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Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*

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Abstract

Plant mitochondria contain non-phosphorylating bypasses of the respiratory chain, catalysed by the alternative oxidase (AOX) and alternative NADH dehydrogenases (NDH), as well as uncoupling (UCP) protein. Each of these components either circumvents or short-circuits proton translocation pathways, and each is encoded by a small gene family in Arabidopsis. Whole genome microarray experiments were performed with suspension cell cultures to examine the effects of various 3 h treatments designed to induce abiotic stress. The expression of over 60 genes encoding components of the classical, phosphorylating respiratory chain and tricarboxylic acid cycle remained largely constant when cells were subjected to a broad range of abiotic stresses, but expression of the alternative components responded differentially to the various treatments. In detailed time-course quantitative PCR analysis, specific members of both *AOX* and *NDH* gene families displayed coordinated responses to treatments. In particular, the co-expression of *AOX1a* and *NDB2* observed under a number of treatments suggested co-regulation that may be directed by common sequence elements arranged hierarchically in the upstream promoter regions of these genes. A series of treatment sets were identified, representing the response of specific *AOX* and *NDH* genes to mitochondrial inhibition, plastid inhibition and abiotic stresses. These treatment sets emphasise the multiplicity of pathways affecting alternative electron transport components in plants.

Abbreviations: AOX, alternative oxidase; NCS, non-chromosomal stripe; NDH, alternative NAD(P)H dehydrogenase; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle; UCP, uncoupling protein

Introduction

Mitochondria play an essential role in diverse metabolic pathways in plants, including the production of ATP and the biosynthesis of carbon skeletons and metabolic intermediates, such as heme and amino acids. They also play roles in pathogen and stress responses and are intimately involved in the process of programmed cell death (Mackenzie and McIntosh, 1999; Lam *et al.*, 2001; Swidzinski *et al.*, 2004). These functions are carried out in a coordinated manner amidst the complex complement of other compartments in the plant cell. Such a coordinated cellular response to environmental challenges requires active organelle-to-nuclear communication and probably also inter-organelle signalling.

In yeast, at least four pathways exist to monitor the functional state of mitochondria. These include carbon-source response pathways, oxygen sensing,

heme synthesis, and the retrograde regulation pathways, which primarily respond to mitochondrial inhibition and tricarboxylic acid (TCA) cycle flux (Butow and Avadhani, 2004). In plants, chloroplast signal transduction, involving tetrapyrroles, carotenoids and the redox state of the plastid, operate as separate retrograde signalling pathways (Surpin *et al.*, 2002; Gray *et al.*, 2003; Jarvis, 2003). Evidence also exists for signalling pathways from mitochondria to the nucleus in plants, but these are not as well characterized (Vanlerberghe and McLntosh, 1996; Maxwell *et al.*, 2002; Vanlerberghe *et al.*, 2002). Several potential signals have been proposed, including reactive oxygen species (ROS), which trigger defence mechanisms, keto-acids that modulate biosynthetic demands, and changes in temperature that trigger cold-tolerant responses (Rhoads and Vanlerberghe, 2004). The pronounced interdependence of plastids and mitochondria is demonstrated by the green–white variegation phenotype, associated with a range of mitochondrial dysfunctions (Hunt and Newton, 1991; Marienfeld and Newton, 1994; Hedtke *et al.*, 1999; Mackenzie and McIntosh, 1999). These variegation mutants provide visual and molecular evidence of the interplay of the metabolic pathways of the chloroplast and the mitochondria, strongly suggesting that inter-organelle signalling pathways in plants await identification (Sakamoto, 2003).

In addition to the major complexes of the mitochondrial respiratory chain common to most eukaryotes, plants possess additional energy-dissipating components including type II NAD(P)H dehydrogenases (NDH), the alternative oxidase (AOX) and uncoupling proteins (UCP) (Picault *et al.*, 2004; Rasmusson *et al.*, 2004). These components form the alternative respiratory pathways, which have been postulated to play a key role in plant stress responses (Moore *et al.*, 2002; Rasmusson *et al.*, 2004). *AOX* expression is often used as a model for retrograde signalling between the mitochondrion and the nucleus (Mackenzie and McIntosh, 1999; Rhoads and Vanlerberghe, 2004). Neither the NDHs nor AOX contribute to proton pumping or ATP synthesis and hence bypass adenylate control and allow for respiration with flexible levels of energy conservation and respiratory control. The modulation of ubiquinone (UQ) redox poise by the activity of these proteins suggests that they can also influence ROS production in mitochondria (Maxwell *et al.*, 1999; Moore *et al.*, 2002). Uncoupling proteins (UCPs) form a subfamily within the mitochondrial carrier protein family and catalyse fatty acid-dependent proton recycling, thereby modulating the degrees of coupling between mitochondrial electron transport and ATP synthesis. UCP only uncouples in the presence of ROS, suggesting that it also monitors and modulates the redox status of the electron transport chain (Echtay *et al.*, 2002; Considine *et al.*, 2003; Smith *et al.*, 2004). In Arabidopsis, AOX is encoded by a five member gene family with each isoform displaying different spatial expression patterns (Saisho *et al.*, 1997; Thirkettle-Watts *et al.*, 2003). Seven type II NAD(P)H dehydrogenases have been identified in the Arabidopsis nuclear genome, based on their similarity to the potato *Nda1* and *Ndb1* enzymes. These can be grouped into three families, A, B and C, with expression of several of them dependent on light and circadian regulation (Michalecka *et al.*, 2003). Two plant-type UCP-encoding genes have been identified in Arabidopsis (*UCP1*, *UCP2*) (Hourton-Cabassa *et al.*, 2004; Picault *et al.*, 2004).

