Identification of Regulatory Pathways Controlling Gene Expression of Stress-Responsive Mitochondrial Proteins in Arabidopsis1[W][OA]

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In this study we analyzed transcript abundance and promoters of genes encoding mitochondrial proteins to identify signaling pathways that regulate stress-induced gene expression. We used Arabidopsis (Arabidopsis thaliana) alternative oxidase AOX1a, external NADP H-dehydrogenase NDB2, and two additional highly stress-responsive genes, At2g21640 and BCS1. As a starting point, the promoter region of AOX1a was analyzed and functional analysis identified 10 cis-acting regulatory elements (CAREs), which played a role in response to treatment with H2O2, rotenone, or both. Six of these elements were also functional in the NDB2 promoter. The promoter region of At2g21640, previously defined as a hallmark of oxidative stress, shared two functional CAREs with AOX1a and was responsive to treatment with H2O2 but not rotenone. Microarray analysis further supported that signaling pathways induced by H2O2 and rotenone are not identical. The promoter of BCS1 was not responsive to H2O2 or rotenone, but highly responsive to salicylic acid (SA), whereas the promoters of AOX1a and NDB2 were unresponsive to SA. Analysis of transcript abundance of these genes in a variety of defense signaling mutants confirmed that BCSI expression is regulated in a different manner compared to AOX1a, NDB2, and At2g21640. These mutants also revealed a pathway associated with programmed cell death that regulated AOX1a in a manner distinct from the other genes. Thus, at least three distinctive pathways regulate mitochondrial stress response at a transcriptional level, an SA-dependent pathway represented by BCSI, a second pathway that represents a convergence point for signals generated by H2O2 and rotenone on multiple CAREs, some of which are shared between responsive genes, and a third pathway that acts via EDS1 and PAD4 regulating only AOX1a. Furthermore, posttranscriptional regulation accounts for changes in transcript abundance by SA treatment for some genes.

The alternative oxidase (AOX) of plant mitochondria is widely used as a model to study the regulation of genes encoding mitochondrial proteins in response to stress or mitochondrial dysfunction (Vanlerbergh and McIntosh, 1997; Finnegan et al., 2004; Clifton et al., 2005, 2006; Rhoads et al., 2006). In addition to AOX several other genes are also induced by treatments that perturb mitochondrial function. A protein encoded at the locus At2g21640 has been shown to be induced by oxidative stress (Sweetlove et al., 2002) and subsequently defined as one of five hallmarks of oxidative stress (Gadjev et al., 2006). An analysis of a large number of microarray data indicated that a gene encoding a protein called BCS1, an ortholog of a protein involved in assembly of cytochrome bc1 in yeast (Saccharomyces cerevisiae; Nobrega et al., 1992), changes transcript abundance in response to at least as many treatments as AOX1a in Arabidopsis (Arabidopsis thaliana; Clifton et al., 2005). In addition, a gene encoding an external NADPH dehydrogenase, NDB2, follows a similar pattern of transcript change to AOX1a in Arabidopsis (Clifton et al., 2005). However, it is unknown if or how the signaling pathways that regulate the transcript abundance for these genes interact and how many signaling pathways exist.

An initial microarray analysis of AOX induction suggested significant overlap in the global pattern of transcript abundance changes between chemical inhibition of mitochondrial function, and abiotic and biotic stresses. This suggests an overlap in the pathways responsible for induction of AOX and stress-responsive genes (Yu et al., 2001). A comprehensive analysis of over 250 arrays supported this initial observation, indicating that induction of AOX transcript occurs under a variety of stress treatments, with a greater response to abiotic stresses (Clifton et al., 2006). In addition, coexpression of NDB2, was observed in almost all cases of AOX1a induction, suggesting co-regulation (Allocco et al., 2004; Clifton et al., 2005, 2006; Elhafez et al., 2006). Biochemical studies suggest there are at least two pathways that signal the induction of

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AOX. These pathways include a reactive oxygen species (ROS) independent pathway, based on the ability of added citrate to induce AOX transcript and protein without any observed increase in ROS in tobacco (Nicotiana tabacum) suspension cells, and an ROS-dependent pathway (Vanlerberghe et al., 1998; Gray et al., 2004). In soybean (Glycine max), a similar conclusion was reached based on the ability of added citrate to induce the expression of AOX1, which was inhibited by the protein kinase inhibitor staurosporine, whereas induction of AOX1 by antimycin A was not sensitive to the addition of staurosporine (Djajanegara et al., 2002).

Although much research has been carried out into the various parameters that can induce AOX (activity, protein, or transcript), there is a scarcity of information of how these varied treatments lead to the induction of AOX at a molecular level (Vanlerberghe and McIntosh, 1997; Finnegan et al., 2004; Rhoads and Subbaiah, 2007). The regulatory regions of genes, generally referred to as promoters, are the end point of any signaling pathway for induction at a transcriptional level. An analysis of the AOX1a promoter from Arabidopsis to determine which regions were responsible for stress response concluded that a 93-bp region, termed the mitochondrial retrograde response (MRR) region, responded to two different treatments, antimycin A and monofluoroacetate (Dojcinovic et al., 2005). This is of interest because antimycin A inhibits complex III and monofluoroacetate inhibits the tricarboxylic acid cycle, thus these two treatments might mimic the ROS and citrate pathways that have been proposed above. It is not known if these treatments act on the same or distinct cis-acting regulatory elements (CAREs) in this 93-bp region and if similar CAREs are present in other genes encoding mitochondrial proteins that are induced under similar circumstances.

In addition to the fact that promoters are the end point of signaling pathways, the response of the promoter reveals the transcriptional response to the treatment, whereas analysis of transcript abundance, protein, or activity levels also incorporates a variety of posttranscriptional regulatory mechanisms. To gain a better understanding of the induction of genes encoding mitochondrial proteins at a transcriptional level, we analyzed the promoter region of Arabidopsis AOX1a to provide a basis to compare the promoters of other stress-responsive genes. AOX1a is the highest expressed AOX gene under untreated conditions in Arabidopsis (Thirkettle-Watts et al., 2003) and the most stress-responsive AOX gene with respect to the number of stresses and the magnitude of response (Clifton et al., 2005). The occurrence and function of CAREs defined in the AOX1a promoter was then analyzed in three other genes encoding mitochondrial proteins that have been observed to be induced with AOX1a after stress treatment (Clifton et al., 2006), NDB2, a gene at locus At2g21640, known as UPOX (up-regulated by oxidative stress), and BCS1. Also the response in transcript abundance of these genes in various defense signaling mutants was determined to investigate which signaling pathways are involved. A cellular context for the induction of AOX1a was obtained by analyzing the occurrence of these elements in the promoters of all genes in Arabidopsis.

