The Transcription Factor ABI4 Is a Regulator of Mitochondrial Retrograde Expression of *ALTERNATIVE OXIDASE1a*[1][C][W][OA]

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Plant cells integrate signals from external sources and from organelles to regulate gene expression, referred to as anterograde and retrograde signaling, respectively. Functional characterization of the promoter of *ALTERNATIVE OXIDASE1a* (*AOX1a*) from Arabidopsis (*Arabidopsis thaliana*), a marker for mitochondrial retrograde response, was carried out by testing the ability of the *AOX1a* promoter to drive expression of the reporter gene GUS. This approach identified a strong repressor element, designated the B element, that was necessary for an increased promoter activity in response to the mitochondrial complex I inhibitor rotenone. This element overlaps with a previously identified potential binding site for the transcription factor ABSCISIC ACID INSENSITIVE4 (ABI4). *AOX1a* promoter activity was fully derepressed in *abi4* mutants and was unresponsive to rotenone. Furthermore, deletion of the B element of the *AOX1a* promoter resulted in increased GUS staining activity compared to the wild-type promoter in transgenic plants. Binding of the ABI4 transcription factor to this region of the *AOX1a* promoter was demonstrated by electromobility shift and yeast one-hybrid assays. Analysis of transcript abundance for *AOX1a* in *abi4* mutant lines revealed significantly increased levels of *AOX1a* mRNA that could not be further induced by rotenone, consistent with the role of ABI4 as a repressor that is derepressed in response to rotenone. These results show that ABI4 plays a central role in mediating mitochondrial retrograde signals to induce the expression of *AOX1a*. Furthermore, they provide a molecular link between mitochondrial and chloroplast retrograde signaling, as ABI4 has been previously shown to act downstream of at least two chloroplast retrograde signaling pathways.

The alternative oxidase (AOX) is a cyanide-insensitive terminal oxidase found in all plants studied to date (Vanlerberghe and McIntosh, 1997). Although the activity was known to exist more than 100 years ago (for review, see Moore and Siedow, 1991), research into the nature of this activity was greatly facilitated by the development of a monoclonal antibody that recognized AOX from a wide variety of species (Elthon et al., 1989) and cloning of the cDNA from *Sauromatum guttatum* (Rhoads and McIntosh, 1991) that facilitated the cloning of cDNA from a variety of other plant species. In higher plants, AOX is encoded by a small gene family, with the number of genes varying between species. AOX has been studied at an activity, protein, and gene level (Finnegan et al., 2004), and these studies reveal that its expression at an mRNA level is induced by a variety of treatments that are commonly referred to as stresses. Expression in a variety of organs at various developmental stages is also observed. At a protein level, AOX in plants can exist as an oxidized dimer (inactive) via intermolecular disulphide cross-linking of conserved Cys residues, and pyruvate and a variety of other α-keto acids act as allosteric activators (Umbach et al., 1994). However, the in vivo implications of these potential posttranscriptional regulatory mechanisms are still unclear.

In addition to the fact that AOX represents a biochemical feature of the plant mitochondrial electron transport chain (mETC), it is also the preeminent model for mitochondrial retrograde regulation in plants (Rhoads and Subbaiah, 2007). This is because a variety of signals, either chemical or genetic, that change or inhibit mitochondrial function stimulate expression of *AOX*. The regulation of gene expression encoding mitochondrial proteins can be broadly divided into anterograde and retrograde regulatory pathways (Rodermel, 2001; Leister, 2005; Woodson and Chory, 2008). Anterograde regulation describes endogenous or external signals that stimulate or control gene expression and is essentially a top-down pathway from the nucleus to the organelle. Retrograde regulation describes the ability of organelles, or rather signals originating in organelles, to modulate nuclear gene

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expression, the products of which may or may not be located within the same organelle, i.e. a bottom-up pathway (Leister, 2005; Liu and Butow, 2006). Anterograde regulation can take place at several levels, from transcriptional to posttranslational events, and the signals that control gene expression for this type of signaling can be developmental, hormonal, or environmental (Rodermel, 2001; Leister, 2005; Nott et al., 2006). In contrast, retrograde regulation occurs in response to changing conditions and allows the cells to modulate anterograde regulatory pathways to affect the functioning of organelles (Butow and Avadhani, 2004; Pesaresi et al., 2007).

In chloroplasts, four distinct retrograde signals have been characterized: signals resulting from the inhibition of plastid gene expression, Mg-protoporphyrin IX (Mg-proto) accumulation, chloroplast-generated reactive oxygen species, and REDOX signals from the photosynthetic electron transport chain (Butow and Avadhani, 2004; Pesaresi et al., 2007; Woodson and Chory, 2008). The plastid gene expression and Mg-proto pathways may converge in the chloroplast, acting upstream of *genomes uncoupled1* (*gun1*). Recently, ABSCISIC ACID INSENSITIVE4 (ABI4), a nuclear-localized APETALA2-type transcription factor, was found to act downstream of both these pathways through binding of a CCAC cis-acting regulatory element (Koussevitzky et al., 2007; Woodson and Chory, 2008). As ABI4 was originally characterized to play a role in abscisic acid (ABA) signaling (Finkelstein et al., 1998) and is involved in both sugar and developmental signaling (Rook et al., 2006), it can act as a point of convergence for both retrograde and anterograde signals.

In yeast, mitochondrial dysfunction leads to activation of a retrograde pathway where a variety of genes are activated by binding of the retrograde regulatory factors 1 and 3 to an R box (GTCAC) in their promoters (Liu and Butow, 1999). The induction of *AOX* is commonly used as a marker for the mitochondrial retrograde response in plants. Treatments that induce *AOX* include inhibition of the mETC with various inhibitors, such as antimycin A or rotenone (Vanlerberghe and McIntosh, 1997; Clifton et al., 2005), and mutations that alter mitochondrial function at the level of the mETC, such as mitochondrially encoded subunits of complex I and complex IV in tobacco (*Nicotiana tabacum*) and maize (*Zea mays*; Gutierres et al., 1997; Karpova et al., 2002; Dutilleul et al., 2003). Although mutants in the retrograde induction of *AOX* expression have been described (Zarkovic et al., 2005) and a 93-bp region of the *AOX1a* promoter from Arabidopsis (*Arabidopsis thaliana*) has been implicated (Dojcinovic et al., 2005), to date no protein components involved in the mitochondrial retrograde response have been identified in plants (Koussevitzky et al., 2007; Woodson and Chory, 2008).

In this study, we show that the transcription factor ABI4 is involved in the retrograde regulation of *AOX1a* in Arabidopsis. A combined approach of functional analysis of cis-acting regulatory elements in the upstream region of *AOX1a*, yeast one-hybrid assays, electro-mobility shift assays (EMSA), and use of ABA-insensitive signaling mutants all reveal a central role for ABA response factors in the regulation of *AOX1a*.