Previous studies on alternative respiratory pathways have encompassed a wide variety of species and approaches (McDonald *et al.*, 2002; Moller, 2002). Although these studies have been informative in elucidating the stress inducible nature of alternative pathway components, the variation in treatments and their duration preclude comparisons of the timing of induction of the various components. Additionally, no direct relationship between activity and gene expression can be deduced from these studies. In some instances, the data from different studies gives apparently contradictory results. For example, treatment of potato leaves with antimycin A resulted in only small increases in AOX protein and no increase in alternative respiratory capacity, compared to large increases in both when tobacco cells were used (Vanlerberghe and McLntosh, 1996; Vanlerberghe *et al.*, 1999; Geisler *et al.*, 2004). Additionally, cold induces AOX in *Vigna* but not in soybean (Gonzalez-Meler *et al.* 1999). Likewise, UCP is reported to be induced by cold in potato and Arabidopsis but not in wheat (Laloi *et al.*, 1997; Maia *et al.*, 1998; Nantes *et al.*, 1999; Murayama and Handa, 2000). Thus an analysis of gene expression of all the alternative respiratory chain components, compared to the classical respiratory

chain components, would provide a clearer picture of the expression and induction of these components in response to a variety of stresses. We have used gene expression analysis, using microarray and quantitative RT-PCR, to examine the expression of the multiple genes encoding alternative pathway components in response to such stresses in Arabidopsis cell cultures. The data obtained identify novel gene responses and are discussed in light of previous roles proposed for the alternative pathways, and signalling mechanisms which may play a role in mitochondrial regulation.

Materials and methods

Plant growth

Arabidopsis suspension cell cultures were maintained under long day conditions (16 h of 100 μE m^{-2} s^{-1} light and 8 h dark), with all other conditions as previously described (Sweetlove *et al.*, 2002).

Chemical treatment of Arabidopsis *cell culture*

Arabidopsis cell cultures were treated two hours into the light cycle 4 days after subculturing into fresh stock solutions. All experimental compounds were obtained from Sigma (Castle Hill, Australia) unless otherwise specified. Treatments involved the addition of the following to 120 ml of suspension cell culture: 12 μl of 50 mM antimycin A (final concentration 5 μM), 480 μl of 10 mM rotenone (final concentration 40 μM), 15 μl of 1 mM oligomycin (final concentration 0.125 μM), 24 μl of 200 g/l triquat (final concentration 0.04 g paraquat/l, made up of 125 g/l paraquat and 75 g/l diquat (marketed as Solicam DF, Crop Care Australia, Sydney, Australia), 60 μl of 5 mM FCCP (final concentration 2.5 μM), 300 μl of 2 M malonate (final concentration 5 mM), 1.2 ml of 1 M citrate (BDH Merck, Victoria, Australia) (final concentration 10 mM), 120 μl of 1 M L-cysteine (final concentration 1 mM), 100 μl of 120 mM norflurazon (Novartis Crop Protection, Sydney, Australia) (final concentration 100 μM), 100 μl of 0.24 M erythromycin (final concentration 200 μM), 100 μl of 0.24 M chloramphenicol (final concentration 200 μM), 1 ml of 2.17 M glucose (final concentration 3% (w/v)), 130 μl of 30% v/v H_2O_2 (final concentration 10 mM), 10 ml of 2.14 M mannitol (final concentration 3% (w/v)), 120 μl of 100 mM salicylic acid (final concentration of 100 μM), 30 μl of 200 μM bongkrekic acid (final concentration 50 μM) (Table 1). All treatments were performed in triplicate, with suspension cell aliquots taken immediately prior

Table 1. Reference list of inhibitors used, mode of action, and method of analysis.

Treatment	Final concentration	Expected affects	qRT-PCR	Microarray
Antimycin A	5 μM	Inhibition of complex III	+	
Anoxia (N_2)	N_2 replacement of air	Oxygen depletion, N_2 induced anoxia		+
Bongkrekic acid	50 μM	Inhibited opening of the permeability transition pore	+	
Chloramphenicol	200 μM	Inhibition of mitochondrial and plastid protein synthesis	+	+
Citrate	10 mM	Retrograde signalling (TCA cycle intermediate)	+	
Cold	10 °C	Induction of alternative respiratory chain components		+
Cysteine	1 mM	Redox and amino acids biosynthesis modulator	+	
Erythromycin	200 μM	Inhibitor of plastid protein synthesis	+	
FCCP	2.5 μM	Uncoupling of membrane potential	+	
Glucose	3% (w/v)	Alternative carbon supply/sugar signalling	+	
H_2O_2	10 mM	ROS induced signalling/damage	+	+
Malonate	5 mM	Inhibition of complex II and TCA cycle turnover	+	
Mannitol	3 % (w/v)	Hyperosmotic stress	+	+
Norflurazon	100 μM	Inhibition of carotenoid biosynthesis	+	
Oligomycin	1.25 μM	Inhibition of chloroplast and mitochondrial ATP synthase	+	+
Oligomycin	0.125 μM	Inhibition of mitochondrial ATP synthase		+
Paraquat	0.04 g/l	Production of superoxide radicals	+	
Rotenone	40 μM	Inhibition of complex I	+	+
Salicylic acid	100 μM	Defense signalling/Uncoupling of respiration	+	+
Salicylic acid	10 μM	Defense signalling/Uncoupling of respiration		+