RESULTS

The Transcript Abundance of Nuclear Genes Encoding Mitochondrial Proteins Change in Response to a Variety of Treatments

The alterations in transcript abundance for AOX1a and NDB2 upon treatment with H2O2, rotenone, salicylic acid (SA), and citrate have been previously documented (Clifton et al., 2005, 2006). Microarray analyses suggest that transcript abundance for genes encoding other mitochondrial proteins are also altered in response to these treatments (Clifton et al., 2006). The changes in transcript abundance upon treatment for UPOX, BCS1, PR1a (pathogen-related protein 1a, a positive control for response to SA [Shah, 2003]) and ubiquitin (UBC, a gene that is unresponsive to a wide variety of treatments [Czechowski et al., 2005]) were analyzed using quantitative reverse transcription PCR (QRT-PCR; Fig. 1). The data for AOX1a and NDB2 are replotted from Clifton et al. (2005) for comparison and the same samples were used to determine the transcript abundance for the genes outlined above. The QRT-PCR data demonstrate that transcript abundance for UPOX and BCS1 increase with these treatments, however, there are differences in the magnitude and kinetics of the response. In the case of AOX1a, the response to all treatments (except citrate; see below) peaked at 3 h, with transcript abundance increasing approximately 6-fold for treatment with H2O2, rotenone, and SA. The transcript abundance for NDB2 was most similar to that of AOX1a, except that it peaked at 12 h, with increases of 4- to 8-fold evident with H2O2, rotenone, and SA. For both AOX1a and NDB2, transcript abundance decreased at 24 h compared to the peak observed at 3 or 12 h, respectively. In the case of UPOX, transcript abundance for treatment with H2O2 and rotenone was not significantly different at 3 h, but increased afterward with a 20-fold increase observed with rotenone at 24 h. BCS1 differed in that treatment with SA caused, by far, the greatest increase in transcript abundance at all time points, with greater than a 25-fold increase observed at 12 and 24 h. Thus, even though analysis of microarray data at single time points suggest that these four genes are all coexpressed in response to a variety of treatments (Clifton et al., 2006), analysis of the kinetics and magnitude suggest significant differences in the response that may be overlooked in global analysis. This suggests that the induction of transcript abundance for these genes is not identical and that a different regulatory mechanism may be involved.
The transcript abundance of all the genes tested was largely unresponsive to citrate (Fig. 1), with only NDB2 showing barely a 2-fold increase in transcript abundance after 24 h.

Identification of Sequence Elements That Play a Role in the Stress Response in the Promoter of AOX1a

The stress-responsive AOX1a promoter (Dojcinovic et al., 2005) was used as a starting point to identify CAREs that play a role in regulating expression under various treatments. Five prediction programs were used to identify putative regulatory cis-sequence elements in the AOX1a promoter: PlantCare (Rombauts et al., 1999), PLACE signal scan (Higo et al., 1999), AthaMap (Steffens et al., 2004), Athena (O’Connor et al., 2005), and AGRIS (Palaniswamy et al., 2006). These approaches predicted numerous putative sequence elements in the AOX1a promoter region (Clifton, 2006), therefore three methods were used to distill this number with the aim of increasing the likelihood of targeting elements involved in the H2O2 and rotenone response. Firstly, lists of genes that were coexpressed with AOX1a were constructed, allowing elements to be predicted from these coexpressed groups (Clifton, 2006). Secondly, we used a biclustering approach (Holt et al., 2006) to form a list of coexpressed genes from which motifs were predicted that could describe the patterns of transcript abundance observed. Finally, a phylogenetic approach compared the promoter regions of AOX1a to the AOX promoter regions of soybean (Thirkettle-Watts, 2004). An outline of the predicted elements for AOX1a is shown in Supplemental Figure S2. Different methods were used to predict the elements and with no significant experimental data relating to CAREs in the AOX1a promoter, it was not possible to rank or prioritize the elements. Element E, predicted by comparison with the soybean AOX promoter sequences and by the coexpression environments was identified as active in a previous study (Dojcinovic et al., 2005). We tested all the elements identified by phylogenetic comparison: elements A1, A2, B1, B2, E, J, K, and L; biclustering elements B1, B2, and H; and six elements predicted using hierarchical clustering coexpression (elements C, D, F, G, I1, and I2; Fig. 2). This group, therefore, consists of elements predicted by more than one approach: B1 and B2, predicted using biclustering and by comparison with soybean; elements predicted in several coexpression environments (I1 and C); and elements predicted by single approaches (F and G), identified only from coexpression environments; H, identified from biclustering; and elements J and K, identified by comparison to soybean.

Figure 1. Transcript abundance of AOX1a, NDB2, BCS1, UPOX, PR1, and UBC. QRT-PCR analysis of transcript abundance for various genes over 24 h in response to addition of citrate, H2O2, rotenone, and SA to Arabidopsis cell cultures. The amount of transcript prior to addition of compounds was set to 1 and changes expressed in a relative manner. An asterisk indicates a significant difference (P ≤ 0.05) Data for AOX1a and NDB2 are redrawn from Clifton et al. (2005).
Altogether, 15 different elements were tested, representing 12 distinct sequences as three occurred twice in the promoter region analyzed.

Ten of the 15 sequence elements were functional in the \textit{AOX1a} promoter, including two found in multiple locations (A and I), resulting in the identification of 12 functional regulatory motifs (Fig. 3; Supplemental Fig. S3; Table I). A positive role in response to H\textsubscript{2}O\textsubscript{2} treatment was defined for 10 elements (B2, C, D, E, F, G, H, I1, I2, and J). Deletion of these elements abolished or suppressed the increase in GUS activity driven by the \textit{AOX1a} promoter in response to H\textsubscript{2}O\textsubscript{2} treatment. The greatest fold change in this study was observed with the deletion of B2 resulting in a 5-fold increase of GUS activity. Deletion of this element also largely abolished induction of GUS activity by H\textsubscript{2}O\textsubscript{2} with an increase in levels only 20\% of the increase observed in the control. This defines element B2 as a strong repressor under untreated conditions, which can be partially derepressed by H\textsubscript{2}O\textsubscript{2} and to a lesser extent rotenone. Elements C, D, E, F, G, H, I1, I2, and J displayed a similar pattern in that the 50\% increase in GUS activity observed with H\textsubscript{2}O\textsubscript{2} treatment was largely abolished upon deletion. However, deleting these elements had little or no effect on basal levels of GUS activity, determined by comparing untreated samples. Thus, these elements were all defined as positive regulators of the response to H\textsubscript{2}O\textsubscript{2}.

Elements A1, D, F, I1, I2, and J were defined as playing a role in the rotenone response in the \textit{AOX1a} promoter (Fig. 3; Table I; Supplemental Fig. S3). Elements A1 and D act as repressors of the rotenone response as deletion of these elements was accompanied by a greater response to rotenone. Elements F, I1, and J were classified as positive response elements to rotenone on the basis that deletion resulted in a loss of induction of GUS activity in response to rotenone treatment.

The elements A2, E, I1, and I2 acted as an activator and B2 as a repressor under untreated conditions. Deletion of the A2, E, I1, and I2 resulted in the loss of GUS activity whereas deletion of the B2 resulted in an increase in GUS activity.

Functional Elements in \textit{AOX1a} Also Function in Other Genes That Are Coinduced with \textit{AOX1a} under Various Treatments

The promoter regions of \textit{NDB2}, \textit{UPOX}, and \textit{BCS1} were searched for the presence of the CAREs that were functional in \textit{AOX1a}, and promoter fragments containing these elements were cloned and tested for function and response to treatments. Six of the functional elements found in \textit{AOX1a} were also found to be present and functional in the 1-kb upstream region of the transcriptional start site (TSS) of \textit{NDB2}; elements B, C, F, G, H, and I (Fig. 2; Table I). Upon deletion of element B, untreated activity was reduced and the increase in GUS activity in response to H\textsubscript{2}O\textsubscript{2} and rotenone was abolished, indicating this element was a positive element under normal conditions and played a role in the H\textsubscript{2}O\textsubscript{2} and rotenone response. Deleting elements C, F, and H showed no effect on untreated activity, but abolished the H\textsubscript{2}O\textsubscript{2} and rotenone stimulation. The deletion of element G increased basal activity by approximately 30\%, defining it as a repressor, but had no effect on induction by H\textsubscript{2}O\textsubscript{2} and rotenone. Deleting element I reduced basal activity to 50\% or less and the induction observed with H\textsubscript{2}O\textsubscript{2} and rotenone was abolished.