RESULTS

The *AOX1a* Promoter Contains a Sequence Element That Represses Promoter Activity

Previous studies on the promoter region of *AOX1a* revealed that it was responsive to the addition of various compounds, including the mETC complex I inhibitor rotenone (Ho et al., 2008), consistent with the use of *AOX1a* as a model for mitochondrial retrograde regulation (Dojcinovic et al., 2005). Of the 10 different elements characterized to be functional in the *AOX1a* promoter, one CGTGAT element, designated B, was the strongest in terms of the potential to regulate expression of *AOX1a* (Fig. 1A). Deleting this element resulted in a 3-fold increase in promoter activity (Ho et al., 2008). This suggested that it was a strong repressor of *AOX1a* expression. As the previous study was carried out in Arabidopsis suspension cells, the function of this element was validated in Arabidopsis leaves (Fig. 1B). To this end, the 1.85-kb region upstream of the *AOX1a* translational start site was transcriptionally fused to a GUS reporter gene in the pLUS vector (Ho et al., 2008). This reporter construct was transiently transformed into wild-type Arabidopsis leaves using biolistic transformation, and the resulting GUS activity was measured and set to 100%. Pretreatment of the leaves with rotenone resulted in a significant increase in promoter activity of the wild-type *AOX1a* promoter. The role of the B element was examined by deletion of this element located −1,589 to −1,594 nucleotides upstream of the transcriptional start site in the *AOX1a*::*GUS* reporter construct, and the biolistic transformation assays demonstrated a 3-fold increase in promoter activity compared to the wild-type promoter (Fig. 1, A and B). Furthermore, deletion of the B element resulted in loss of induction in response to rotenone treatment (Fig. 1B), confirming that the region of the B element is repressing the expression of *AOX1a* in the absence of inducing conditions. Finally, a role of the B element in vivo was analyzed in stably transformed transgenic plants where the *AOX1a* promoter was used to drive the expression of GUS. GUS staining of plants driven by the wild-type *AOX1a* promoter was barely detectable under normal conditions (Fig. 2), whereas deleting the B element resulted in plants with strong GUS staining patterns (Fig. 2). Together, these experiments indicate that the *AOX1a* promoter is constitutively repressed by transcription factors that bind to the region around the B element.

The *AOX1a* Promoter Region Contains cis-Acting Regulatory Elements That Are Potential Binding Sites for ABA Response Factors

In the context of the *AOX1a* promoter, the B element overlaps with a potential binding site for ABI4, the

