Running Title: Regulation of stress induced mitochondrial gene expression

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Identification of regulatory pathways controlling gene expression of stress responsive mitochondrial proteins in Arabidopsis

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Abbreviations

ABA  abscisic acid
AOX  alternative oxidase
BCS1 cytochrome bc₁ synthesis
CARE cis-acting regulatory elements
GUS  β-glucuronidase
H₂O₂ Hydrogen peroxide
LUC  luciferase
MRR  mitochondrial retrograde response region
NDB2 external alternative NAD(P)H dehydrogenase b2
PR  pathogen related
QRT-PCR Quantitative reverse transcriptase PCR
ROS reactive oxygen species
SA  salicylic acid
UBC  ubiquitin
UPOX up-regulated by oxidative stress
yeast Saccharomyces cerevisiae
Abstract

In this study we analysed transcript abundance and promoters of genes encoding mitochondrial proteins to identify signalling pathways that regulate stress-induced gene expression. We used Arabidopsis alternative oxidase AOX1a, external NAD(P)H-dehydrogenase NDB2 and two additional highly stress-responsive genes, At2g21640 and BCS1. As a starting point, the promoter region of AOX1a was analysed and functional analysis identified ten 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 signalling 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), while the promoters of AOX1a and NDB2 were unresponsive to SA. Analysis of transcript abundance of these genes in a variety of defence signalling mutants confirmed that BCS1 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 BCS1, 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, post-transcriptional regulation accounts for changes in transcript abundance by SA treatment for some genes.
Introduction

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 (Vanlerberghe and McIntosh, 1997; Finnegan et al., 2004; Clifton et al., 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 orthologue of a protein involved in assembly of cytochrome bc1 in yeast (Nobrega et al., 1992), changes transcript abundance in response to at least as many treatments as AOX1a in Arabidopsis (Clifton et al., 2006). Also a gene encoding an external NAD(P)H 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 signalling pathways that regulate the transcript abundance for these genes interact and how many signalling 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, co-expression of NDB2, was observed in almost all cases of AOX1a induction, suggesting co-regulation (Allocco et al., 2004; Clifton et al., 2005; Clifton et al., 2006; Elhafez et al., 2006). Biochemical studies suggest there are at least two pathways that signal the induction of 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 suspension cells, and a ROS dependent pathway (Vanlerberghe et al., 1998; Gray et al., 2004). In soybean, 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 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 signalling 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 region (MRR), responded to two different treatments, antimycin A and monofluoroacetate (Dojcinovic et al., 2005). This is of interest as antimycin A inhibits Complex III and monofluoroacetate inhibits the TCA 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 signalling pathways, the response of the promoter reveals the transcriptional response to the treatment, whereas analysis of transcript abundance, protein or activity levels also incorporate a variety of post-transcriptional regulatory mechanisms. To gain a better understanding of the induction of genes encoding mitochondrial proteins at a transcriptional level, we analysed 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 magnitude of response (Clifton et al., 2005). The occurrence and function of CAREs defined in the AOX1a promoter was then analysed 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 “up-regulated by oxidative stress” (UPOX) and BCS1. Also the response in transcript abundance of these genes in various defence signalling mutants was determined to investigate which signalling pathways are involved. A cellular context for the induction of AOX1a was obtained by analysing 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; Clifton et al., 2006). Microarray analyses suggests 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 analysed using quantitative RT-PCR (QRT-PCR) (Figure 1). The data for AOX1a and NDB2 are re-plotted 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 afterwards 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 co-expressed 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 different regulatory mechanism may be involved.