Analysis of the promoter region of \textit{UPOX}, containing overlapping B and I elements, revealed it is responsive to H\textsubscript{2}O\textsubscript{2}, increasing reporter activity by 2.5-fold, however, it was not responsive to rotenone and only slightly responsive to SA (Fig. 4A). Removal of the overlapping B + I element reduced reporter activity to 50\% and induction by H\textsubscript{2}O\textsubscript{2} was abolished. The \textit{BCS1} promoter only contained one common element, H, and the approximately 700-bp region upstream of the TSS of \textit{BCS1} containing this element was cloned and tested. A 2.5-fold response to SA was observed, along with a small but significant response to rotenone but no response to H\textsubscript{2}O\textsubscript{2} (Fig. 4B). Deletion of the H element revealed that it plays no role in driving expression.

The \textit{AOX1a} and \textit{NDB2} promoters showed no response to SA (Fig. 4C). To test for a repressor to SA
induction in the AOX1a and NDB2 promoters, extensive analysis of the effects of SA on the AOX1a and NDB2 promoters was performed. Each predicted CARE was deleted and various lengths of the upstream region were tested, but no response to this treatment was observed (data not shown). Thus, it was concluded using the promoters’ regions that were responsive to H₂O₂ and rotenone that no SA response regions were present. Finally, we tested the effect of citrate on the AOX1a promoter and also detected no increase in promoter activity (data not shown).

H₂O₂ and Rotenone Have Overlapping But Distinct Effects on Transcript Abundance

From the above results, two points suggest that the response to rotenone and H₂O₂ is overlapping but not identical. Firstly, the promoter region of UPOX does not respond to rotenone, yet displays a strong response to H₂O₂. Secondly there are CAREs in the AOX1a promoter that repress the response to rotenone but not to H₂O₂. To investigate this further we reexamined microarray data from Arabidopsis cell cultures treated with rotenone and H₂O₂ (Clifton et al., 2005) to determine the similarity of the response to each treatment. We also carried out microarray analysis of plant leaves treated with H₂O₂ and rotenone for 3 h to identify overlapping and/or distinct responses. This was carried out as there are some differences in alterations of transcript abundances between suspension cells and leaf tissue with reference to the abundance of transcripts from genes that encode proteins involved in ROS metabolism (Umbach et al., 2005). Studies with tobacco suspension cultures report alterations in some antioxidant components associated with mitochondria (Maxwell et al., 1999; Amirsadeghi et al., 2006), whereas with Arabidopsis leaf tissue no changes were observed (Umbach et al., 2005). Additionally tobacco suspension cultures grown under low inorganic phosphate have increased AOX, but leaves from tobacco plants grown under low inorganic phosphate was tested with H₂O₂ (green) and rotenone (blue) treatment. Activities for untreated (light), mock-treated (medium), and treated (dark) samples are shown for H₂O₂ and rotenone. A red asterisk indicates a significant difference (P ≤ 0.05) between the GUS activities of untreated samples with unmutated versus mutated promoter fragments indicating the presence of a constitutive element (positive or negative). A black asterisk indicates a significant difference (P ≤ 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 green asterisk indicates a significant difference (P ≤ 0.05) between the GUS activities of treated samples with unmutated versus mutated promoter fragments indicating that when the element was absent the stress response was compromised. The latter two tests define a significant stress response when the element was present (black asterisk), which is affected when the element was deleted (green asterisk). Data for all six elements are shown for NDB2, the data for elements A1, A2, B1, and B2 are shown for AOX1a, and the data for the other elements tested are shown in Supplemental Figure S3.

**Figure 3.** Functional analysis and stress response of predicted CAREs in the AOX1a and NDB2 promoters. The regulatory characteristics of each predicted CARE was tested by comparing the GUS activity driven by the AOX1a unmutated promoter to the promoter with the element deleted. The unmutated promoter represents the 1.85-kb and 1.0-kb regions upstream of the TSS in AOX1a and NDB2, respectively. The labeling of the element above the graph indicates the sequence element that was deleted from this fragment, as listed in Table I. The activity of the wild-type promoter region (normalized value set to 100%) and corresponding deletion was tested with H₂O₂ (green) and rotenone (blue) treatment. Activities for untreated (light), mock-treated (medium), and treated (dark) samples are shown for H₂O₂ and rotenone. A red asterisk indicates a significant difference (P ≤ 0.05) between the GUS activities of untreated samples with unmutated versus mutated promoter fragments indicating the presence of a constitutive element (positive or negative). A black asterisk indicates a significant difference (P ≤ 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 green asterisk indicates a significant difference (P ≤ 0.05) between the GUS activities of treated samples with unmutated versus mutated promoter fragments indicating that when the element was absent the stress response was compromised. The latter two tests define a significant stress response when the element was present (black asterisk), which is affected when the element was deleted (green asterisk). Data for all six elements are shown for NDB2, the data for elements A1, A2, B1, and B2 are shown for AOX1a, and the data for the other elements tested are shown in Supplemental Figure S3.
Table 1. Summary of the elements tested in the promoters analyzed in this study

The CAREs in the AOX1a promoter are designated by a letter. If a sequence element occurs more than once this is indicated by a number. The position of the sequence element is given upstream of the TSS. A “+” or “−” a positive of negative element in untreated conditions, deleting a positive elements results in a loss of activity, whereas deleting a negative element results in an increase of activity. An “H,” “R,” or “SA” indicates a response to H2O2, rotenone, or SA, respectively, with a “−” or “+” indicating a positive or negative regulator. The position of the as1 element characterized to be responsive to SA is indicated in the BCS1 promoter, but this element was not tested for activity in this study.

<table>
<thead>
<tr>
<th>Motif Designation</th>
<th>Sequence</th>
<th>AOX1a</th>
<th>NDB2</th>
<th>UPOX</th>
<th>BCS1</th>
<th>Previously Reported</th>
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<tbody>
<tr>
<td>A1</td>
<td>TGAAGC</td>
<td>R−</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A2</td>
<td>TGAAGC</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>CGTGAT</td>
<td>1623−1618</td>
<td>+; H+; R+ (846−841)</td>
<td>+; H+; SA+ (95−90)</td>
<td>Rogers et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>CGTGAT</td>
<td>−; H+ (1581−1576)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>ATCCG</td>
<td>H+ (660−656)</td>
<td>H+; R+ (601−597)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>CGGCTTT</td>
<td>H+ (223−217)</td>
<td>H+; R+ (38−378)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E</td>
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<td>H+; R+ (183−177)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>F</td>
<td>TCTCT</td>
<td>H+ (169−165)</td>
<td>− (19−23)</td>
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<tr>
<td>G</td>
<td>GTATC</td>
<td>H+ (143−138)</td>
<td>H+; R+ (876−871)</td>
<td>(463−458)</td>
<td>Eulgem (2005); Kilian et al. (2007)</td>
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<tr>
<td>H</td>
<td>ACGTG</td>
<td>+; H+; R+ (97−93)</td>
<td>+; H+; R+ (52−48)</td>
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<tr>
<td>I1</td>
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<tr>
<td>I2</td>
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<tr>
<td>L</td>
<td>ATAAAC</td>
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<td>as1</td>
<td>TGACG</td>
<td>(34−36)</td>
<td>Lam et al. (1989); Jupin and Chua (1996)</td>
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Regulation of Stress-Induced Mitochondrial Gene Expression