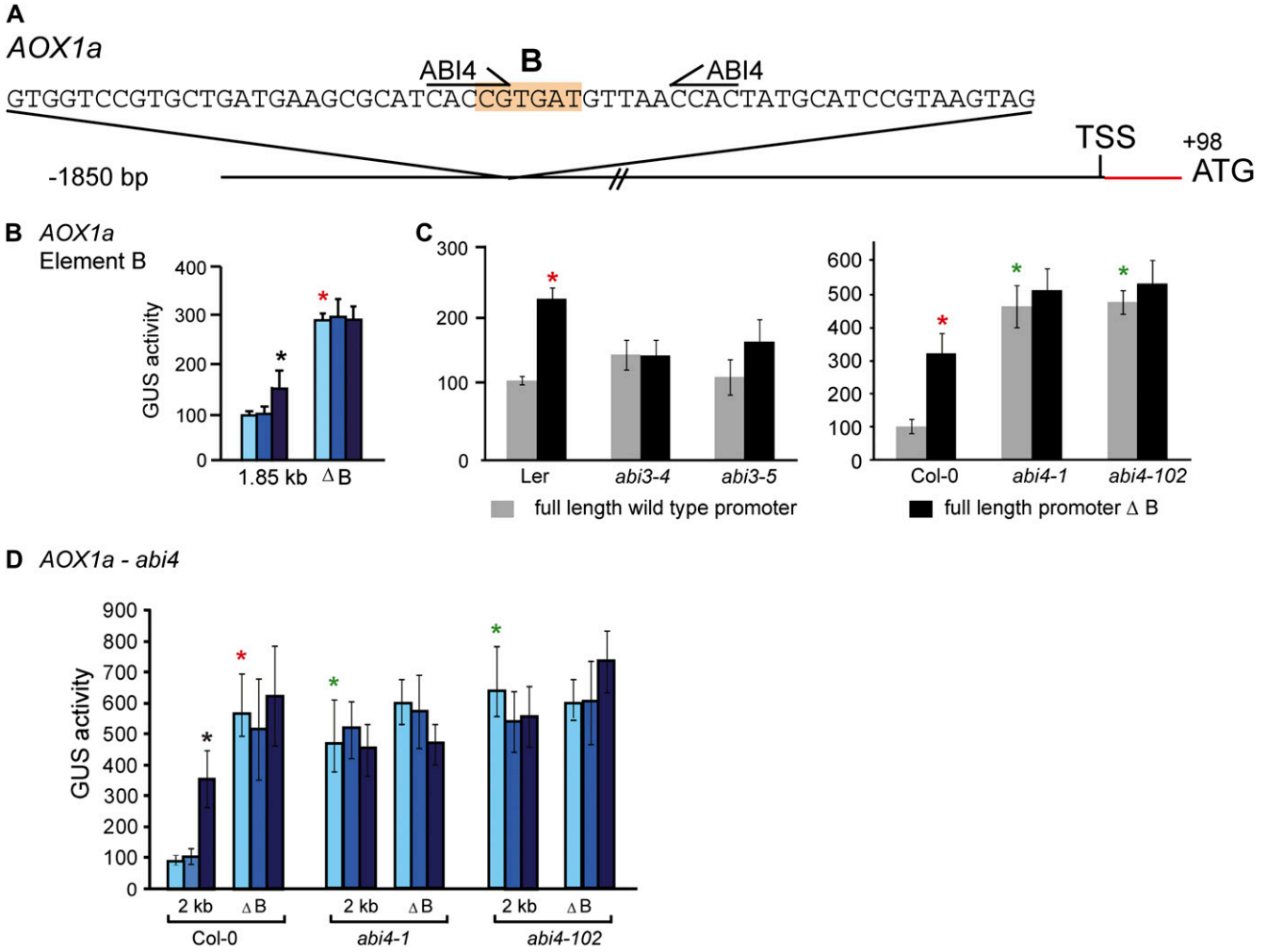

Figure 1. Functional analysis and rotenone response of regulatory element B in the *AOX1a* promoter region. A, The position of the B element (−1,589 to −1,594) in *AOX1a* is shown along with the position of two predicted ABI4 binding sites. Numbering is from the transcriptional start site (TSS) with the start ATG codon for translation indicated. B, The regulatory characteristics of element B were tested by comparing the GUS activity driven by the *AOX1a* promoter to the promoter with the B element deleted. The ability of the *AOX1a* promoter region to drive expression of the reporter gene GUS (normalized value set to 100%) and corresponding deletion was tested with rotenone treatment. Activities for untreated (light), mock-treated (medium), and treated (dark) samples are shown. A black asterisk indicates a significant difference ($P \leq 0.05$) between the GUS activity of mock-treated versus treated samples, indicating a rotenone response is associated with the promoter fragment being tested. A red asterisk indicates a significant difference ($P \leq 0.05$) between the GUS activities of untreated samples with unmutated versus mutated promoter fragments, indicating presence of a constitutive activity. C, The GUS activity under the control of the *AOX1a* promoter with element B deleted in the ABA signaling mutants, *abi3* and *abi4*. The activity of the promoter in the corresponding wild-type background was set to 100% and other values expressed in a relative manner. A red asterisk indicates a significant difference ($P \leq 0.05$) between mutated and unmutated promoter. A green asterisk indicates a significant difference ($P \leq 0.05$) between the GUS activities with unmutated promoter fragments between the different genetic backgrounds. D, The effects of rotenone treatment and deletion of element B on GUS activity in the ABA signaling mutant *abi4*. A black asterisk indicates a significant difference ($P \leq 0.05$) between the GUS activity of mock-treated versus treated samples, indicating a stress response is associated with the promoter fragment being tested. A red asterisk indicates a significant difference ($P \leq 0.05$) between the GUS activity of the wild-type promoter and the mutated promoter; this determined if the element has any regulatory function in the absence of any stress treatment. A green asterisk indicates a significant difference ($P \leq 0.05$) between the GUS activities with unmutated promoter fragments between the different genetic backgrounds.

coupling element CE1 (CACCG; Niu et al., 2002), and just 5 bp downstream from the predicted B element a second putative ABI4 binding site (CCAC), as defined in the *Lhcb* promoter, was evident (Koussevitzky et al., 2007; Fig. 1A). These findings prompted us to determine if ABI4 was involved in regulation of *AOX1a* promoter function. The activity of the wild-type 1.85-kb *AOX1a* promoter and the *AOX1a* promoter with the

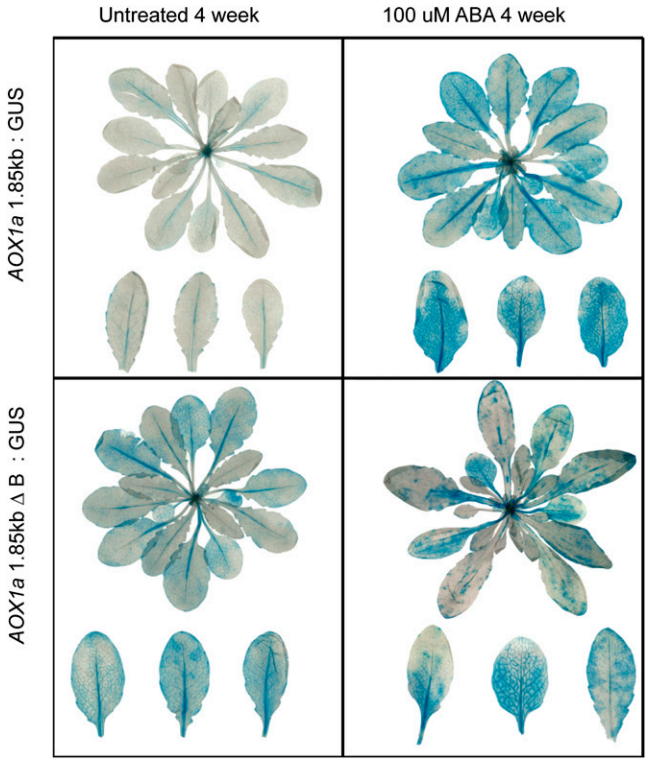

Figure 2. ABA treatment of transgenic lines containing *AOX1a* promoter:GUS fusions. GUS staining for transgenic Arabidopsis lines carrying the *AOX1a* wild-type promoter or the *AOX1a* promoter with the B element deleted driving the expression of GUS under normal conditions or after 3 h treatment with 100 μM ABA, applied by painting the treatment solution on the leaves every 30 min during treatment time. Staining for whole plants is shown for each condition, along with representative leaves from three independent replicates.

B element deleted were tested in two independent mutant lines for the ABI4 transcription factor, *abi4-1* and *abi4-102* (Finkelstein, 1994), and compared to wild-type plants in order to determine if a lack of ABI4 had any effect on activity. Analysis of the *AOX1a* promoter in the *abi4* mutant background revealed that it was constitutively derepressed, as activity was >4-fold that observed in the wild-type (Columbia-0 [Col-0]) background (Fig. 1C). Furthermore, deletion of the B element in the *abi4* mutant background resulted in no further increase in promoter activity as was observed in the wild-type (Col-0) background (Fig. 1C). Thus, deletion of the B element and activity of the *AOX1a* promoter in *abi4* mutants has the same effect in that the promoter was derepressed.

A possible role for the transcription factor *abi3* in regulating *AOX1a* promoter activity was also examined, as it has also been shown to bind an ACGTG promoter motif containing the same CGTG core sequence as the B element (Roschzttardtz et al., 2009). In the *abi3* mutant backgrounds (*abi3-4* and *abi3-5*; Ooms et al., 1993), activity of the *AOX1a* promoter did not differ from wild-type plants (Fig. 1C). Deletion of the B element in the *abi3* mutant backgrounds resulted in no increase in promoter activity (Fig. 1C), suggesting that ABI3 was required for an increase in *AOX1a* promoter activity but that it did not directly play a role in the repression of the *AOX1a* promoter observed under normal conditions as observed with ABI4. A possible explanation for the lack of induction is that ABI3 binds elsewhere in the *AOX1a* promoter region and is involved in activation of expression above normal levels. The ability of the *AOX1a* promoter to respond to mitochondrial retrograde signals induced by rotenone treatment was tested in the *abi4* mutant backgrounds. While rotenone increased the *AOX1a* promoter activity in wild-type plants, rotenone could not further induce the *AOX1a* promoter in the *abi4* mutant background, likely because it was already fully derepressed, the untreated activity in the *abi4* backgrounds was higher than with rotenone in the wild-type background (Fig. 1D).

The *AOX1a* Promoter Is Regulated by ABA Treatment

As *abi3* and *abi4* were originally characterized as having alterations in ABA responses and since ABA is a known stress hormone, the ability of the *AOX1a* promoter to respond to ABA treatment was tested. The ability of the *AOX1a* promoter (wild-type and with the B element deleted) to drive GUS reporter gene expression was tested by transient transformation of Arabidopsis leaf tissue after 3 h of pretreatment with 100 μM ABA. However, a negative response of the promoter was observed (data not shown). This may be due to technical limitations of the transient transformation process; leaves are detached from the plant affecting stomatal closure, which has been linked to ABA signaling and assayed for activity 27 h later. The transformation process in itself can be a significant stress on the plant (Ho et al., 2008), complicating the response to ABA in this system. To overcome these limitations, transgenic lines containing the *AOX1a* promoter (the wild type or with the B element deleted) as translational fusions to GUS were treated with 100 μM ABA for 3 h and then stained for GUS activity (Fig. 2). Treatment was applied by painting treatment solution on the leaves every 30 min to avoid wounding stress. Plants containing the wild-type promoter driving GUS expression showed a considerable increase in GUS staining after treatment with ABA. Plants containing the *AOX1a* promoter with the B element deleted driving GUS expression showed much higher levels of staining even under untreated conditions; a further response to ABA treatment in these plants was not evident.