The transcript abundance of all the genes tested was largely unresponsive to citrate (Figure 1), with only *NDB2* showing barely a two-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 distil this number with the aim of increasing the likelihood of targeting elements involved in the H$_2$O$_2$ and rotenone response. Firstly, lists of genes that were co-expressed with *AOX1a* were constructed, allowing elements to be predicted from these co-expressed groups (Clifton, 2006). Secondly, we used a bi-clustering approach (Holt et al., 2006) to form a list of co-expressed 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 Supplementary Figure 2. 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 prioritise the elements. Element E, predicted by comparison with the soybean AOX promoter sequences and by the co-expression 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; bi-clustering elements B1, B2, H; and six elements predicted using hierarchical clustering co-expression: elements C, D, F, G, I1, and I2 (Figure 2). This group therefore consists of elements predicted by more than one approach: B1 and B2, predicted using bi-clustering and by comparison with soybean; elements predicted in several co-expression environments: I1 and C; and elements predicted by single approaches: F and G, identified only from co-expression environments; H, identified from bi-clustering; and elements J and K, identified by comparison to soybean. Altogether, fifteen different elements were tested, representing twelve distinct sequences as three occurred twice in the promoter region analysed.

Ten of the fifteen sequence elements were functional in the AOX1a promoter, including two found in multiple locations (A and I), resulting in the identification of twelve functional regulatory motifs (Figure 3, Supplementary Figure 3, Table 1). A positive role in response to H2O2 treatment was defined for ten 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 AOX1a promoter in response to H2O2 treatment. The greatest fold change in this study was observed with the deletion of B2 resulting in a five fold increase of GUS activity. Deletion of this element also largely abolished induction of GUS activity by H2O2 with an increase in levels only 20% of the increase observed in the control. This defines element B2 as a strong repressor under normal conditions, which can be partially de-repressed by H2O2 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 hydrogen peroxide 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$_2$O$_2$.

Elements A1, D, F, I1, I2 and J were defined as playing a role in the rotenone response in the AOX1a promoter (Figure 3, Table 1, Supplementary Figure 3). 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 AOX1a also function in other genes that are co-induced with AOX1a under various treatments

The promoter regions of NDB2, UPOX and BCS1 were searched for the presence of the CAREs that were functional in AOX1a, and promoter fragments containing these elements were cloned and tested for function and response to treatments. Six of the functional elements found in AOX1a were also found to be present and functional in the 1 Kb upstream region of the transcriptional start site of NDB2; elements B, C, F, G, H and I (Figure 2, Table 1). Upon deletion of element B, untreated activity was reduced and the increase in GUS activity in response to H$_2$O$_2$ and rotenone was abolished, indicating this element was a positive element under normal conditions and played a role in the H$_2$O$_2$ and rotenone response. Deleting elements C, F and H showed no effect on untreated activity, but abolished the H$_2$O$_2$ 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$_2$O$_2$ and rotenone. Deleting element I reduced basal activity to 50% or less and the induction observed with H$_2$O$_2$ and rotenone was abolished.

Analysis of the promoter region of UPOX, containing overlapping B and I elements, revealed it is responsive to H$_2$O$_2$, increasing reporter activity by
2.5 fold, however it was not responsive to rotenone and only slightly responsive to SA (Figure 4A). Removal of the overlapping B+I element reduced reporter activity to 50% and induction by H₂O₂, was abolished. The BCS1 promoter only contained one common element, H and the ~700 bp region upstream of the transcriptional start site of 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 H₂O₂, (Figure 4B). Deletion of the H element revealed that it plays no role in driving expression.

The AOX1a and 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).

Hydrogen peroxide 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 re-examined 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 anti-oxidant 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 do not (Gonzalez-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 (Figure 6, supplementary table 1). The overlap between H$_2$O$_2$ 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 signalling 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).

**Analysis of transcript abundance of genes encoding mitochondrial proteins induced under stress in signalling mutant backgrounds**

An alternative approach to characterise the pathways that regulate the expression of mitochondrial proteins under stress is to use mutants compromised in various defence signalling pathways. Transcript abundance of genes encoding mitochondrial proteins was assessed in a variety of these lines, representing the major phytohormone signalling pathways. Specifically; *pad4* (phytoalexin deficient mutant), which acts upstream of SA and is essential for SA-dependent defence pathways (Glazebrook and Ausubel, 1994; Jirage et al., 2001; Glazebrook et al., 2003); *npr1* (non-expressor of *PR1* gene) required for the expression of defence 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); *etr1* which confers ethylene insensitivity in Arabidopsis (Chang et al., 1993); *jar1* which is deficient in jasmonic acid signalling, particularly associated with defence 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 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 (Figure 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 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, while PR1 was just detectable – both reduced by > 100 fold compared to the ~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 signalling mutants npr1, jar1, abi3 and etr1.