to treatment, then 30 min, 1, 3, 12 and 24 h post-treatment. Aliquots were vacuum filtered and snap frozen. The pH of the suspension cells was sampled 3 h post-treatment, as was the cell viability using acridine orange and ethidium bromide stains. Additional treatments were performed for microarray analysis only, including the addition of 12 μl of 100 mM salicylic acid (final concentration 10 mM), 150 μl of 1 mM oligomycin (final concentration 12.5 μM). To simulate anoxia, flasks were treated with N_2 gas to replace the air above the cell culture, N_2 was added at time 0 and refreshed every hour thereafter. The absence of oxygen was monitored using Anaerobic Indicator Solution (Don Whiley Scientific, West Yorkshire, England) in an open microcentrifuge tube suspended above the cells within the flask. For cold treatment flasks were placed at 10 °C.

Primers for quantitative RT-PCR

See supplementary Table 3.

Isolation of total RNA and cDNA synthesis for quantitative RT-PCR

Total RNA isolation, and cDNA synthesis were carried out as described previously (Murcha *et al.*, 2003; Lister *et al.*, 2004).

Quantitative RT-PCR analysis of transcript levels

Transcript levels were assayed using the iCycler™ and iQ Supermix and iQ SYBR Supermix (Bio-Rad) as described previously (Thirkettle-Watts *et al.*, 2003; Lister *et al.*, 2004). From the three independent cDNA preparations, each transcript was analysed twice. The standard error was calculated for every data point. The data for each gene was expressed as a ratio or fold change from pre-treatment values (time = 0, before the addition of chemical) by setting this data point to 1 and expressing other time points relative to it. Students *t*-tests were employed to determine if ratio to pre-treatment values obtained for each time point following treatment were statistically different to the values seen at that time point in the untreated control cells. The effects of the treatment were considered significant if difference between the treated sample and the control at that time point is greater than the critical *t*-value according to a Students *t*-test (where d.f. = 2, $p = 0.05$). An analysis of variance was performed to determine if the variances of the data points compared using a Students *t*-test were or were not significantly different, based on the ratio of the variances of each sample exceeding the critical F_{max} (d.f. = 4) according to a test for homogeneity of variance.

Microarray analysis

Microarray analysis of the changes in transcript abundance in Arabidopsis cell culture was performed using Affymetrix GeneChip® Arabidopsis ATH1 Genome Arrays (Affymetrix, Santa Clara, CA). For each treatment, four flasks of Arabidopsis cell culture were treated as indicated above, with samples taken and snap frozen 3 h post-treatment. For sample preparation for microarray analysis, two flasks were combined for each treatment resulting in two separate cRNA preparations, with the exception of the untreated sample for which eight flasks were used to prepare four cRNA samples. cRNA was prepared and analysed as described previously (Lister *et al.*, 2004). For each ATH1 GeneChip® the raw signal data was extracted using the Affymetrix Microarray Suite 5.0, and averaged for replicate treatments, with the exception of mannitol treatment were data is derived from one GeneChip®. The data set was filtered for stringency: ambiguous probe sets were discarded, genes with an average raw signal value below 50 in the untreated sample were discarded, and genes called absent in more than 11 out of the 24 chips analyzed were discarded. The average signal data for each treatment was expressed as a ratio of the average signal data from the untreated sample.

Analysis of gene expression data using GeneCluster2.0, CLUSFAVOR v.6 and TMeV

GeneCluster 2.0 (http://www-genome.wi.mit.edu/cancer/software/genecluster2/gc2.html) (Golub *et al.*, 1999; Tamayo *et al.*, 1999) was used in the analysis of quantitative RT-PCR data, all inputs used data expressed as a fold change of pre-treatment values. To look for genes responding in a common pattern to a single treatment a 4 column by 16 row GeneCluster 2.0 matrix was constructed for each treatment analysed. Each row represents a separate gene and the 4 columns