It was concluded that element B played an essential role in the ability of the *AOX1a* promoter to respond to rotenone. A functional ABI4 appeared to be necessary for this response, as in the absence of ABI4, the *AOX1a* promoter displayed a very high activity. Thus, under normal growth conditions *AOX1a* was under negative regulation.

The B Element of *AOX1a* Interacts with ABI4 in Binding Assays

To test if the ABI4 transcription factor binds to the region of the B element in the *AOX1a* promoter, a 50-bp region surrounding the B element as shown in Figure 1A was used as a bait for binding assays in the yeast one-hybrid system (Vidal and Legrain, 1999). The interaction between transcription factor and DNA sequence was tested by growth on media lacking Trp, Leu, and His. Increasing concentrations of the His synthase inhibitor 3-aminotriazole (3AT) were added to the media to suppress background activation and assess the strength of the interaction (Fig. 3). From these assays it was concluded that ABI4 and ABI3 were able to bind the 50-bp *AOX1a* B element region as well as the 3 x B element synthetic construct, with similar strength as the p53 positive control interaction.

To further assess the binding of ABI4 and ABI3 to the *AOX1a* B element region, EMSAs were performed. A biotin-labeled double-stranded DNA probe containing the 50-bp region surrounding the *AOX1a* B element was synthesized, whereas the transcription factors were expressed in an in vitro translation system. The labeled probe was incubated with and without transcription factor protein extracts and separated on native polyacrylamide gels. In a third reaction, an excess of unlabeled DNA probe competitor was added to the incubation mixture to verify if the interaction and observed shifts were specific. For ABI3, we were unable to show specific shifts using EMSAs and could not further confirm binding to the B element region (data not shown). For ABI4, shifted bands were routinely observed with the ABI4 and *AOX1a* B element oligonucleotide that could be abolished using a competitor against the whole 50-bp region (Fig. 4). Assays were also performed using specific competitors for each of both ABI4 binding sites. As each competitor was able to abolish one specific shift, these assays confirmed the specific binding of ABI4 to both putative ABI4 binding sites in the promoter of *AOX1a*. Thus, it was concluded that the ABI4 transcription factor binds to the B element region in the *AOX1a* promoter.

Transcript Abundance of *AOX1a* Is Responsive to ABA and Affected by ABI4

As both transcription factors ABI3 and ABI4 were originally isolated for their reduced sensitivity to ABA application, it was investigated if transcript abundance of *AOX1a* is directly responsive to ABA and if the increase in transcript abundance of *AOX1a* observed in response to rotenone is affected by the absence of ABI3 or ABI4. Transcript abundance of *AOX1a* was analyzed by quantitative reverse transcription-PCR (QRT-PCR) and was found to be strongly induced after treatment with ABA (Fig. 5). To confirm that *AOX1a* gene expression in response to ABA and rotenone is subjected to regulation by ABA signaling factors in planta, transcript levels were analyzed in the *abi3* and *abi4* signaling mutants using two different mutant alleles of each gene. In the case of *abi4*

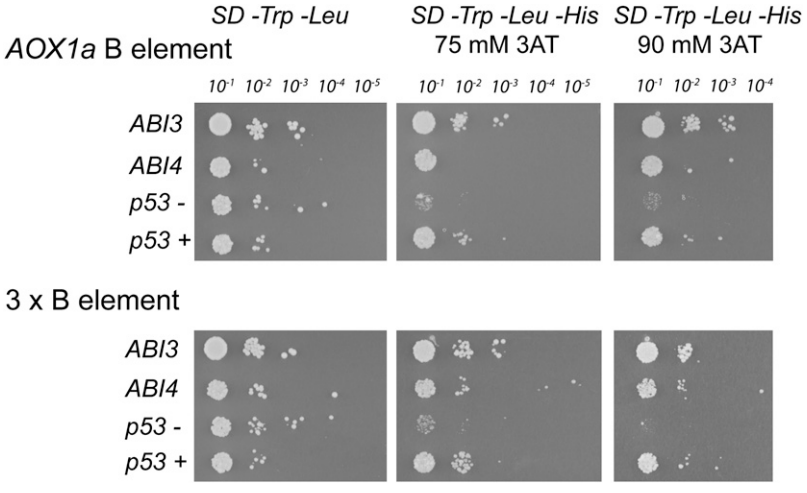

Figure 3. Yeast one-hybrid binding assays of ABI3 and ABI4. Yeast cells were cotransformed with a bait vector, containing a DNA sequence of interest fused to a *HIS3* reporter gene, and a prey vector, containing a transcription factor coding sequence fused to a *GAL4* activation domain. Binding assays were performed for ABI3 and ABI4 against the 50-bp region surrounding the *AOX1a* B promoter element (*AOX1a* B element) and against a synthetic sequence containing three consecutive repeats of the B element (3 x B element). Binding of p53 to a sequence containing or not containing a p53 binding motif were used as a positive (p53 +) and negative control (p53 −), respectively. Cells were grown in liquid media to OD_{600} of 0.1 (10^{-1}) and diluted in a 10× dilution series (10^{-2} to 10^{-5}). Of each dilution, 5 μL was spotted on media selecting for both plasmids (SD –Trp –Leu) and selecting for interaction (SD –Trp –Leu –His), supplemented with 75 or 90 mM 3AT to suppress background growth and test the strength of the interaction.