A predicted co-regulatory 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 6 or more of the elements defined as functional in this study in their promoters (AGI, 2000), resulting in a list of 1141 genes. As with any CARE, there is an expected random occurrence of these elements throughout promoter regions genome wide. Assuming there is an equal chance of the occurrence of the 4 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 genome wide, 31764 promoter sequences (www.arabidopsis.org). The observed occurrence of these elements was significantly less (11108, 15674, 16328, 8268 and 17074 respectively), with a p value < 0.01 according to a
chi-squared test, indicating that these elements may be employed to regulate only a subset of genes. Elements H and D are expected to occur in 15332 promoter regions, with actual occurrences of 9225 and 13014, respectively, significantly less than expected, at a p value <0.01. The other elements E, F and J do not show significant over or under representation. However, these analyses need to be interpreted with caution as the assumption of equal occurrence for each of the 4 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 1141 genes, containing 6 or more elements, in response to treatments designed to induce oxidative stress were investigated in a number of publicly available microarray data sets (Supplementary Table 2). These included treatment of Arabidopsis plants with cyclohexamide, N-octyl-3-nitro-2,4,6-trihydroxybenzamide (PNO8), ABA, heat, osmotic stress, salt, ozone, UV light, quartz filtered UV light or tri-iodobenzoic acid (TIBA), along with Arabidopsis cell culture treated with rotenone, H$_2$O$_2$ or SA. Data files were downloaded from (ftp://ftp.arabidopsis.org/home/tair/Microarrays/Datasets/), normalised and the fold-changes in transcript abundance for these 1141 genes were determined. Transcripts were considered to be responsive to oxidative stress if they showed a greater than 1.5 fold-change in response to 5 or more of the treatments investigated. Of these 1141 genes, 255 are not represented on the Arabidopsis ATH1 gene chip, leaving a set of 886 genes. Considering the criteria for defining a transcript as stress responsive, 601 out of these 886 genes were responsive to oxidative stress (Supplementary Table 2). These include genes encoding proteins previously reported to play roles in biotic and abiotic stress responses, such as leucine rich repeat transmembrane protein kinase genes (Ausubel, 2005), several protein kinase genes 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) (Supplementary Table 2). Of the 285 genes that contained 6 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 4, 3, 2 or 1 treatment (Supplementary Table 2). Analysis of these 121 genes revealed that 106 of them have expression levels too low to be detected across all microarrays experiments, while the other 15 genes were too low to be detected in 75% of the microarray experiments. Also it is possible that some combinations of 6 of the 12 elements do not act in a combinatorial manner to regulate gene expression in planta.