Analysis of Transcript Abundance of Genes Encoding Mitochondrial Proteins Induced under Stress in Signaling Mutant Backgrounds

An alternative approach to characterize the pathways that regulate the expression of mitochondrial proteins under stress is to use mutants compromised in various defense signaling pathways. Transcript abundance of genes encoding mitochondrial proteins was assessed in a variety of these lines, representing the major phytohormone signaling pathways. Specifically, pad4 (phytoalexin-deficient mutant), which acts upstream of SA and is essential for SA-dependent defense pathways (Glazebrook and Ausubel, 1994; Jirage et al., 2001; Glazebrook et al., 2003); npr1 (non-expressor of the PR1 gene) is required for the expression of defense genes and acts downstream of SA (Cao et al., 1994; Shah, 2003); eds4 (enhanced disease susceptibility), which acts downstream of SA (Gupta et al., 2000); ctr1, which confers ethylene insensitivity in Arabidopsis (Chang et al., 1993); jar1, which is deficient in jasmonic acid signaling, particularly associated with defense against insects and necrotrophic pathogens (Staswick et al., 1992; Beckers and Spoel, 2006); abi3, which encodes a viviparous-like transcription factor that interacts with other abscisic acid (ABA)-responsive transcription factors (Giraudat et al., 1992; Nambara et al., 2002); and NahG, plants that express bacterial salicylate hydroxylase that breaks down SA (Lawton et al., 1995; Bowling et al., 1997).

Three expression patterns were observed for the stress-responsive genes encoding mitochondrial proteins (Fig. 6). BCS1 was different from all the other genes, decreasing in eds1, pad4, and NahG plants, consistent with a role for SA signal induction, but

phosphate do not (González-Meler et al., 2001). This analysis indicated that there were more distinct responses for each treatment compared to common responses in both leaves and cells (Fig. 5; Supplemental Table S1). The overlap between H2O2 and rotenone treatments in leaves was not significantly different from what is expected in a random distribution (P < 0.05), but the overlap in cell cultures is significantly higher than expected (P < 0.0001). Altogether, this suggests that the signaling pathways triggered by both treatments may overlap, but are not identical. The difference in response between leaves and suspension cells may be due to the fact that cells may have a different metabolic equilibrium with reference to plant tissue (Umbach et al., 2005), particularly in terms of oxidative stress (Halliwell, 2003).
notably this does not depend on NPR1, defining its induction as SA dependent but NPR1 independent, distinguishing it from PR1 (Uquillas et al., 2004). It was notable that the reduction in transcript for PR1a and BCS1 was also greater in magnitude than for AOX1a, NDB2, and UPOX. In NahG plants, BCS1 transcripts were essentially absent, whereas PR1 was just detectable—both reduced by greater than 100-fold compared to the approximately 5-fold reduction for AOX1a, NDB2, and UPOX. This difference is reflected in the induction of the promoters by SA, the promoter of PR1a previously described to be SA responsive (Yang et al., 2000; Uquillas et al., 2004), and BCS1 in this study. AOX1a differed to NDB2 and UPOX in that it was up-regulated in abundance in pad4 and eds1. However, the increase in transcript abundance of AOX1a, NDB2, and UPOX was similar in some the signaling mutants npr1, jar1, abi3, and etr1.

A Predicted Coregulatory Context for AOX1a

To explore the association between stress response and presence of stress-responsive elements identified in AOX1a, we compiled a list of the genes that contain six or more of the elements defined as functional in this study in their promoters (AGI, 2000), resulting in a list of 1,141 genes. As with any CARE, there is an expected random occurrence of these elements throughout promoter regions genomewide. Assuming there is an equal chance of the occurrence of the four bases in a promoter region (A, T, C, or G) under the search conditions used in this study, there would be an expected occurrence of elements A, B, C, G, and I of at least once in each promoter region genomewide,

Figure 4. Functional analysis of the stress response of promoters of genes coinduced with AOX1a. A, Analysis of the response of the promoter region of At2g21640 (UPOX) to H2O2, rotenone, and SA. The effect of removing the overlapping B and I element was also tested. B, Analysis of the response of the promoter region of At3g50930 (BCS1) to H2O2, rotenone, and SA. The affect of removing the H element was also tested. C, The response of the AOX1a and NDB2 to SA was tested. The labeling and asterisk are as for Figure 3.

Figure 5. Analysis of genes whose transcript abundance changes in response to H2O2 and rotenone in Arabidopsis suspension cell cultures and leaves. The number of significant changes in transcript abundance upon treatment were determined using Arabidopsis ATH1 microarrays. The number of treatment-specific and common changes are indicated. Positive or negative refers to an increase or decrease in transcript abundance.
31,764 promoter sequences (www.arabidopsis.org). The observed occurrence of these elements was significantly less (11,108, 15,674, 16,328, 8,268, and 17,074, respectively), with a P value <0.01 according to a \( \chi^2 \) test, indicating that these elements may be employed to regulate only a subset of genes. Elements H and D are expected to occur in 15,332 promoter regions, with actual occurrences of 9,225 and 13,014, respectively, significantly less than expected, at a P value <0.01. The other elements E, F, and J do not show significant over- or underrepresentation. However, these analyses need to be interpreted with caution as the assumption of equal occurrence for each of the four bases in promoter regions is not necessarily accurate, as promoters are AT rich, and thus, promoters are difficult to model statistically.

Changes in transcript abundance of these 1,141 genes, containing six or more elements, in response to treatments designed to induce oxidative stress were investigated in a number of publicly available microarray data sets (Supplemental Table S2). These included treatment of Arabidopsis plants with cyclohexamide, N-octyl-3-nitro-2,4,6-trihydroxybenzamide, ABA, heat, osmotic stress, salt, ozone, UV light, quartz-filtered UV light, or 2,3,5-triiodobenzoic acid, along with Arabidopsis cell culture treated with rotenone, 

\[ \text{H}_2\text{O}_2, \text{or light, or 2,3,5-triiodobenzoic acid, along with Arabidopsis cell culture treated with rotenone, } \text{H}_2\text{O}_2, \text{ABA, heat, osmotic stress, salt, ozone, UV light, quartz-filtered UV light, or 2,3,5-triiodobenzoic acid, along with Arabidopsis cell culture treated with rotenone,} \]

including members of the MAPKK and MEKK families (Nakagami et al., 2005; Pitzschke and Hirt, 2006), and transcription factors (Chen et al., 2002; McGrath et al., 2005). However, the list of 601 genes lacks other classes of genes associated with stress, such as enzymes involved in oxidative stress, with the exception of one glutathione-S transferase (At2g29440; van Loon et al., 2006; Supplemental Table S2). Of the 285 genes that contained six or more elements that did not show a 1.5-fold change in five treatments, only 121 genes did not respond to any treatment; the others responded to four, three, two, or one treatments (Supplemental Table S2). Analysis of these 121 genes revealed that 106 of them have expression levels too low to be detected across all microarray experiments, whereas the other 15 genes were too low to be detected in 75% of the microarray experiments. In addition, it is possible that some combinations of six of the 12 elements do not act in a combinatorial manner to regulate gene expression in planta.