house the fold change expression data derived by quantitative RT-PCR over the four time points, 0, 3, 12 and 24 h post-treatment. The 'find classes' tool in GeneCluster 2.0 was used under default settings with the cluster range varied. To look for classes of treatments inducing common response patterns over the complement of genes analysed a GeneCluster 2.0 matrix was initially constructed in which the matrix rows comprised of expression data for all genes with four time points (64 data points per row), with the data from each treatment in a separate row (17 rows = 16 treatments plus control, Table 1). For analysis of the *AOX* and *NDH* encoding gene family expression data matrices were constructed as above consisting of the data only from the three *AOX* genes, or the six *NDH* genes, or the nine genes representing both the *AOX* and *NDH* genes. The 'find classes' function of GeneCluster 2.0 was used to generate unsupervised self-organising maps to extract classes in the dataset representing common response trends to different treatments. These maps were then dissected into single gene responses and scaled for each gene to represent the fold changes in the quantitative RT-PCR data. CLUSFAVOR v.6 (Peterson, 2002) was used to produce hierarchical dendograms revealing natural groupings in microarray data generated from Arabidopsis suspension cells 3 h post a range of treatments. Colour scheme based on sorted fold change data with distance measures reflecting expression values (Euclidean distance). Subsets of the whole genome data were entered. Firstly the array data from the alternative respiratory genes; *AOX1a*, *AOX1c*, *AOX2*, *NDA1*, *NDA2*, *NDB1*, *NDB2*, *NDB4*, and *NDC1* under six treatment conditions was entered. Secondly, the array data for the 18 genes analysed by quantitative RT-PCR under 12 treatment conditions was entered. Dendrograms displaying the 'natural' groupings within the data are displayed with colour based on sorted fold change data, and distances between tree branches determined using a Euclidean function. Hierarchical clustering of the whole transcriptomic dataset was performed using the TIGR Multiexperiment viewer v3.0.3 (TMeV) using the average linkage settings clustering both genes and treatments, dendrograms displaying the treatment clusters are shown (Eisen *et al.*, 1998; Saeed *et al.*, 2003).

Comparison of the 1000 bp upstream region of AOX1a *and* NDB2

Conserved sequence elements in the *AOX1a* and *NDB2* 1000 bp upstream sequences were identified using publicly available software, including Improbizer (http://www.cse.ucsc.edu/~kent/improbizer/improbizer.html), MEME (Bailey and Elkan, 1994) and the TAIR Motif Analysis tool (http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp). Previously described motifs were identified using Softberry nsiteM (Shahmuradov *et al.*, 2003), PlantCARE (Lescot *et al.*, 2002), PLACE (Higo *et al.*, 1999), and MotifViz (Frith *et al.*, 2004; Haverty *et al.*, 2004). The number of *Arabidopsis* genes possessing the identified conserved sequence elements within the 1000 bp upstream of their transcription start site was determined using the TAIR Patmatch V1.1 tool (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl).

Results

Stress treatments of cell cultures

Arabidopsis suspension cell batch-cultures were maintained under a 16 h light/8 h dark photoperiod (Sweetlove *et al.*, 2002). Under these conditions, the cells are light green in colour and their plastids contain thylakoid membranes with small grana stacks (not shown). Cells were subjected to the various treatments (Table 1) 2 h into the light cycle and 4 days after subculturing. To ensure that the treatments imposed did not induce cell death over the time course of the experiments, cell viability was examined using acridine orange/ethidium bromide fluorescent microscopy and cell respiration was measured using an O_2-electrode. These measurements revealed that the cells survived all treatments without major changes in whole cell respiration rate after 24 h (data not shown).

Alternative respiratory pathways rapidly respond to stress treatments while the classical respiratory chain is non-responsive

To investigate the response of the classical and alternative respiratory pathways to various environmental stimuli, whole-genome expression

profiling was undertaken using Arabidopsis ATH1 GeneChip® microarrays (Affymetrix) (Table 1). For this investigation, cell cultures were sampled 3 h post-treatment and compared to untreated cells at the same time point. Transcript levels of the genes encoding proteins present in complexes I–V of the classical respiratory chain were stable across all treatments, with few exceptions, where a transcript is described as stable if it displays fold-changes of less than 2 in the microarray experiments (supplementary Table 1). Interestingly this trend was also observed for genes encoding components of TCA cycle complexes and uncoupling proteins (supplementary Table 1). In contrast, the transcript abundance of genes encoding members of the *NDH* and *AOX* gene families displayed large (>2-fold) and varied response to the treatments examined (supplementary Table 1).