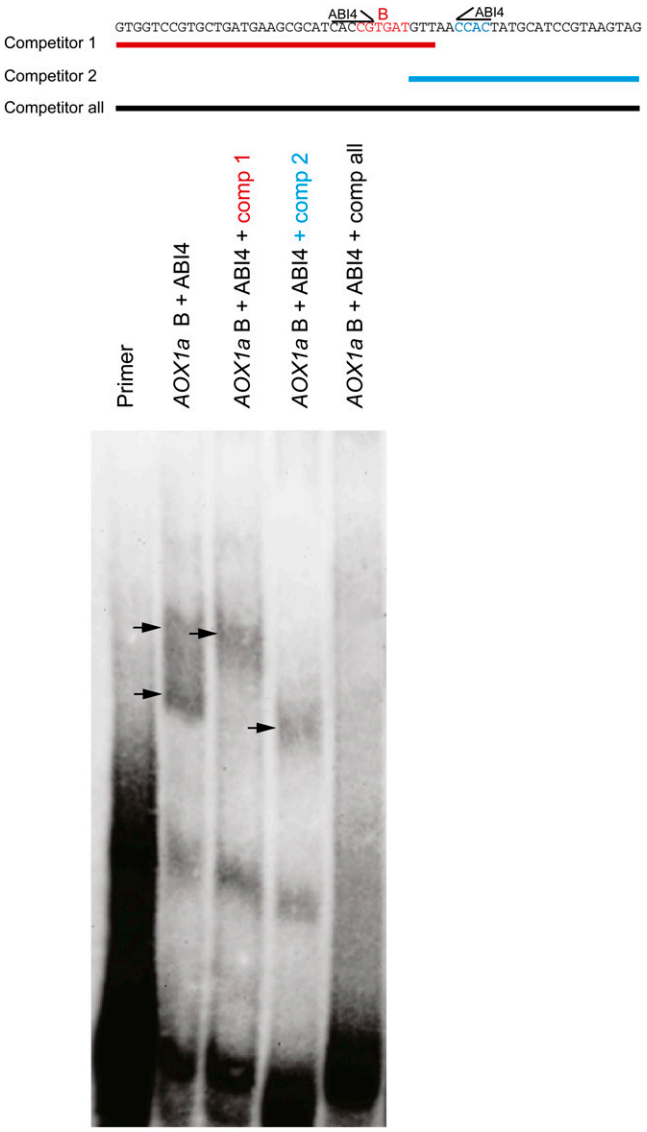

Figure 4. Binding of ABI4 transcription factor to the promoter region of *AOX1a*. EMSAs using in vitro-synthesized proteins and biotin-labeled probes of promoter regions of *AOX1a* containing the B element. Proteins were synthesized using the Roche RTS Wheatgerm RTS-100 CECF system as described in "Materials and Methods." Probes were incubated with synthesized proteins in 20-μL reactions. Lane 1, 40 fmol of labeled wild-type probe alone. Lane 2, 40 fmol of labeled wild-type probe with 1 μg synthesized protein. Lane 3, 40 fmol of labeled wild-type probe, 1 μg synthesized protein with 4 pmol unlabeled competitor 1 targeting the first potential ABI4 binding site. Lane 4, 40 fmol of labeled wild-type probe, 1 μg synthesized protein with 4 pmol unlabeled competitor 2 targeting the second potential ABI4 binding site. Lane 5, 40 fmol of labeled wild-type probe, 1 μg synthesized protein with 4 pmol unlabeled competitor "all" targeting the both potential ABI4 binding sites. Black arrows indicate protein:probe complexes. [See online article for color version of this figure.]

mutant backgrounds, *AOX1a* transcripts displayed a 2-fold increase in abundance (Fig. 5). Furthermore, the significant increase in transcript abundance by rotenone or ABA treatment was abolished, likely due to the fact that the uninduced transcript abundance in the *abi4* mutant backgrounds was as high as the induced levels of transcript abundance in the Col-0 background, indicating the expression could not be further derepressed in the mutant upon stress treatment. Transcript abundance in *abi3* mutant backgrounds was not significantly different compared to the wild type (Landsberg *erecta* [L*er*]), in agreement with the promoter activity (Fig. 1B). Furthermore, an increase in transcript abundance for *AOX1a* with rotenone and ABA treatment was not abolished in the *abi3* mutant backgrounds. In summary, *AOX1a* transcript abundance is induced by ABA and by rotenone, and ABI4 appears to play an essential role in the response to these signals.

DISCUSSION

Multiple lines of evidence point to a role for the ABI4 transcription factor in the regulation of *AOX1a* in

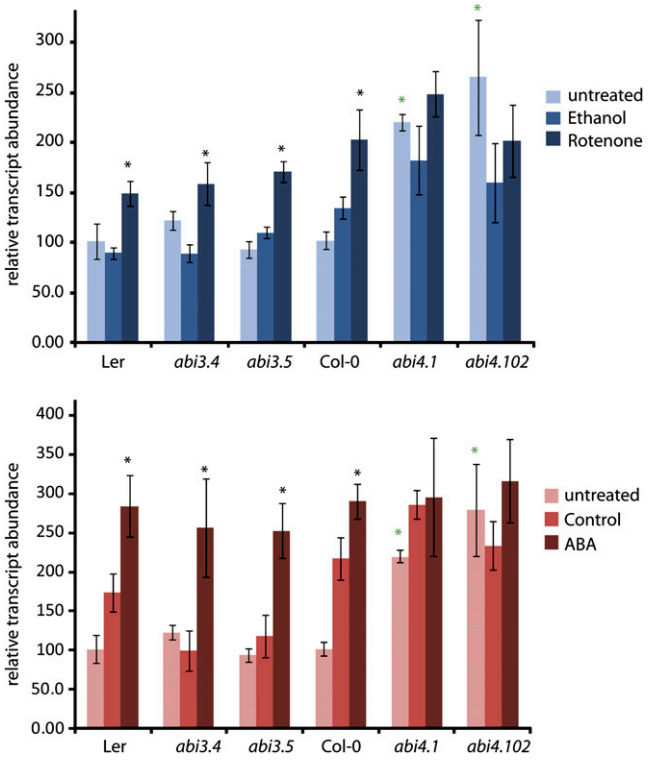

Figure 5. Transcript abundance of *AOX1a* in response to rotenone and ABA and in abscisic acid signaling mutants. The transcript abundance of *AOX1a* and in lines mutated in *abi3* or *abi4* (two mutant alleles per gene) was determined using QRT-PCR. Seedlings were harvested at growth stage 1.04 in biological triplicates. Values are averaged per line and normalized to their respective wild-type ecotypes (L*er* for *abi3* and Col-0 *for abi4*). Error bars indicate SE. Asterisks indicate significant changes in transcript abundance as calculated by a Student's *t* test, black for a significant difference between mock-treated and stress-treated, i.e. a stress response, and green for a significant difference between abundances of untreated samples from wild-type plants compared to mutant plants.

response to retrograde signals. Deletion of the B element in the *AOX1a* promoter and the absence of the ABI4 transcription factor have similar effects on promoter activity: the promoter is derepressed and loses ability to respond to rotenone. Additionally, transcript abundance for *AOX1a* increased in *abi4* mutant lines and was unresponsive to rotenone. The ABI4 transcription factor was shown to interact with *AOX1a* in two binding assays, EMSA and yeast one-hybrid assay, respectively, indicating that is directly involved in *AOX1a* transcription.