To investigate any percentile distribution differences in sub-cellular localisation 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 (Supplementary Table 2). Lists of 1025 mitochondrial and 1407 chloroplastic proteins were isolated, corresponding to 3.22% and 4.48% of the whole genome 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-squared 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 ten genes to drive the expression of GUS under H$_2$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 ten candidate genes revealed six were up-regulated and four were down regulated in response to both H$_2$O$_2$ and rotenone (Figure 7) and analysis of the promoter regions of the ten candidates revealed that they were all significantly responsive to H$_2$O$_2$ treatment in a manner consistent with changes in their transcript abundance (Figure 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 mitochondrial retrograde response (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 (Parsons et al., 1999; Yip and Vanlerberghe 2001; Escobar et al., 2006). A number of genes are co-expressed 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 how this may relate to a wider cellular stress response. The promoter regions of four genes were analysed 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 five-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 characterised 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 ABI4 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 photosystem-I and analysis of the promoter activity in ABA biosynthetic or ABA insensitive mutants reveal cross-talk between redox and ABA signalling 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 (Shaikhali 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 anti-oxidant 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 promoter 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 characterised were responsive to both H2O2 and rotenone. The response of the UPOX promoter to H2O2 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 H2O2 response and that all CAREs in AOX1a and UPOX are not equally responsive to rotenone and H2O2 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 oxidise 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 hydrogen peroxide using microarrays reveal 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 signalling 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 response 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* (that were reduced by approximately 5-fold) compared to *BCS1* (that was reduced over one hundred fold) support a different mode of action of SA on the respective transcripts. The promoter region of *BCS1* was responsive to SA application, indicating a direct effect on transcription initiation, while regulation seems to be post-transcriptional for the other genes tested. Similarly, in *Sauromatum guttatum*, application of SA can increase transcript abundance of *AOX* several fold, but analysis of transcription with isolated nuclei indicated no change in the rate of transcription, also leading to the conclusion that post-transcriptional 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
characterised 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 post-transcriptional 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., 1993; 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 signalling pathways which 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 signalling 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 signalling (Brodersen et al., 2006), the fact that only AOX1a transcript increases, suggests an SA independent induction of AOX1a in these mutants. Such a pathway has been described for 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 which have altered AOX levels (Ordog et al., 2002; Robson and Vanlerberghhe, 2002; Amirsadeghi et al., 2006). Thus, the up-regulation of AOX1a transcript abundance suggests that this is a unique signalling pathway that regulates 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 signalling pathways are possible (Van Aken et al., 2007).
Conclusion

The integrative use of promoter activity information, transcript analysis and mutants in specific signalling 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 (Figure 8): a SA dependent pathway, a second pathway that converges on a number of CAREs, previously characterised to bind ABA responsive transcription factors, and a third pathway that acts via EDS1 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 post-transcriptional mechanism (Figure 8), while BCS1 is under direct transcriptional control. \( \text{H}_2\text{O}_2 \) and rotenone can act at a transcriptional level, acting on an overlapping set of CAREs. As 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 \( \text{H}_2\text{O}_2 \). Additionally, as the transcript abundance of AOX1a is affected differently compared to NDB2 and UPOX in the pad4 and eds1 signalling 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, as identical or similar CAREs have been previously characterised in these pathways.
Materials and Methods

Cloning of Arabidopsis promoter regions

The promoter regions were cloned using standard protocols and subcloned into pLUS. The numbering of Arabidopsis promoters are given from the transcriptional start site as determined from the SIGAL database (Yamada et al., 2003). Initially the 1.85 Kb region upstream of the transcriptional start site (TSS) of AOX1a was used based on the fact that small regions of this promoter had previously been shown to be responsive (Dojcinovic 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, 442 bp and 689 bp 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 promoter regions also contained different lengths of 5' untranslated regions, 98 bp for AOX1a (At3g22370), 131 bp for NDB2 (At4g05020), 49 bp\(^1\) for UPOX (At2g21640) and 291 bp for BCS1(At3g509300). In the case of the other ten promoters analysed, 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 bp and 995 bp upstream of the TSS, At1g13990 contain 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, At1g72200 contained a 172 bp 5' UTR and 1.2 Kb upstream of the TSS, At2g23320 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 TSS, At2g17850 contained 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 Supplementary Table 3.

\(^{1}\) 49 bp comprises UTR, an additional 478 bp comprises an intron.
Construction of pLUS

The pLUS vector was created as follows. The LUC+ gene, flanked by the omega translational enhancer (5') and E93' terminator (3') (Welsh et al., 2005), was cloned downstream of the 35S CaMV promoter in pGEM3Zf(+) (Promega. Melbourne, Australia). 35S CaMV β-glucuronidase (GUS) from pCAMBIA1301 (http://www.cambia.org/daisy/cambia/585.html) was then cloned into pGEM3Zf(+)–LUC+ downstream of the multiple cloning site, in the opposite orientation to 35S CaMV–LUC(+) (Supplementary Figure 1).