To investigate any percentile distribution differences in subcellular localization between the list of 601 stress-responsive genes and the whole genome, lists of genes encoding proteins targeted to the mitochondria and the chloroplast were generated (Supplemental Table S2). Lists of 1,025 mitochondrial and 1,407 chloroplastic proteins were isolated, corresponding to 3.22% and 4.48% of this list, respectively. Both mitochondrial and chloroplastic proteins are significantly enriched in the list of 601 genes, at a P value <0.0001 according to a \( \chi^2 \) test, making up 10.98% and 8.4% of this list, respectively.

To verify the role of the promoters of these genes in the transcript response we tested the ability of the promoters of a candidate set of 10 genes to drive the expression of GUS under \( \text{H}_2\text{O}_2 \) treatment. The only criterion used to select genes was that they encode proteins involved in a variety of functions. The promoters tested were for genes encoding transcription factors involved in stress responses (Eulgem, 2005), a ring finger E3 ligase that has been reported to play a role in the hypersensitive response (Kawasaki et al.,
2005), stress-induced proteins predicted to be targeted to mitochondria and chloroplasts, and NIMIN-1, a protein that negatively regulates the activity of NPR1 (Weigel et al., 2005). Transcript analysis of the 10 candidate genes revealed six were up-regulated and four were down-regulated in response to both H$_2$O$_2$ and rotenone (Fig. 7), and analysis of the promoter regions of the 10 candidates revealed that they were all significantly responsive to H$_2$O$_2$ treatment in a manner consistent with changes in their transcript abundance (Fig. 7). Thus, it was concluded that the AOX1a promoter region contains stress-responsive regulatory elements that occur in the promoters of several other stress-responsive genes.

**DISCUSSION**

The expression/induction of AOX is a widely used model to study the MRR (Rhoads and Subbaiah, 2007), as it is induced by a wide variety of perturbations, including application of chemicals that directly affect mitochondrial function (Finnegan et al., 2004), oxidative stress (Rhoads et al., 2006), genetic lesions (Karpova et al., 2002), and the availability of various nutrients (Farsons et al., 1999; Yip and Vanlerberghhe, 2001; Escobar et al., 2006). A number of genes are coexpressed with AOX1a under a variety of conditions; however, their mechanism of induction in relation to AOX1a remains largely unknown (Clifton et al., 2006). The aim of this study was to gain insight into the induction of stress-induced genes encoding mitochondrial proteins, to determine if induction overlapped, and to learn how this may relate to a wider cellular stress response. The promoter regions of four genes were analyzed to determine their ability to respond to a number of treatments and the role of the predicted sequence elements in the various promoters played in this response. The functional elements identified represent a molecular fingerprint of sequence elements that play a role in the response. The element identified may only represent a portion of the sequence element that mediates the response, or interact with other undefined sequence elements to mediate the response. Defining the promoter regions is a somewhat reiterative process and it cannot be ruled out that regulatory regions are missed. However, comparative studies carried out within a single promoter with various treatments and analysis of CAREs and between promoters with differential responses to treatments is informative on the nature of the signals that affect those regions.

Under normal conditions, the AOX1a promoter is under strong repression, as evidenced by the 5-fold increase in activity upon removal of element B2. Removal of this element resulted in a promoter activity that dwarfed the effect of the other elements. It appears that application of stress relieves the repression of AOX1a and allows the positive regulatory activity of the other elements characterized to be exerted, consistent with the combinatorial nature of gene regulation. Elements B and I identified in this study contain an ABRE core, but differ in the flanking sequences that may give different binding specificities. Element B2 and E in AOX1a overlap with an ABD binding site (Koussevitzky et al., 2007) and I1 overlaps with a G-box binding site, defined as a light responsive cis-element (Menkens et al., 1995; Terzaghi and Cashmore, 1995). This provides a direct mechanism through which AOX1a can be regulated and/or co-regulated with photosynthetic function, consistent with previous reports that AOX expression is under diurnal control (Dutilleul et al., 2003). This I1 element overlaps with an ABRE core, and is in close proximity to another ABRE element, I2, forming an ABRE-ABRE pair (Gomez-Porras et al., 2007), that is proposed to mediate ABA-dependent gene regulation. It has been demonstrated that the expression of 2-cys peroxiredoxin, a well-studied model for transcriptional regulation of a gene encoding a chloroplast antioxidant enzyme, correlates with the acceptor availability of PSI and analysis of the promoter activity in ABA-biosynthetic or ABA-insensitive mutants reveals cross talk between redox and ABA signaling downstream of ABI1 and ABI2 (Baier et al., 2004). Subsequently it was shown that this is mediated by a CE3 element in this promoter that binds Rap2.4a (Shaikhkali et al., 2008). The presence of the ABRE-ABRE core in the AOX1a promoter (I1 and I2) and ABRE elements in the functional B2 element provide a means to link the expression of chloroplast antioxidant enzymes and AOX1a, providing a molecular link between expression of AOX1a and chloroplast function that has been revealed in many studies (Noguchi and Yoshida, 2008). As the elements in the 2-cys peroxiredoxin differ in sequence to those in the AOX1a promoter, different ABA response factors may be involved in binding AOX1a.

Several of the 10 distinct promoter elements (CAREs) found to be active in controlling AOX1a gene expression were also functional in the NDB2 and UPOX promoters. Many but not all of the elements characterized were responsive to both H$_2$O$_2$ and rotenone. The response of the UPOX promoter to H$_2$O$_2$ is consistent with it being defined as a marker to oxidative stress (Gadjev et al., 2006), yet this promoter was largely unresponsive to rotenone. The findings that the promoter of AOX1a has CAREs that suppress rotenone but not the H$_2$O$_2$ response and that all CAREs in AOX1a and UPOX are not equally responsive to rotenone and H$_2$O$_2$ suggests that they illicit at least some different responses. Furthermore, the AOX1a promoter contains CAREs not found in UPOX or NDB2 and two of these CAREs, A1 and D, repress the response to rotenone. As inhibition of complex I requires alternative routes to oxidize NADH but not necessarily a dramatic increase in AOX activity, the regulating elements are consistent with the biological functions of the encoded proteins. The analysis of transcriptome changes in response to rotenone and
The response of promoter regions that contain sequence elements defined as functional in AOX1a. Determination of the promoter-driven response to H$_2$O$_2$ of 10 genes, each containing six or more sequence elements defined as functional in AOX1a. Six genes whose transcript abundance is up-regulated after H$_2$O$_2$ treatment and four genes whose transcript abundance is down-regulated after H$_2$O$_2$ were tested. All promoters displayed a significant response in agreement with the trend observed in transcript abundance. A black asterisk indicates a significant difference ($P \leq 0.05$) when comparing the GUS activity between mock-treated and treated samples. Activities for untreated (light), mock-treated (medium), and treated (dark) samples are shown for H$_2$O$_2$. A schematic representation of each promoter is shown with the region in base pair or kilobase pair upstream of the TSS shown. The relative position of the occurrence of the different sequence elements in each promoter tested are indicated. The heat map displays the changes in transcript abundances for the genes whose promoters were tested for responsiveness to H$_2$O$_2$. A red color indicates up-regulation and green indicates down-regulation; the transcript changes for AOX1a, NDB2, and UPOX are included as a comparison. The genes are arranged numerically by AGI code. The At1g02450, At3g50870, and At1g72200 transcript levels were called absent after MAS5.0 normalization.