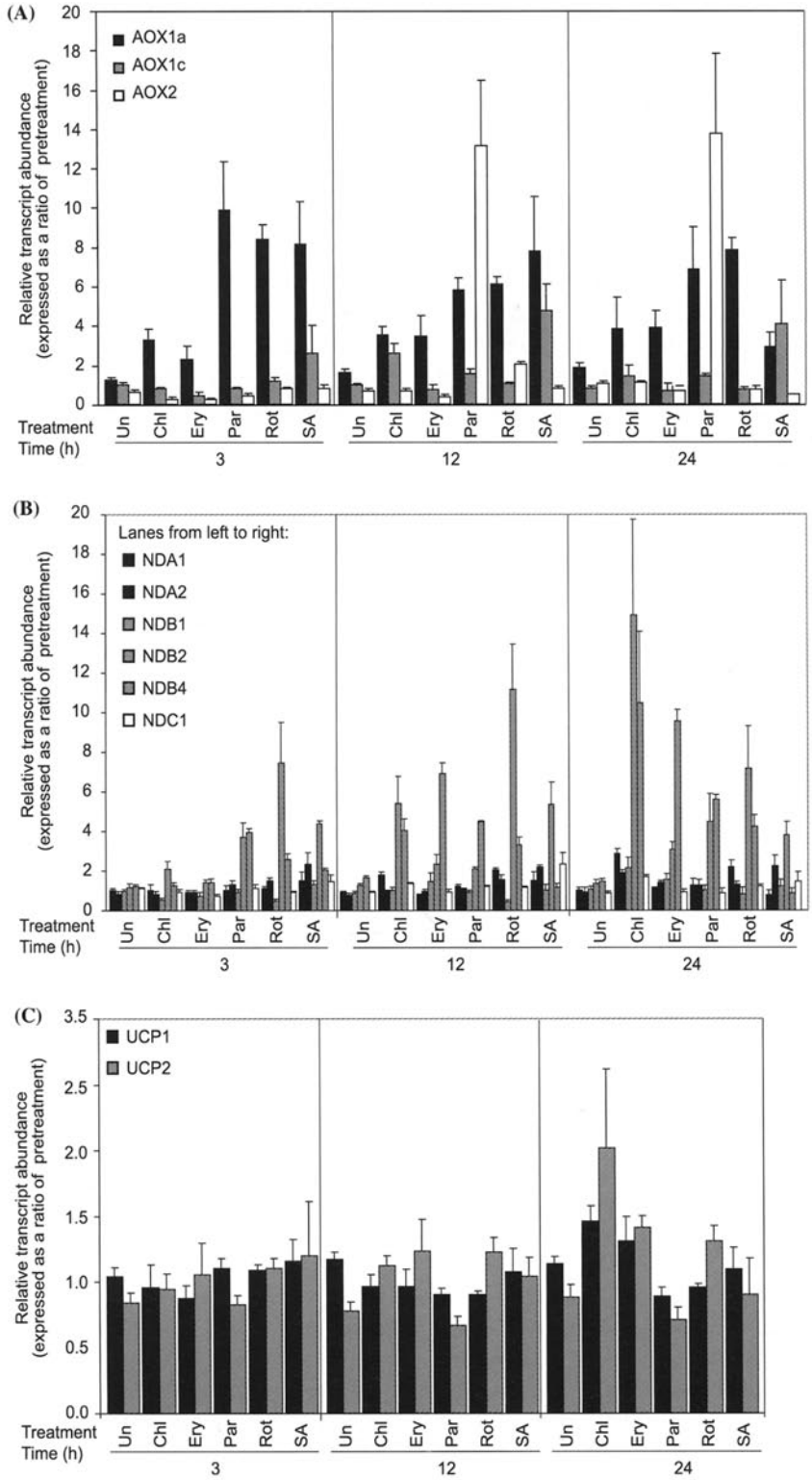
Individual members of the gene families encoding alternative respiratory enzymes respond differently to stress treatments

To further examine the transcript responses of genes encoding mitochondrial proteins, quantitative RT-PCR (qRT-PCR) was used to analyse the transcript abundance of members of the *AOX*, *UCP* and *NDH* gene families, as well as selected genes encoding some TCA cycle and classical respiratory chain components (supplementary tables 2 and 3). Compounds were added to an Arabidopsis suspension cell culture with sampling at 0, 3, 12 and 24 h (Table 1). This enabled validation of results from the microarray studies and allowed the time course of gene induction to be closely monitored and compared. The treatments used can be described broadly as compounds that affect metabolism, i.e. glucose and cysteine (Heldt, 1997; Vanlerberghe *et al.*, 2002); that have been associated with stress signalling, i.e. mannitol, salicylic acid and H_2O_2 (Kreps *et al.*, 2002; Overmyer *et al.*, 2003; Shah, 2003); compounds that affect mitochondrial function (rotenone, malonate, antimycin A, oligomycin, FCCP, bongkrekic acid, citrate and chloramphenicol) (Newton and Walbot, 1985; Maxwell *et al.*, 2002; Siedow and Day, 2002); or compounds that affect chloroplast function (erythromycin, norflurazon, paraquat and chloramphenicol) (Newton and Walbot, 1985; Surpin *et al.*, 2002). Citrate treatment has been included in the compounds that affect mitochondrial function group as it has been previously shown to induce AOX in soybean and tobacco cells, albeit via a different pathway compared to antimycin A (Vanlerberghe and McLntosh, 1996; Djajanegara *et al.* 2002). Whilst paraquat will affect mitochondrial function it has been described as a compound primarily affecting plastid function due to its well characterized role as an PSII electron acceptor acting in the thylakoid membrane (Vicente *et al.*, 2001; Peixoto *et al.*, 2004).

Figure 1. Stress response patterns are not conserved among members of gene families. Relative transcript abundance as measured by quantitative RT-PCR in Arabidopsis cell cultures over a 24 h time course in untreated cells or following treatments with 200 μM chloramphenicol (Chl), 200 μM erythromycin (Ery), 0.04 g/l paraquat (Par), 40 μM rotenone (Rot), or 100 μM salicylic acid (SA). (A) Members of the alternative oxidase gene family, *AOX1a* (indicated in black), *AOX1c* (indicated in grey), and *AOX2* (indicated in white). (B) Members of the alternative NADH dehydrogenase gene family, from left to right, A class *NDA1* and *NDA2* (indicated in black), B class *NDB1*, *NDB2* and *NDB4* (indicated in grey), and C class *NDC1* (indicated in white). (C) Members of the uncoupling gene family, *UCP1* (indicated in black), *UCP2* (indicated in grey). For each gene transcript abundance expressed as a ratio of the pre-treated levels for each treatment (time = 0), error bars represent SE.