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 seven day intervals to maintain growth. Four day old cells were treated 1 hr prior to collection for transient biolistic transformation, as described in (Clifton et al., 2005). A final concentration of 40 μM was used for rotenone, 100 μM for SA and 10 mM for H₂O₂ (Clifton et al., 2005). All Arabidopsis mutant lines were obtained from ABRC at TAIR; defence signalling mutants pad4 – CS3806, eds4 – CS3799, npr1 – CS3726, abi3 – CS24, jar1 – CS8072, etr1 – CS6374. NahG seed was kindly donated by Prof. Xing Wang Deng from Yale University.

Biolistic 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 (Biorad, Sydney Australia). After transformation, Arabidopsis suspension cell culture was incubated at 22 °C for 24 h under long day 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. Transiently 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, Sydney, Australia). Luciferase activity assays were carried out according to the manufacturer’s instructions and
activity was measured at 2 second intervals over 20 seconds, using the
Polarstar Optima (BMG Labtechnologies, Offenburg, Germany). GUS activity
was determined using the fluorimetric GUS assay (Jefferson et al., 1987).
Assay samples were taken over an hour at 3 minute intervals. Fluorescence
was measured using the Polarstar Optima (BMG Laboratories, Offenburg,
Germany) with excitation at 355 nm and emission at 460 nm. The normalised
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 normalised to control
conditions.

In the case of the AOX1a promoter, the region 1.85 Kb upstream of the
transcriptional start site was taken as previously 1.3 Kb upstream of the
transcriptional start site had been shown to be responsive to some treatments
(Dojcinovic et al., 2005). To test the function of various elements the 5 to 8 bp
element were deleted. In the case of the other promoters regions that
contained the elements defined as functional in AOX1a were cloned and
elements deleted.

For comparison of GUS activities of the motif deletions with that of the
un-mutated 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 normalised 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 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.
A comparison between the GUS activities of treated samples with un-mutated 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 AOX1a**

Three approaches were used to predict functional elements in the promoter region of *AOX1a*, defined as the sequences upstream of the transcriptional start site. These approaches were: 1) defining a common co-expression environment for *AOX1a* and *NDB2* (marked in red, Supplementary Figure 2), for *AOX1a* (marked in blue, Supplementary Figure 2) and using the promoter regions of the genes within the co-expression environments to predict putative sequence elements. Briefly, six co-expression environments were created using 3 linkage methods (average, centroid and complete) and 2 distance metrics (Euclidean and Pearson). Elements were identified in these co-expression environments using the PLACE signal scan (Higo et al., 1999) and PlantCare (Lescot et al., 2002). Additionally, the promoter regions of *AOX1a* and *NDB2* alone were also used to predict elements (marked in green, Supplementary Figure 2). This analysis was restricted to the 1000 bp upstream of the transcriptional start site to restrict the number of elements predicted. 2) using a bi-clustering approach with a list of genes encoding mitochondrial proteins (Holt et al., 2006) and 3) a phylogenetic approach comparing the promoter region of *AOX1a* to the promoter regions for soybean *AOX* genes (Thirkettle-Watts et al., 2003). A comparative genomics approach was used 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 non-functional 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.

**Quantitative RT-PCR analysis of gene expression**

Quantitative RT-PCR (QRT-PCR) was performed on Arabidopsis leaf tissue from various signalling mutant lines, *pad4*, *npr1*, *abi3-5*, *NahG*, *jar1*, *etr1* and *eds4*. Leaf tissue was excised from 3 week old mutant and 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, Sydney, Australia). From each of the independent cDNA preparations, each transcript was analysed twice. The standard error was calculated for every data point. Transcript abundance for Col-0 untreated sample was normalised to one for each gene with all other values presented as relative transcript abundance. QRT-PCR primers used for the genes *AOX1a* (At3g22370) and *NDB2* (At4g05020) have been previously described (Clifton et al., 2005). QRT-PCR primers used for the genes *UPOX* (At2g21640), *BCS1* (At3g50930), *PR1* (At2g14610) and *UBC* (At5g25760) have been described previously (Giraud et al., 2008).