The finding that *AOX1a* is repressed by ABI4 is consistent with previous studies on AOX. Arabidopsis plants with a T-DNA insertional mutation of *aox1a* display increased sensitivity to stress and have altered levels of several genes involved in anti-oxidant defense (Giraud et al., 2008). Two other studies on *aox1a* plants also reveal increased stress sensitivity and alteration in the transcript abundance of several genes (Watanabe et al., 2008; Strodtkötter et al., 2009). Analysis of transcript abundance for *ABI4* in these plants reveals that it is reduced severalfold (Giraud et al., 2008); in fact, an analysis of all mutants in the GENEVESTIGATOR web tool reveal that the *aox1a* T-DNA insertional mutants display among the strongest reduction in *ABI4* expression (Zimmermann et al., 2004). Thus, in *aox1a* plants, it appears that one consequence is to reduce expression of ABI4 likely in an attempt of the plants to increase *AOX1a* levels. This may help to explain the observed sensitivity of *aox1a* plants to moderate light conditions, as ABI4 has been shown to compete for binding to inhibit G-box-mediated, light-induced expression of photosynthesis related genes, including *Lhcb1.2* (Koussevitzky et al., 2007). Thus, if ABI4 is down-regulated, repression of genes encoding light-harvesting chlorophyll *a* and *b* components is compromised. Furthermore, in microarray experiments that were performed on samples treated with rotenone, the increase in *AOX1a* expression was accompanied by a reduction in *ABI4*, consistent with a strong connection at a regulatory level between ABI4 and *AOX1a* (Clifton et al., 2005). In a variety of plants, it has been observed that the induction of *AOX* transcript in response to stress is rapid (Clifton et al., 2005). In fact, even using the transcriptional inhibitor Actinomycin D, the levels of *AOX1a* transcript initially increase before declining (Narsai et al., 2007). This is consistent with a gene under strong negative regulation, as upon treatment, transcription can occur before full inhibition of transcription initiation is effective.

Previous characterization of the *AOX1a* promoter from Arabidopsis identified a region that contained putative binding sites for WRKY and Dof transcription factors (Dojcinovic et al., 2005). The B element in *AOX1a* lies outside this region, suggesting that other pathways may also interact to control expression of *AOX1a*. However, one element characterized in this previous study, mut8 motif, also overlaps with a putative ABI4 binding site (Dojcinovic et al., 2005). Another study that identified 10 different elements in the *AOX1a* promoter from Arabidopsis also identified two functional elements that contain the core binding site for ABA response element (ABRE) binding factors ACGTG, which contains a similar CGTG motif as the B element studied here (Ho et al., 2008). This suggests that additional ABA signaling transcription factors are involved in the regulation of *AOX1a* in Arabidopsis. Thus, the regulation of *AOX1a* by ABA response factors is likely to be complex. Application of ABA leads to an increase in *AOX1a* promoter activity (Fig. 2) and transcript abundance (Fig. 5). In addition to ABI4, it was observed that ABI3 played some role in the regulation of *AOX1a*, as deletion of the B element in the *abi3* mutant backgrounds did not result in derepression; thus, ABI3 appears to play some role in the positive regulation of *AOX1a*. This also suggests that while ABI4 binds two sites (Fig. 1A), one that overlaps with the B element and one just downstream to repress the *AOX1a* promoter, ABI3 may bind elsewhere to act as a positive regulator. Thus, the regulation by ABA is likely to be a balance of positive and negative interactions. These interactions are also likely to be integrated with other signals, as several other elements have previously been characterized in the *AOX1a* promoter (Dojcinovic et al., 2005; Ho et al., 2008). ABREs containing the core binding site of ACGT have been previously shown to be present and active in the promoter regions of genes encoding proteins of the mETC, specifically from *COX5b-1* (for cytochrome oxidase subunit 5b; Comelli et al., 2009) and *SDH2-3* (for succinate dehydrogenase subunit 2-3; Roschzttardtz et al., 2009), which bind AREB2/ABF4 and ABI3, respectively. In this study, the binding of ABI3 to the *AOX1a* B element region in the yeast one-hybrid assays may be explained by the fact that both the B element and the ABRE element (one of the ABI3 binding sites; Hattori et al., 1995) contain a CGT(G) core sequence. Interestingly, in other reports, ABI3 binding to an ABRE-RY could also be detected using an ELISA-type test but not by EMSA (Roschzttardtz et al., 2009). Thus, it appears that ABA response factors are involved in regulation of both the cytochrome and alternative electron transport chains in Arabidopsis. Interestingly, application of ABA was still able to induce *AOX1a* transcripts in the *abi3* mutant background, yet not in *abi4* mutants. Although the fact that the induction of *AOX1a* in response to ABA is not abolished in the ABA-insensitive *abi3* background seems counterintuitive, similar reports for other genes, such as cold-regulated genes and a gene, *ATHB-7*, encoding a homeobox protein, have been made previously (Gilmour and Thomashow, 1991; Söderman et al., 1996), indicating a complex interplay between ABA signaling factors.

Regulation of the expression of *AOX1a* via ABI4 provides a direct link between chloroplast and mitochondrial retrograde signaling (Koussevitzky et al., 2007; Woodson and Chory, 2008; Fig. 6). The ABI4 transcription factor has been characterized to act

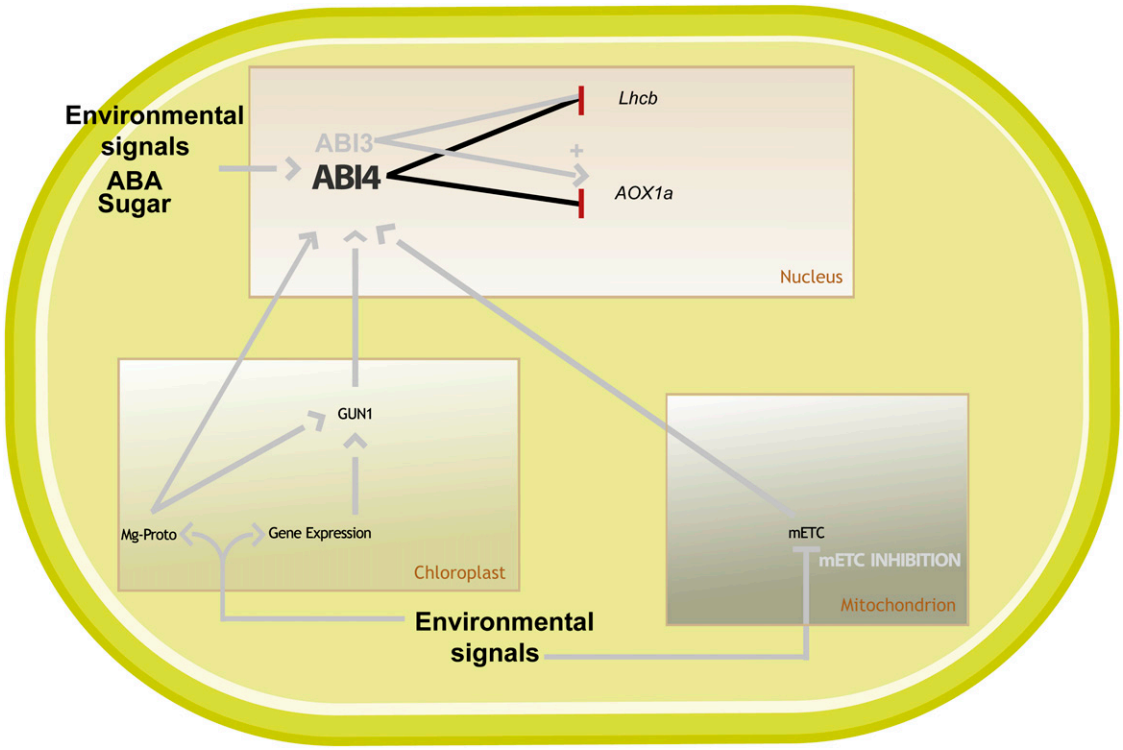

Figure 6. Interaction of signaling pathways controlling expression of AOX1a with chloroplast and anterograde regulation. Two of the four chloroplast retrograde signaling pathways elucidated to date act through ABI4 to regulate expression of *Lhcb*. Additionally, responses to many environmental stresses and sugar signaling are mediated via ABA. Regulation of *AOX1a* by ABI4 provides a point of convergence for these pathways. In contrast with chloroplast retrograde signaling, ABI3 appears to be a positive regulator of *AOX1a* expression (Koussevitzky et al., 2007; Bossi et al., 2009).