Following initial experiments, genes for *AOX1b*, *AOX1d* and *NDB3*, were not analysed further as their transcripts levels in the cell culture system were below the level of confident detection, even after stress treatments (data not shown). For clarity, time points where the transcript level in response to the addition of treatment is statistically different to the untreated sample are referred to as significant, time points where transcript levels vary from untreated levels by $\pm$2-fold are referred to as exhibiting a large change, and by more than $\pm$5-fold as exhibiting a dramatic change. Transcript levels determined by qRT-PCR are referred to as stable when variation was less than $\pm$2-fold untreated samples.

Supporting the micoarray data, qRT-PCR also showed that the majority of genes encoding proteins belonging to the classical respiratory chain and TCA cycle displayed stable transcript abundance in response to the majority of treatments. Two exceptions were cytochrome *c* and *IDH2*, the former displaying large fold increases 24 h after most treatments (9 out of 16), and the latter displaying large decreases 3 h after most treatments (11 out of 16).

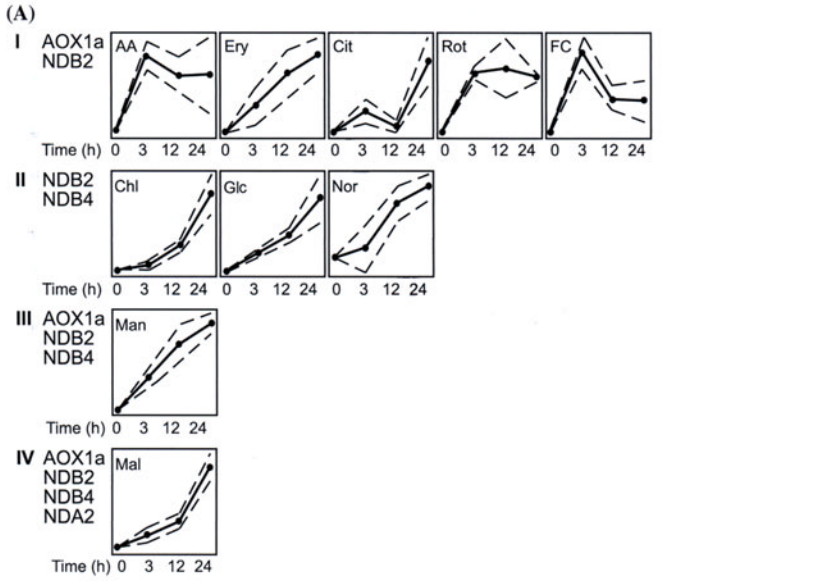

(A)
AOX1a
AOX1c
AOX2
Relative transcript abundance (expressed as a ratio of pretreatment)
Treatment
Time (h)
Un Chl Ery Par Rot SA
3 12 24
(B)
Lanes from left to right:
NDA1
NDA2
NDB1
NDB2
NDB4
NDC1
Relative transcript abundance (expressed as a ratio of pretreatment)
Treatment
Time (h)
Un Chl Ery Par Rot SA
3 12 24
(C)
UCP1
UCP2
Relative transcript abundance (expressed as a ratio of pretreatment)
Treatment
Time (h)
Un Chl Ery Par Rot SA
3 12 24

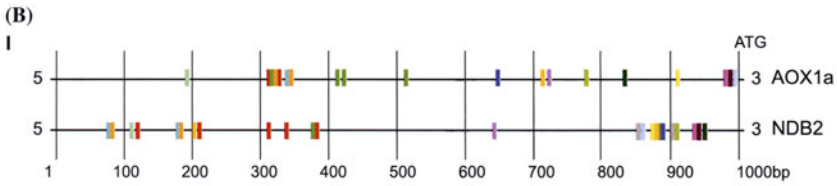
Individual members of the *NDH* and *AOX* gene families responded differently to the various treatments (Figure 1, supplementary Table 2). *AOX1a* transcript abundance exhibited dramatic increases in response to the majority of treatments (12 out of 16) and its induction profile generally followed one of two patterns, either peaking 3 h after treatment and then declining, as observed with H_2O_2, paraquat, rotenone and salicylic acid treatment, or continuing to increase over the entire 24 h (albeit at a lower level), as observed with malonate and erythromycin (Figure 1A, supplementary Table 2). *AOX2* transcript abundance increased dramatically 12 h after treatment with paraquat (19-fold increase), and with cysteine (9-fold increase) (supplementary Table 2). Interestingly, *AOX2* transcript abundance displayed large decreases in response to the majority of other treatments (9 out of 16) (Figure 1A, supplementary Table 2). *AOX1c* transcript levels were stable in response to the majority (11 out of 16) of treatments analysed, with significant induction only observed in response to glucose, cysteine, and mannitol. The largest induction of this transcript was seen in response to SA treatment (Figure 1A, supplementary Table 2).

The transcript response profiles of members of the *NDH* gene family also varied, even within the three previously characterized classes (Michalecka *et al.*, 2003). Members of the external B-class genes (*NDB2*, *NDB4*) displayed the most dramatic transcript increases in response to a 24 h chloramphenicol treatment, yet also displayed significant transcript increases at 3 h following several treatments including SA and cysteine (supplementary Table 2). In contrast, *NDB1* transcript levels were stable in response to the majority of treatments, a trend also seen for the internal A-class genes (*NDA1*, *NDA2*) and *NDC1* (Figure 1B, supplementary Table 2). Interestingly, both the A-class genes displayed large increases following 24 h treatment with cysteine, glucose and malonate (supplementary Table 2). Unlike the *NDH* and *AOX* genes, the transcript profiles of the two *UCP* genes were stable under the majority of treatments. Noteworthy exceptions were *UCP1* transcript abundance decreasing significantly after 12 h in response to FCCP, malonate and mannitol treatments, and *UCP2* transcript abundance increasing significantly after 12 h in response to glucose, cysteine and malonate (supplementary Table 2).