**Defining a putative co-regulatory environment for AOX1a**

For each functional sequence element identified in the *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 website (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl). Each element nucleotide sequence was searched against Locus Upstream Sequences – 1000 bp (DNA) with the exception of elements A and B which were searched against Locus Upstream Sequences – 3000 bp (DNA) as these elements occurred more than 1000 bp upstream of the transcriptional start site in *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.plantenergy.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: \( \frac{\text{length of DNA region searched}}{4 \times \text{length of element in bp}} \) multiplied by two, 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 H\( _2 \)O\( _2 \) or 40 \( \mu \)M rotenone was performed using Affymetrix GeneChip™ Arabidopsis ATH1 Genome Arrays (Affymetrix, Santa Clara, CA). Leaves excised from Col-0 plants were submerged in solutions of H\( _2 \)O\( _2 \) or 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 RNeasy Plant Mini Protocol (QIAGEN, Clifton Hill, Australia). The quality of the RNA was verified using a Bioanalyzer (Agilent Technologies, Palo Alto, CA) and spectrophotometric analysis to determine the \( A_{260} \) to \( A_{280} \) ratio. Preparation of labelled cRNA from 5 \( \mu \)g of total RNA, target hybridisation, 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 an 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 Pvt Ltd) for further analysis. As an additional comparison, raw signal data from
microarrays performed previously for Arabidopsis cell culture treated with 10 mM H$_2$O$_2$ or 40 µM rotenone along with an untreated control (Clifton et al., 2005) were analysed using the program AVADIS$^\text{TM}$ Prophetic (Version 4.3, Strand Life Sciences Pvt Ltd). All CEL files were subjected to GC-RMA normalisation, a variation of the robust multi-array average normalisation algorithm (RMA). Fluorescence intensities were log transformed and correlation plots were examined between replicates using the Scatter Plot function, and in all cases $r \geq 0.9$ (data not shown). Fold changes for untreated/mock treated verses 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 (FDR) in multiple comparisons. A method of FDR correction that is based on the Benjamini and Hotchberg method was used (Benjamini and Hochberg, 1995; Nettleton, 2006). This method utilises 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 H$_2$O$_2$ or rotenone for cells and plants were plotted on 2-way venn-diagrams such that the overlap in transcripts with altered abundance could be determined. A chi-square statistical test using the expected overlap in a random distribution (# significantly changed probesets in set 1 x # significantly changed probesets in set 2 / # probesets 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 website (ftp://ftp.arabidopsis.org/home/tair/Microarrays/Datasets/) for the treatments, PNO8, cyclohexamide, ABA, TIBA, osmotic, salt, heat and UV-B, which are found in the following directories ME00363, ME00361, ME00333, ME00358, ME00327, ME00328, ME00339 and ME00329 respectively. Access to the microarray data for treatment of Arabidopsis cell culture with rotenone, SA and H$_2$O$_2$ is outlined in Clifton et al., (2005) and 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 normalisation to generate present/absent calls and a GC-RMA normalisation to summarise fluorescent intensity values for further analysis. Probe sets which 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 6 or more motifs. Additionally, a fold-change cut-off of 1.5 fold was applied and changes less dramatic than this were removed.
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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, hydrogen peroxide, rotenone and salicylic acid 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).

Figure 2. Summary of the predicted cis-acting regulatory elements in the AOX1a and NDB2 promoter regions. Putative cis-acting regulatory elements were identified in the AOX1a promoter region using a variety of prediction methods. Numbering is from the transcription start site (TSS), bases 5’ are indicated with a “-” and bases after the TSS with a “+”. The predicted translational start codon ATG is indicated. The twelve elements tested were designated A to L and if the element occurred more than once it was designated by both a letter and a number. A region termed the mitochondrial retrograde response region (MRR) defined in a previous study is indicated that contain element E analysed in this study (Dojcinovic et al., 2005).