**Motifs and Sequence Elements**
- **motif A**: TGAAGC
- **motif B**: CGTAT
- **motif C**: ATCCG
- **motif D**: CACACA
- **motif E**: CGGCTTT
- **motif F**: TCGTAAA
- **motif G**: TCTCT
- **motif H**: GTCATC
- **motif I**: ACGTG
- **motif J**: TTCGATCA

**Legend**
- Rotenone
- H$_2$O$_2$
- Unknown Predicted Chloroplast
- Unknown Predicted Mitochondria
- Glycosyl Hydrolase
- Senescence Associated protein
- Wound-responsive protein-related
- UPOX
- AOX1a
- NDB2
- WRKY15
- AOX1a
- UPOX
- Wound-responsive protein-related


Figure 8. Summary of signaling pathways that regulate stress-induced transcript abundance for genes encoding mitochondrial proteins. Three distinctive pathways regulate mitochondrial stress response at a transcriptional level, an SA-dependent pathway represented by BCS1. A second pathway that represents a convergence point for signals generated by H$_2$O$_2$ and rotenone that act via the same CAREs. 

H$_2$O$_2$ using microarrays reveals overlapping and distinct effects. Thus, these pathways may overlap at two levels; firstly, rotenone may trigger ROS production (van der Merwe and Dubery, 2006) and secondly, the signaling pathways converge to act on shared CAREs. As transcription factors are encoded by large gene families that can bind similar or identical CAREs, it cannot be concluded that these pathways act on the same transcription factors, only that the transcriptional responses share CAREs.

Although the application of the phytohormone SA increases transcript abundance to the same extent as H$_2$O$_2$ and rotenone (Clifton et al., 2005), the AOX1a and NDB2 promoters were unresponsive to this treatment. The small but significant response of the UPOX promoter to SA may be due to the fact that addition of SA to cells results in an inhibition of electron transport via both the alternative and cytochrome pathways (Norman et al., 2004) and is therefore likely to produce a level of oxidative stress. The sensitivity of UPOX is consistent with the promoter being the most responsive to H$_2$O$_2$, and its definition as a marker for oxidative stress (Gadjev et al., 2006). The difference in the changes in transcript abundance in NahG plants between AOX1a, NDB2, and UPOX (which were reduced by approximately 5-fold) compared to BCS1 (which was reduced greater than 100-fold) support a different transcriptional level, with isolated nuclei indicated no change in the rate of transcription, also leading to the conclusion that posttranscriptional mechanisms were involved (Rhoads and McIntosh, 1992). Examination of other regions upstream of the AOX1a promoter region used in these studies revealed no evidence of any characterized SA-responsive elements, such as the as1 element that is present in the BCS1 promoter region (Lam et al., 1989; Jupin and Chua, 1996). It is well established in mammalian systems that hormones have transcriptional and posttranscriptional effects on regulating transcript abundance (Ing, 2005) and that zinc finger transcription factors can bind both DNA and RNA in mammalian systems (Clemens et al., 2003; Lu et al., 2003). Finally, it cannot be ruled out that there are other elements that control the SA response for AOX1a, NDB2, and UPOX.

A similar response of AOX1a, NDB2, and UPOX in the mutants etr1, abi3, jar1, and npr1 further support the model that they share common components in signaling pathways that regulate their expression, distinct from BCS1. The exact effects of these mutants on altering transcript abundance cannot be determined from these studies due to the complexity of interactions between various phytohormone signaling pathways (Fujita et al., 2006). However, it was evident that the transcript abundance of AOX1a only increased in the pad4 and eds1 mutants, indicating that it is regulated distinctly by these components compared to the other genes examined in this study. Although PAD4 and EDS1 act upstream of SA to regulate SA signaling (Brodersen et al., 2006), the fact that only the AOX1a transcript increases, suggests an SA-independent induction of AOX1a in these mutants. Such a pathway has been described for the regulation of various transcripts in pad4 and eds1 (Bartsch et al., 2006). Notably,
these proteins play a central role in mediating programmed cell death conditioned by Toll-like receptors. A role for AOX in modulating programmed cell death is well described in tobacco lines that have altered AOX levels (Ordog et al., 2002; Robson and Vanlerberghe, 2002; Amirsadeghi et al., 2006). Thus, the up-regulation of AOX1a expression compared to NDB2 and UPOX. In plants deficient in the prohibitin AtPHB3, an inner mitochondrial membrane protein involved in mitochondrial respiration and morphology, the expression of AOX1a, UPOX, and BCS1, but not NDB2, is strongly induced, further demonstrating that multiple combinations of signaling pathways are possible (Van Aken et al., 2007).

CONCLUSION

The integrative use of promoter activity information, transcript analysis, and mutants in specific signaling cascades provides a solid base for dissecting the intricate mechanisms underlying gene expression under stress conditions. Based on our results, we propose that the mitochondrial stress response is mediated by at least three distinct pathways at the transcriptional level (Fig. 8): an SA-dependent pathway, a second pathway that converges on a number of CAREs, previously characterized to bind ABA-responsive transcription factors, and a third pathway that acts via ED51 and PAD4 regulating AOX1a. Furthermore, we propose from the analysis of the promoters and transcripts in this study that SA acts to increase transcript abundance for AOX1a, NDB2, and UPOX via a posttranscriptional mechanism (Fig. 8), whereas BCS1 is under direct transcriptional control. H2O2 and rotenone can act at a transcriptional level, acting on an overlapping set of CAREs. Because rotenone cannot induce the promoter of UPOX, and transcript abundance for UPOX peaks at 24 h after treatment with rotenone, this suggests that different sets of transcription factors are involved in the targeting of CAREs by rotenone and H2O2. Additionally, because the transcript abundance of AOX1a is affected differently compared to NDB2 and UPOX in the pad4 and eds1 signaling mutants, it suggests an additional pathway, possibly involved in regulating programmed cell death. The identification of CAREs involved in the expression of these genes provides links to the expression of chloroplast antioxidant enzymes and to cellular stress responses because identical or similar CAREs have been characterized previously in these pathways.

