblotting was performed using Immun-Star HRP Chemiluminescent Detection Kits (Bio-Rad Laboratories). Antibodies against Hsp101, Hsp25.3-P, and sHSP-CI were purchased from Agrisera (product codes AS07 253, AS08 285, and AS07 254, respectively). Ponceau-S staining and immunoblotting using the antibody against the large subunit of Rubisco (AS03037) were used to confirm even loading and transfer (Agrisera).

Microarray data from this article were submitted to the public National Center for Biotechnology Information Gene Expression Omnibus database (GEO accession no. GSE16222).

Supplemental Data

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

Supplemental Figure S1. Effects of anoxia, heat, and combined heat + anoxia treatments on ADH activity and ethanol production.

Supplemental Figure S2. Heat map of transcription factors strongly up-regulated under anoxia.

Supplemental Figure S3. Ethanol production in anoxic Col-0 and 35S::HsfA2 seedlings.

Supplemental Figure S4. Stability of HSP transcripts and proteins under aerobic and anoxic conditions.

Supplemental Table S1. List of up-regulated genes.

Supplemental Table S2. List of down-regulated genes.

Supplemental Table S3. List of primers used in this work.

Supplemental Table S4. MapMan mapping file for anoxia genes reported in Figure 1C.

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