Figure 3. Functional analysis and stress response of predicted cis-acting regulatory elements (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 un-mutated promoter to the promoter with the element deleted. The un-mutated promoter represents the 1.85 Kb and 1.0 Kb regions upstream of the transcriptional start site in AOX1a and NDB2, respectively. The labelling of the element above the graph indicates the sequence element that was deleted from this fragment, as listed in Table 1. The activity of the wild-type promoter region (normalised 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 un-mutated versus
mutated promoter fragments indicating presence of a constitutive element (positive or negative). 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 * (green asterisk) indicates a significant difference ($p \leq 0.05$) between the GUS activities of treated samples with un-mutated 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, the data for the other elements tested are shown in supplementary figure 3.

**Figure 4. Functional analysis of the stress response of promoters of genes co-induced with AOX1a.**

A) Analysis of the response of promoter region of At2g21640 (UPOX) to hydrogen peroxide, rotenone and salicylic acid. The effect of removing the overlapping B and I element was also tested. B) Analysis of the response of promoter region of At3g50930 (BCS1) to hydrogen peroxide, rotenone and salicylic acid. The affect of removing the H element was also tested. C) The response of the AOX1a and NDB2 to salicylic acid was tested. Labelling and asterisk are as for Figure 3.

**Figure 5. Analysis of genes whose transcript abundance changes in response to hydrogen peroxide 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.

**Figure 6. Expression of AOX1a, NDB2, UPOX, BCS1, PR1a and UBC in defence signalling mutants.** The transcript abundance was determined by
QRT-PCR in the defence signalling mutants etr1, abi3, pad4, npr1, jar1 and eds1 and NahG and compared to Col-0 where expression was set to 100%.

**Figure 7.** The response of promoter regions that contain sequence elements defined as functional in AOX1a. Determination of promoter driven response to H2O2 of 10 genes, each containing 6 or more sequence elements defined as functional in AOX1a. Six genes whose transcript abundance is up-regulated after H2O2 treatment and four genes whose transcript abundance is down-regulated after H2O2 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 H2O2. A schematic representation of each promoter is shown with the region in bp or Kb upstream of the transcriptional start site (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 H2O2. A red colour indicates up-regulation and green indicates down-regulation, the transcript changes for AOX1a, NDB2, UPOX are included as a comparison. The genes are arranged numerically by AGI code. At1g02450, At3g50870 and At1g72200 transcript levels were called absent after MAS 5 normalisation.

**Figure 8.** Summary of signalling 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 H2O2 and rotenone that on CAREs. H2O2 and rotenone act at a transcriptional level, with some differences evident between genes, namely rotenone also has a repressive effect on specific regions of the AOX1a promoter, while rotenone has no direct effect on the promoter of UPOX. A third pathway that
acts via EDS1 and PAD4 regulates only AOX1a. The AOX1a promoter is strongly repressed under normal conditions and is de-repressed upon treatment. The regulation of transcript abundance upon treatment with SA is regulated at a transcriptional level for BCS1 and post-transcriptionally for AOX1a, NDB2 and UPOX. SA has a small but significant effect on the UPOX promoter and this may be indirect by triggering oxidative stress (see text for details).
Supplementary Figure 1. Diagrammatic representation of the biolistic transformation vector pPLUS. The vector contains two reporter genes, *Luc* and *GUS*, driven by two promoters. The 35S CaMV driving the expression of *GUS* was replaced with the promoter of interest (*AOX1a, NDB2, UPOX or BCS1*).

Supplementary Figure 2. The 1850 bp upstream of the *AOX1a* transcriptional start site (TSS). Putative cis-acting regulatory elements were identified in the *AOX1a* promoter region using a variety of prediction methods. For the elements identified from predictions based on co-expression environments the number of times each element was identified is indicated by the thickness of the line, the exact sequence element tested using this prediction are highlighted in grey. The twelve elements tested were designated A to L and if the element occurred more than once it was designated by both a letter and a number.