To identify genes co-expressed in response to each treatment, we analysed the patterns of transcript abundance over time, obtained by quantitative RT-PCR, using the GeneCluster 2.0 software package. The greater the number of treatments and time points at which genes display co-expression, the greater the probability of identifying genes that are co-regulated (Yeung *et al.*, 2004). We used the self-organising map (SOM) function as an unsupervised learning tool to reveal classes in the data set, selecting the 'find classes' tool with default parameters except for variation of cluster range. The expression data over the four time points for the 3 *AOX* genes, 6 *NDH* genes, 2 *UCP* genes, 2 genes encoding aconitase, 2 genes encoding ATP synthase subunit, 2 genes encoding isocitrate dehydrogenase, and a gene encoding cytochrome *c*, were entered under each treatment separately. In the resulting outputs, several genes consistently clustered under particular treatments (Figure 2A). In each graph, a solid mean data line is shown along with broken lines, which indicate the range of data fitted to form each cluster. The transcript response profiles of *AOX1a* and *NDB2* clustered in response to several treatments, including antimycin A, erythromycin, citrate, rotenone, and FCCP (Figure 2A, I). Likewise *NDB2* and *NDB4* clustered in response to chloramphenicol, glucose, and norflurazon (Figure 2A, II). In response to mannitol treatment, a cluster consisting of *AOX1a*, *NDB2* and *NDB4* was seen (Figure 2A, III), while a cluster of *AOX1a*, *NDB2*, *NDB4* and *NDA2* expression was identified in response to malonate treatment (Figure 2A, IV). Thus a number of genes encoding alternative respiratory chain components clustered in response to specific treatments. Transcript response profiles of the two genes encoding ATP synthase subunit also clustered in response to a number of treatments (data not shown).

In an attempt to determine if the co-expression of *AOX1a* and *NDB2* could be a result of co-regulation, we examined the sequence 1000 bp upstream of the translational start site of the two genes for common sequence elements. It is generally held that expression of co-regulated genes is directed by at least one common transcription factor, which binds to a specific *cis*-acting regulatory element (CARE) and contributes to the initiation of transcription (Yeung *et al.*, 2004). CAREs are short conserved motifs of 5–20





II

Key	Sequence element	Occurence	Detection program	Motif
	TCGATA	8935	TAIR Motif Analysis	GT1CORE
	AAACCGGA	1570	IMPROBIZER	MYB PLANT
	GATCC[AG]A[AC]CC	310	IMPROBIZER	LTRECORE
	CC[GA]A[CAT]CCG	2732	IMPROBIZER	MYB PZM
	AAC[GC][GA]AA[CA]C	1196	IMPROBIZER	MYB PLANT
	CCA[AC]AACG	1483	MEME	
	GGTCAA	10704	TAIR Motif Analysis	
	CTTGGC[GC]ACC	29	IMPROBIZER	
	CT[CA]CACCG[TG]C	137	IMPROBIZER	DPBF CORE
	CTCTCTC	8980	MEME	
	TACACG	6056	TAIR Motif Analysis	DPBF CORE
	CACGTG	8634	IMPROBIZER	G-Box
	CCT[GT]GC[CT]C	746	IMPROBIZER	
	CTC[GT]AGA[CT]CT	164	IMPROBIZER	
	GCGTGT	3697	TAIR Motif Analysis	

Figure 2. Several of the alternative pathway genes display common transcript responses revealed by GeneCluster 2.0. (A) cluster analysis was conducted using GeneCluster2.0 (Golub *et al.*, 1999) using quantitative RT-PCR data for all genes examined, expressed as fold changes relative to pre-treatment in Arabidopsis cell cultures over a 24 h time course following treatment, with each treatment analysed individually. Self-organising maps (SOMs) were generated where expression responses of certain genes were classed together in response to several treatments. (A-I) The expression responses of *AOX1a* and *NDB2* were placed in the same class in response to treatment with 5 μM antimycin A (AA), 200 μM erythromycin (Ery), 10 mM citrate (Cit), 40 μM rotenone (Rot) and 2.5 μM FCCP (FC), (A-II) the expression responses of *NDB2* and *NDB4* were placed in the same class in response to treatment with 200 μM chloramphenicol (Chl), 3 (% w/v) glucose (Glc), 100 μM norflurazon (Nor), (A-III) the expression responses of *AOX1a*, *NDB2* and *NDB4* were grouped in response to treatment with 3% (w/v) mannitol (Man), and (A-IV) the expression responses of *AOX1a*, *NDB2*, *NDB4* and *NDA1* were grouped in response to treatment with 5 mM malonate (Mal). The black line with filled circles represents the mean expression, and the area between the dashed lines represents the range of values within the cluster. (B) Analysis of the 1000 bp region of the upstream region of *AOX1a* and *NDB2* for common sequence elements that may control co-expression. Panel I shows the position of common elements found in each promoter region. Panel II describes the sequence element, the number of genes with the element in the upstream region, the program used to identify this sequence, and its description if it has been previously characterized as a CARE. The [ ] on the sequence element indicate that the base at that site can be any of the shown nucleotides.