downstream of both the plastid gene expression and Mg-proto retrograde pathways from the chloroplasts (Koussevitzky et al., 2007). Thus, ABI4 plays a role in regulating genes that are responsive to retrograde signals from the mitochondrion and chloroplast. The identification of a molecular link between mitochondrial and chloroplast retrograde signaling pathway provides a platform to understand how perturbation of photosynthesis or alternative respiratory activity can feed back to affect each other (Giraud et al., 2008). Inactivating components that are regulated by the same master switch may result in compensations in the regulatory network that have widespread effects outside the immediate biochemical activity of the components that have been inactivated. Thus, changes in chloroplast function when *AOX1a* is inactivated (Giraud et al., 2008) and activation of *AOX1a* when chloroplast function is altered may (Yoshida et al., 2007, 2008) in part be explained by perturbation in interacting regulatory pathways, rather than in terms of pure biochemical mechanisms alone.

ABA signaling factors have also been found to be closely associated with sugar signaling, for example, *abi4* mutants are allelic to multiple Glc-insensitive mutants (Rook et al., 2006; Bossi et al., 2009), and ABI3 has also been found to function in Glc signaling (Dekkers et al., 2008). Glc is an early inducer of *AOX1a* expression (Li et al., 2006), suggesting that not only ABA but also carbohydrate signals could be integrated through ABI3-4 regulation. As photosynthesis supplies plants with sugars, it seems quite useful that regulation of chloroplast function, carbohydrate metabolism, and mitochondrial respiration share common regulatory factors. Although ABI3 appears to be a negative regulator of photosynthetic genes, and possibly displays a GUN phenotype (our interpretation of data presented in Fig. 3A in Koussevitzky et al., 2007), ABI3 appears to be a positive regulator of *AOX1a* (Fig. 6), as evidenced by the fact that it was necessary for the increase in *AOX1a* promoter activity when the B element was deleted (Fig. 1C).

A connection between mitochondrial and chloroplast retrograde signaling also indicates that signals from both organelles interact. A mutation in Arabidopsis prolyl-tRNA synthetase that is targeted to mitochondria and plastids that results in decreased rates of organelle translation causes a decrease in transcript abundance for nuclear genes encoding proteins involved in photosynthesis (Pesaresi et al., 2006). As mutations in genes encoding plastid or mitochondrial ribosomal proteins alone do not elicit the same response, it is a combination of affecting translation in

both organelles that causes the response (Kleine et al., 2009). Furthermore, in this study, we show that mitochondrial retrograde signaling acts via ABI4, an ABA-responsive transcription factor. We also showed that ABA can induce *AOX1a* expression, and this is compromised in *abi4* mutants. Although ABA is not synthesized in plastids or mitochondria, it may represent an important intermediate or signaling molecule to mediate and integrate plastid and mitochondrial retrograde signals.

MATERIALS AND METHODS

Plant Material Growth and Treatments

Arabidopsis (*Arabidopsis thaliana*) mutant lines were obtained from the Arabidopsis Biological Resource Center at The Arabidopsis Information Resource: *abi3-4*, CS6130; *abi3-5*, CS6131; *abi4-1*, ABI4-1BPS1-2; *abi4-102*, CS3837. Arabidopsis plants, ecotype Col-0, L*er*, and corresponding mutant lines were grown at 22°C for 16 h at 100 μE m^{-2} s^{-1} light conditions and 8 h of dark. For treatments, Arabidopsis leaf tissue was taken from 4-week-old plants. Stems of each leaf were submerged into the mock (same volume of water or ethanol solvent for stress solution added) or treatment solution for 3 h. A final concentration of 40 μM was used for rotenone and 100 μM for ABA.

QRT-PCR Analysis of Gene Expression

QRT-PCR was performed on Arabidopsis tissue from *abi3* (*abi3-4* and *abi3-5*) and *abi4* (*abi4-1* and *abi4-102*) mutant lines, and their respective wild-type ecotypes L*er* and Col-0. Seedlings were cultivated in vitro on Murashige and Skoog media and harvested at developmental stage 1.04 (Boyes et al., 2001). Seedlings were treated with rotenone or ABA as mentioned above and collected after 3 h along with untreated and mock-treated control samples. Several seedlings were pooled per sample, and all samples were taken in biological triplicate and snap frozen in liquid nitrogen. Total RNA isolation and cDNA synthesis was carried out as described previously (Lister et al., 2004). Transcript levels were assayed using the LightCycler 480 and the LightCycler 480 SYBR Green I Master (Roche). From each of the independent cDNA preparations, each transcript was analyzed twice and normalized to *UBC* as a housekeeping gene. QRT-PCR primers used for the genes *AOX1a* (At3g22370) and *UBC* have been previously described (Clifton et al., 2005; Supplemental Table S1). Transcript abundance for *abi3* lines were normalized to L*er* and *abi4* to Col-0 samples, with all other values presented as relative transcript abundance. Standard errors are shown, and a Student's *t* test was performed to determine significant changes ($P < 0.05$) in transcript abundance. Significance is shown by the presence of an asterisk, black for a significant difference between mock-treated and stress-treated, i.e. a stress response, and green for a significant difference between abundances of untreated samples from wild-type plants compared to mutant plants.

Cloning of Arabidopsis Promoter Regions

The promoter regions were cloned using standard protocols and subcloned into pLUS (see Supplemental Table S1 for primer sequence). The numbering of Arabidopsis *AOX1a* promoter is given from the transcriptional start site as determined from the SIGAL database (Yamada et al., 2003). The construct was made as a translational fusion with GUS, with the first ATG of the gene used as the start codon for GUS. Thus, the promoter region also contained a 5′ untranslated region of 99 bp for *AOX1a* (At3g22370). The element to be tested, the B element located −1,589 to −1,594 nucleotides upstream of the transcriptional start site (Fig. 1), was deleted from the promoter via site-directed mutagenesis using the Stratagene Quikchange II XL site-directed mutagenesis kit as outlined previously (Ho et al., 2008).