Supplementary Figure 3. Functional analysis and stress response of predicted cis-acting regulatory elements in the *AOX1a*. The regulatory characteristics of each predicted cis-acting regulatory element were tested by comparing the GUS activity driven by the *AOX1a* un-mutated promoter to the promoter with the element deleted. The activity of the promoter region (normalised 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 un-mutated versus mutated promoter fragments indicating 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 un-mutated versus mutated promoter fragments indicating that when the element was absent the
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QRT-PCR analysis of transcript abundance for various genes over 24 h in response to addition of citrate, hydrogen peroxide, rotenone and salicylic acid 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).
Figure 2. Summary of the predicted cis-acting regulatory elements in the AOX1a and NDB2 promoter regions. Putative cis-acting regulatory elements were identified in the AOX1a promoter region using a variety of prediction methods. Numbering is from the transcriptional start site (TSS), bases 5’ are indicated with a “-“ and bases after the TSS with a “+”. The predicted translational start codon ATG is indicated. The twelve elements tested were designated A to L and if the element occurred more than once it was designated by both a letter and a number. A region termed the mitochondrial retrograde response region (MRR) defined in a previous study is indicated that contain element E analysed in this study (Dojcinovic et al., 2005).
Figure 3. Functional analysis and stress response of predicted cis-acting regulatory elements 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 un-mutated promoter to the promoter with the element deleted. The un-mutated promoter represents the 1.85 Kb and 1.0 Kb regions upstream of the transcriptional start site in AOX1a and NDB2, respectively. The labelling of the element above the graph indicates the sequence element that was deleted from this fragment, as listed in Table 1. The activity of the wild-type promoter region (normalised 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 un-mutated versus mutated promoter fragments indicating 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 un-mutated 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, the data for the other elements tested are shown in supplementary figure 3.
Figure 4. Functional analysis of the stress response of promoters of genes co-induced with AOX1a. A) Analysis of the response of promoter region of At2g21640 (UPOX) to hydrogen peroxide, rotenone and salicylic acid. The effect of removing the overlapping B and I element was also tested. B) Analysis of the response of promoter region of At3g50930 (BCS1) to hydrogen peroxide, rotenone and salicylic acid. The affect of removing the H element was also tested. C) The response of the AOX1a and NDB2 to salicylic acid was tested. Labelling and asterisk are as for Figure 3.
Figure 5. Analysis of genes whose transcript abundance changes in response to hydrogen peroxide 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.
Figure 6. Expression of AOX1a, NDB2, UPOX, BCS1, PR1a and UBC in defence signalling mutants. The transcript abundance was determined by QRT-PCR in the defence signalling mutants etr1, abi3, pad4, npr1, jar1, eds1 and NahG and compared to Col-0 where expression was set to 100%.
Figure 7. The response of promoter regions that contain sequence elements defined as functional in $AOX1a$. Determination of promoter driven response to $H_2O_2$ of 10 genes, each containing 6 or more sequence elements defined as functional in $AOX1a$. Six genes whose transcript abundance is up-regulated after $H_2O_2$ treatment and four genes whose transcript abundance is down-regulated after $H_2O_2$ 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 $H_2O_2$. A schematic representation of each promoter is shown with the region in bp or Kb upstream of the transcriptional start site (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 $H_2O_2$. A red colour indicates up-regulation and green indicates down-regulation. The genes are arranged numerically by AGI code. At1g02450, At3g50870 and At1g72200 transcript levels were called absent after MAS 5 normalisation.
Figure 8. Summary of signalling 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 on several CAREs. H$_2$O$_2$ and rotenone act at a transcriptional level, with some differences evident between genes, namely rotenone also has a repressive effect on specific regions of the AOX1a promoter, while rotenone has no direct effect on the promoter of UPOX. A third pathway that acts via EDS1 and PAD4 regulates only AOX1a. The AOX1a promoter is strongly repressed under normal conditions and is de-repressed upon treatment. The regulation of transcript abundance upon treatment with SA is regulated at a transcriptional level for BCS1 and post transcriptionally for AOX1a, NDB2 and UPOX. SA has a small but significant effect on the UPOX promoter and this may be indirect by triggering oxidative stress (see text for details).