MATERIALS AND METHODS

Cloning of Arabidopsis thaliana Promoter Regions

The promoter regions were cloned using standard protocols and subcloned into pLUS. The numbering of Arabidopsis (Arabidopsis thaliana) promoters are given from the TSS as determined from the SIGAL database (Yamada et al., 2003). Initially the 1.85-kb region upstream of the TSS of AOX1a was used based on the fact that small regions of this promoter had previously been shown to be responsive (Dojcimovic et al., 2005). The lengths of the other promoter regions used were based on the presence of overlapping CAREs with AOX1a. Thus, a 1-kb region, 442-bp region, and 689 bp region upstream of the TSS was used for NDB2, UPOX, and BCS1, respectively. The constructs were made as translational fusions with GUS, with the first ATG of the gene of interest used as the start codon for GUS. Thus, the different promoters regions also contained different lengths of 5’ untranslated regions (UTRs), 98 bp for AOX1a (At3g22370), 131 bp for NDB2 (At4g5020), 49 bp for UPOX (At2g21640), and 291 bp for BCS1 (At3g50930; 49 bp comprises a UTR, an additional 478 bp comprises an intron). In the case of the other analyzed 10 promoters that contained six or more CAREs in common with AOX1a, a similar criterion was used to determine the size of the region upstream of the TSS cloned. At1g02460 contained a 5’ UTR of 36 and 995 bp upstream of the TSS, At1g13990 contained a 54-bp 5’ UTR and 1.4 kb upstream of the TSS, At1g70420 contained a 104-bp 5’ UTR and 1.3 kb upstream of the TSS, At1g27200 contained a 172-bp 5’ UTR and 1.2 kb upstream of the TSS, At2g23220 contained a 65-bp 5’ UTR and 1.6 kb upstream of the TSS, At3g50870 contained a 122-bp 5’ UTR and 1.1 kb upstream of the TSS, At1g75730 contained a 684-bp 5’ UTR and 1.8 kb upstream of TSS, At2g01630 contained a 168-bp 5’ UTR and 1.1 kb upstream of the TSS, At2g17580 contained a 35-bp 5’ UTR and 983 bp upstream of TSS, and At4g28240 contained a 108-bp 5’ UTR and 1.6 kb upstream of the TSS. A full list of primers is shown in Supplemental Table S3.

Construction of pLUS

The pLUS vector was created as follows. The LUC+ gene, flanked by the omega translational enhancer (5’ ) and E93’ terminator (3’; Walsh et al., 2005), was cloned downstream of the 35S CaMV promoter in pGEM3Zf(+) (Promega). 35S CaMV GUS from pCAMBIA1301 (http://www.cambia.org/daisey/cambia/585.htm) was then cloned into pGEM3Zf(+) downsteam of the multiple cloning site, in the opposite orientation to 35S CaMV-LUC(+) (Supplemental Fig. S1).

Plant Material Growth and Treatments

Suspension cell culture from Arabidopsis (ecotype Landsberg erecta) leaf tissue was obtained from (Sweetlove et al., 2002). Cells were grown under 16-h light conditions (100 μE m-2 s-1) and 8-h dark. Cells were subcultured (1:6 [v:v]) at 7-d intervals to maintain growth. Four-day-old cells were treated 1 h prior to collection for transient biosilic acid transformation, as described in Clifton et al. (2005). A final concentration of 40 μM was used for rotenone, 100 μM for SA, and 10 μM for H2O2 (Clifton et al., 2005). All Arabidopsis mutant lines were obtained from the Arabidopsis Biological Resource Center at The Arabidopsis Information Resource (TAIR). Defense signaling mutants are: pad4, CS5806; edd4, CS3799; npr1, CS3726; ab13, CS24; jar1, CS8072; and ctr1, CS6374. NAG seed was kindly donated by Professor Xing Wang Deng from Yale University.

Biologic Transformation and Assays for Luc and GUS

Transformation was performed using the PDS-1000 system using the Hepta adaptor according to the manufacturer’s instructions (Bio-Rad). After transformation, Arabidopsis suspension cell culture was incubated at 22°C for 24 h under long light conditions of 16 h at approximately 100 μE m-2 s-1 light conditions and 8 h of dark on paper discs on osmoticum media. Temporarily transformed cells were harvested 24 h after bombardment, disrupted by grinding in a mortar and pestle under liquid nitrogen and cellular contents extracted with the lysis buffer and protocol supplied with the luciferase assay system kit (Roche). Luciferase activity assays were carried out according to the manufacturer’s instructions and activity was measured at 2-s intervals over 20 s, using the Polarstar Optima (BMG Labtech). GUS activity was determined using the fluorometric GUS assay (Jefferson et al., 1987). Assay samples were taken over 1 h at 3-min intervals. Fluorescence was measured using the Polarstar Optima (BMG Labtech) with excitation at 355 nm and emission at 460 nm. The normalized GUS activity was determined by dividing the GUS fluorescence value by the luciferase activity value for each sample, thus eliminating variation due to transformation efficiency. A minimum of
nine replicate bombardments were carried out per construct. GUS activities measured for each construct were determined and expressed as a percentage and normalized to control conditions.

In the case of the \textit{AOX1a} promoter, the region 1.85 kb upstream of the TSS was taken as previously 1.3 kb upstream of the TSS had been shown to be responsive to some treatments (Dojcinovic et al., 2005). To test the function of various elements the 5- to 6-bp elements were deleted. In the case of the other promoters, regions that contained the elements defined as functional in \textit{AOX1a} were cloned and elements deleted.

For comparison of the GUS activities of the motif deletions with those 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 had 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 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 GUS activities of treated samples with unmutated versus mutated promoter fragments indicating that when the element was absent the stress response was compromised. A green asterisk indicates a significant difference (\( P \leq 0.05 \)).

### Predicting Functional Elements in the Promoter Region of Arabidopsis \textit{AOX1a}

Three approaches were used to predict functional elements in the promoter region of \textit{AOX1a}, defined as the sequences upstream of the TSS. These approaches were: (1) defining a common coexpression environment for \textit{AOX1a} and \textit{NDB2} (marked in red; Supplemental Fig. S2), for \textit{AOX1a} (marked in blue; Supplemental Fig. S2) and using the promoter regions of the genes within the coexpression environments to predict putative sequence elements. Briefly, six coexpression environments were created using three linkage methods (average, centroid, and complete) and two distance metrics (Euclidean and Pearson). Elements were identified in the coexpression environments using the PLACE signal scan (Higo et al., 1999) and PlantCare (Lesco et al., 2002). Additionally, the promoter regions of \textit{AOX1a} and \textit{NDB2} alone were also used to predict elements (marked in green; Supplemental Fig. S2). This analysis was restricted to 1,000 bp upstream of the TSS to restrict the number of elements predicted. (2) A biclustering approach was used with a list of genes encoding mitochondrial proteins (Holt et al., 2006); and (3) a phylogenetic approach compared the promoter region of \textit{AOX1a} to the promoter regions for soybean (\textit{Glycine max}) \textit{AOX} genes (Thirkettle-Watts et al., 2003). A comparative genomics approach was used to compare the soybean and Arabidopsis promoter regions to identify putative regulatory sequence elements using MotifSampler (Thijs et al., 2002; http://www.esat.kuleuven.ac.be/\~{}thijs/work/motifSampler.html) and Improbizer (http://www.cse.ucsc.edu/\~{}kent/improbizer/improbizer.html). By limiting the comparisons to functional regions previously defined in the soybean promoter regions (Thirkettle-Watts et al., 2003), the background noise created by large amounts of nonfunctional DNA sequence is reduced, increasing the likelihood of identifying functional regulatory sequences (Zheng et al., 2003). These comparisons predicted different but some overlapping elements, which are shown in Figure 2.