Construction of Transgenic Plants

Promoter regions were subcloned into pCAMBIA1301 (http://www.cambia.org/daisy/cambia/585.html), using standard protocols, as a translational fusion with GUS. Six-week-old Arabidopsis plants were transformed using *Agrobacterium tumefaciens*-mediated transformation of the flowers (Clough and Bent, 1998). T1 generation seeds were selected for resistance on medium (Gamborg, 1968) containing 20 mg/mL hygromycin, selected for a single T-DNA insertion locus, and propagated to homozygosity. Four-week-old homozygous plants, grown under normal growth conditions described above, were either untreated or treated with 100 μM ABA by painting the treatment solution on the leaves every 30 min for 3 h. Whole plants were then harvested and stained for GUS activity as described previously (Thirkettle-Watts et al., 2003). Stained and bleached plants were mounted between transparency slides with 40% (v/v) glycerol and scanned. Staining for whole plants is shown along with representative leaves from three independent replicates.

Biolistic Transformation and Assays for Luciferase and GUS

Transformation was performed using the PDS-1000 system using the Hepta adaptor according to the manufacturer's instructions (Bio-Rad) as previously described (Ho et al., 2008), except that leaf tissue was used in place of Arabidopsis suspension cell cultures.

Standard errors are shown, and to determine statistical significance, a Student's *t* test was performed assuming unequal variances. For comparison of GUS activities of the motif deletions with that of the unmutated promoter, a two-sample *t* test assuming unequal variances was also performed. Significance was defined as $P \leq 0.05$. The following comparisons were carried out to determine the activity of each element tested: (1) A comparison of the normalized GUS activity between the wild-type promoter and the mutated promoter; this determined if the element has any regulatory function in the absence of any stress treatment. Significance for this is indicated with a red asterisk. (2) A comparison between the mock-treated and stress-treated GUS values. This determines if the promoter fragment was stress responsive and if deleting the element resulted in a loss of a significant effect. Significance for this is indicated with a black asterisk. (3) A comparison between the treated GUS values of the wild-type promoter in wild-type plants compared to mutant plants. This determines if promoter activity is altered in a mutant. Significance for this is indicated with a green asterisk.

Construction of Vectors for Yeast One-Hybrid Analysis

The coding regions of the transcription factors ABI3 (At3g24650) and ABI4 (At2g40220) were cloned from Arabidopsis Col-0 cDNA using standard protocols with the Roche Expand High Fidelity PCR system (Roche), using gene specific primers (Invitrogen). The PCR products were then cloned into the pGADT7-rec2 prey vector (CLONTECH), creating a translational fusion between the GAL4 activation domain and the transcription factor. As a control system, the binding capacity of the p53 transcription factor to a DNA sequence containing (p53 +) or not containing (p53 −) a p53-binding motif was used. For construction of the pHIS2 bait vectors, forward and reverse oligonucleotides (Supplemental Table S1) were annealed and subcloned into *Eco*RI/*Sac*I linearized pHIS2 vector. The 50-bp sequence surrounding the B element was cloned into the pHIS2 reporter vector upstream of the minimal *HIS3* promoter region and *HIS3* nutritional reporter gene. Similarly, a synthetic construct containing three consecutive repeats of the B element was cloned into the pHIS2 vector.

Yeast One-Hybrid Screen

Competent yeast cells were prepared according to the CLONTECH Yeast Protocols Handbook using the Y187 yeast strain. Yeast one-hybrid transformation screens were performed using the CLONTECH Matchmaker One Hybrid Library Construction and Screening kit. For each yeast one-hybrid transformation screen, 100 μL of competent yeast cells were incubated with 100 ng of pHIS2 bait vector and 100 ng of pGADT7-Rec2 prey vector, 100 μg Herring Testes Carrier DNA (CLONTECH), and 0.6 mL PEG/LiAc solution. Cells were transformed according to the manufacturer's instructions. Transformations were plated onto SD media –Leu –Trp to select for cotransformed cells and incubated at 28°C for 4 d. The pGADT7-rec2-p53 prey vector in combination with p53HIS2 was used as a positive control transformation and pGADT7-rec2-p53 in combination with pHIS2 as negative control. Transformed yeast cells were subsequently grown in SD –Trp –Leu liquid media to OD_{600} of 0.1 and diluted in a 10× dilution series. From each dilution, 5 μL was spotted on SD –Trp –Leu and on SD –Trp –Leu –His media plates supple-

mented with 75 or 90 mM 3AT (Sigma-Aldrich). The plates were then incubated for 3 d at 28°C.

Cloning of Transcription Factors for in Vitro Expression

The coding regions of the transcription factors were cloned using standard protocols with the Roche Expand High Fidelity PCR system using gene-specific primers (Supplemental Table S1), with appropriate restriction sites incorporated. Transcription factors were then subcloned into pIVEX1.3 WG (Invitrogen) according to the manufacturer's instructions.

In Vitro Translation of Transcription Factors

In vitro translation of transcription factors was performed using the RTS 100 Wheatgerm CECF kit (Roche) using pIVEX1.3WG-transcription factor constructs. Translations were performed according to the manufacturer's instructions. For each reaction, 900 μL of feeding solution, 80 μL of amino acids, and 20 μL of Met was added to the feeding compartments of the CECF module. To the reaction compartment, 15 μL of reaction mix, 4 μL amino acids, 1 μL Met, 15 μL of wheat germ lysate, 15 μL RNase and DNase free sterile water, and 2 to 4 μg of plasmid DNA were added. The CECF module was loaded into the RTS Proteomaster instrument (Roche) and set to 24°C, shaking at 900 rpm for up to 24 h. Reactions were run on SDS-PAGE gels and detected with anti-His_6-tag antibody to identify the translated protein.

EMSAs

EMSAs were performed using the Lightshift Chemilluminescent EMSA kit (Pierce) using biotinylated oligonucleotides (Supplemental Table S1; Sigma-Aldrich). Oligonucleotides were annealed at 95°C for 5 min, with the temperature decreasing by 1°C for each minute and thereafter until holding 4°C. EMSAs were performed according to the manufacturer's instructions with the LightShift Chemiluminescent EMSA kit (Thermo), with final concentrations of 1× binding buffer, 50 ng/μL poly dl-dC, 2.5% (v/v) glycerol, 0.05% (v/v) Nonidet P-40, 5 mM $MgCl_2$, 40 fmol biotinylated oligonucleotides, 1 μg expressed protein, and 4 pmol competitor oligonucleotides, if applicable. Reactions were incubated at room temperature for 20 min before loading onto a 5% native polyacrylamide gel in 0.5× TBE. The gel was electrophoresed for 7 h at 4°C before transferring onto Hybond N^+ membrane (GE Healthcare) at 150 mA for 2 h using a semidry blotting apparatus (Millipore). The membrane was fixed with 120 mJ/cm UV-C radiation using a UV-light cross-linker (UVItec). Blocking, washing, and detection were performed using the Chemiluminescent Nucleic Acid Detection Module (Thermo) according to the manufacturer's instructions.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At3g22370 (*AOX1a*), At3g24650 (*ABI3*), and At2g40220 (*ABI4*).

Supplemental Data

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

Supplemental Table S1. List of primers used in this study.

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