### QRT-PCR Analysis of Gene Expression

QRT-PCR was performed on Arabidopsis leaf tissue from various signal-mutant lines, \textit{pad4}, \textit{npr1}, \textit{abi3-5}, \textit{NahG}, \textit{jar1}, \textit{etr1}, and \textit{eds4}. Leaf tissue was excised from 3-week-old mutant and ecotype Columbia of Arabidopsis (Col-0) plants. Samples were taken in biological triplicate and snap frozen under 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. The \( \delta \) was calculated for every data point. Transcript abundance for Col-0 untreated sample was normalized to one for each gene with all other values presented as relative transcript abundance. QRT-PCR primers used for the genes \textit{AOX1a} (At3g22370) and \textit{NDB2} (At4g50920) have been previously described (Clifton et al., 2005). QRT-PCR primers used for the genes \textit{UPOX} (At2g21640), \textit{BCSI} (At3g50930), \textit{PRI} (At2g14610), and \textit{UBC} (At1g25760) have been described previously (Giraud et al., 2008).

### Defining a Putative Coregulatory Environment for \textit{AOX1a}

For each functional sequence element identified in the \textit{AOX1a} promoter in this study, a gene list was generated containing all Arabidopsis genes with these sequences in their upstream regions using the Patmatch function on the TAIR Web site (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl). Each element nucleotide sequence was searched against Locus Upstream Sequences 1,000 bp (DNA) with the exception of elements A and B, which were searched against Locus Upstream Sequences 3,000 bp (DNA) because these elements occurred more than 1,000 bp upstream of the TSS in \textit{AOX1a}. Elements were searched in both DNA strands (forward direction and reverse complement), with no mismatch allowed and a maximum of one hit per sequence. Once gene lists were generated, they were compiled such that the overlap, i.e. genes containing more than one element, could be identified. Lists of mitochondrial and chloroplast targeted proteins were generated using the SUBA database (http://www.planetenergy.uwa.edu.au/applications/suba2/index.php), searching for organellar proteins confirmed via experimental methodology.

### Calculating Expected Random Occurrences of Motifs

Assuming each base, A, T, C, or G, has an equal chance of being incorporated into an element within the promoter region, the expected random occurrence of any motif of defined length in a given promoter region, as searched in this study would be: 

\[
\text{Expected random occurrence} = \frac{\text{Length of motif in bp}}{4^{\text{Length of element in bp}}} 
\]

\( \times 2 \), as the elements were searched in either direction in double-stranded DNA.

### Microarray Analysis

Analysis of the changes in transcript abundance in 4-week-old Arabidopsis Col-0 plants treated with 10 mM \( \text{H}_2\text{O}_2 \) or 40 \( \mu \text{M} \) rotenone was performed using Affymetrix GeneChip Arabidopsis ATH1 genome arrays (Affymetrix). Leaves excised from Col-0 plants were submerged in solutions of \( \text{H}_2\text{O}_2 \), rotenone, or a water control for 1 h before being snap frozen. All samples were collected in triplicate. For each replicate, total RNA was isolated from the leaves of two plants using the RNaseasy Plant Mini Protocol (QUIAGEN). The quality of the RNA was verified using a Bioanalyzer (Agilent Technologies) and spectrophotometric analysis to determine the \( A_{260} \) to \( A_{280} \) ratio. Preparation of labeled cRNA from 5 \( \mu \)g of total RNA, target hybridization, as well as washing, staining, and scanning of the arrays was carried out exactly as described in the Affymetrix GeneChip Expression Analysis Technical Manual, using an Affymetrix GeneChip Hybridization Oven 640, an Affymetrix Fluidics Station 450, and a GeneChip Scanner 3000 7G at the appropriate steps. Data quality was assessed using GCOS 1.4 before CEL files were exported into AVADIS Prophetic (Version 4.3; Strand Life Sciences) for further analysis. As an additional comparison, raw signal data from microarrays performed previously for Arabidopsis cell culture treated with 10 mM \( \text{H}_2\text{O}_2 \) or 40 \( \mu \text{M} \) rotenone along with an untreated control (Clifton et al., 2005) were analyzed using the program AVADIS Prophetic (Version 4.3; Strand Life Sciences). All CEL files were subjected to GC-robust multirarray average (GC-RMA) normalization, a variation of the RMA normalization algorithm. Fluorescence intensities were log transformed and correlation plots were examined between replicates using the Scatter Plot function, and in all cases \( r = 0.9 \) (data not shown). Fold changes for untreated/mock-treated versus stress-treated cell culture and leaf tissue were calculated using the Differential Expression Analysis function and \( P \) values were calculated using an unpaired \( t \) test. \( P \) values were given as uncorrected and after \( P \)-value correction for estimation of false discovery rate in multiple comparisons. A method of false discovery rate correction that is based on the Benjamini and Hochberg method was used (Benjamini and Hochberg, 1995; Nettleton, 2006). This method utilizes an add-on in the software package R to calculate \( q \) values based on \( P \)-value distributions (http://www.r-project.org; Storey, 2002). Changes were considered significant with a \( q \) value \(< 0.1 \). Transcripts significantly up- or down-regulated after treatment with \( \text{H}_2\text{O}_2 \) or rotenone for cells and plants were plotted on two-way
Venn diagrams such that the overlap in transcripts with altered abundance could be determined. A χ-square statistical test using the expected overlap in a random distribution (no. of significantly changed probe sets in set 1 × no. of significantly changed probe sets in set 2 / no. of probe sets on ATH1 chips) or by random sampling was applied to test the significance of overlap between the two treatments. For the analysis of publicly available microarray data, data was downloaded from the TAIR Web site (ftp://ftp.arabidopsis.org/home/tair/microarrays/datasets/) for the treatments N-octyl-3-nitro-2,4,6-trihydroxybenzamide, cyclohexamide, ABA, 2,3,5-triodobenzoic acid, osmotic, salt, heat, and UV-B, which are found in the following directories: ME00363, ME00333, ME00338, ME00327, ME00328, ME00339, and ME00329, respectively. Access to the microarray data for the treatment of Arabidopsis cell culture with rotenone, SA, and H2O2 is outlined in Clifton et al. (2005) and the treatment of plants with quartz-filtered UV light is outlined in Ulm et al. (2004). CEL files for the treatment of plants with ozone were kindly provided by Alan D Shirras (Lancaster University). Details of the treatments and growth conditions used can be obtained from the original studies. CEL files were subjected to a MAS5.0 normalization to generate present/absent calls and a GC-IRA normalization to summarize fluorescent intensity values for further analysis. Probe sets that were determined as absent across all of the chips were removed and fold-change values were calculated as described above for each control-versus-treatment condition, for a set of 886 genes with six or more motifs. Additionally, a fold change cutoff of 1.5-fold was applied, and changes less dramatic than this were removed.

Supplemental Data

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

Supplemental Figure S1. Diagrammatic representation of the biotic stimulation transformation vector pLUS.

Supplemental Figure S2. The 1,850 bp upstream of the AOX1a TSS.

Supplemental Figure S3. Functional analysis and stress response of predicted CAREs in the AOX1a.

Supplemental Table S1. Transcripts that are significantly changing at a q value of <0.1 in Arabidopsis leaf tissue treated with either H2O2 or rotenone compared to mock-treated plants.

Supplemental Table S2. Analysis of changes in transcript abundance for the list of 1,441 genes with six or more elements in the upstream regions in response to treatments designed to induce oxidative stress.

Supplemental Table S3. Primers for cloning of promoter regions and deletion of CAREs.

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LITERATURE CITED


Regulation of Stress-Induced Mitochondrial Gene Expression

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