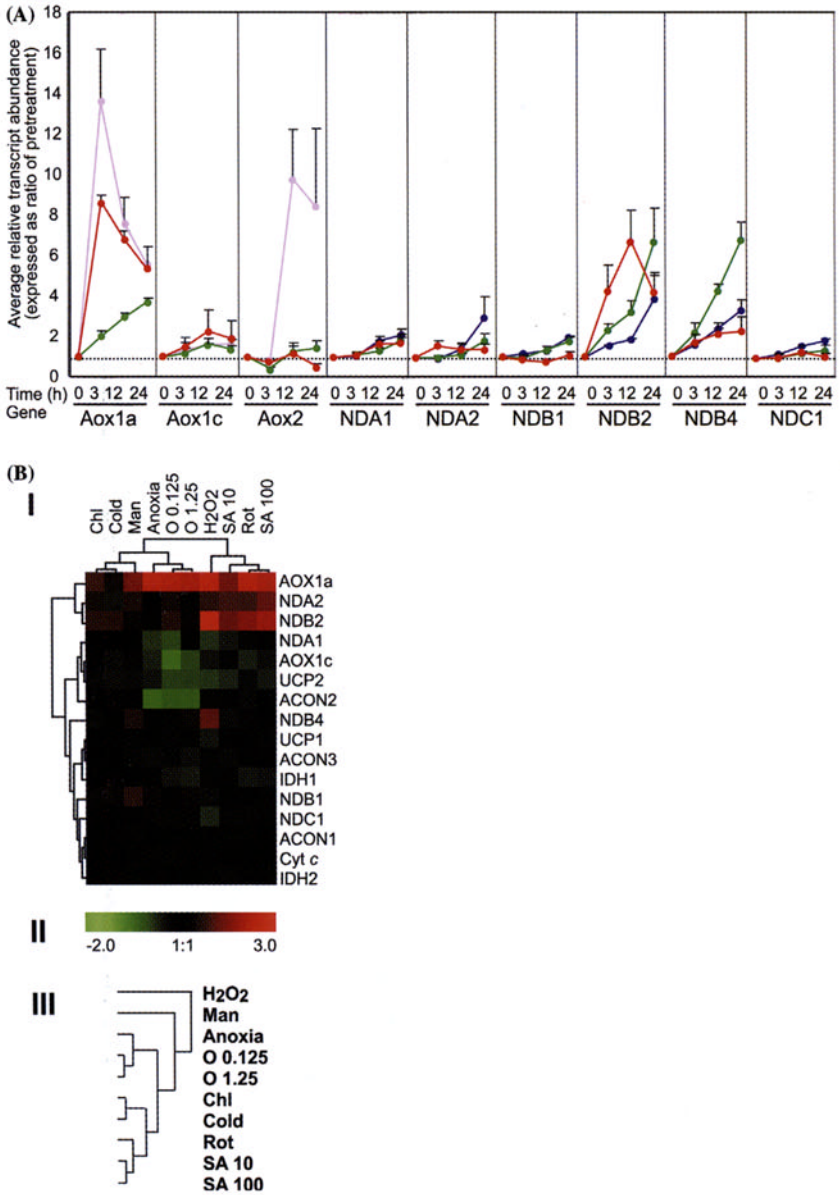
nucleotides located upstream of the transcription start site (Reichmann, 2002). *In vivo* regulatory patterns can be the result of the interplay of multiple CAREs and the relative organization of these binding elements within a promoter is thought to provide specificity of promoter-controlled gene regulation, in a manner described as a 'modular hierarchy'. According to this model, individual CAREs are grouped into islands of composite elements, themselves organised into modules that confer specific expression of a gene (Fessele *et al.*, 2002; Rombauts *et al.*, 2003). Therefore, we attempted to identify a common 'modular hierarchy' defined by a combination of elements with similar spacing for *AOX1a* and *NDB2*.

Several different pattern match tools were used to search for common elements, which were then examined for relative positional occurrence in both promoters, and the number of Arabidopsis genes possessing such elements in their 1000 bp upstream sequences was determined. Using the criteria described above, we identified a number of common elements with a similar localisation within the promoters (Figure 2B, part I). All of these elements were detected within 1000 bp upstream of approximately one third or less of the total complement of Arabidopsis genes, with one element associated with only 29 genes (Figure 2B, part II). Because a combination of different elements is likely to be responsible for co-regulation under different circumstances, it is unlikely that all elements would be involved under any one condition. Furthermore, elements that occur in a relatively large number of upstream regions (e.g. CACGTG, CTCTCTC – Figure 2B, part II) may play important roles in more general facets of gene regulation, such as chromatin remodelling (Kornberg and Lorch, 2002; Rombauts *et al.*, 2003). The results shown in Figure 2B suggest co-regulation of *AOX1a* and *NDB2*.

The response of alternative pathway components to stress can be grouped in sets

To investigate the number of pathways that regulate the expression of alternative respiratory components, we analysed the expression profiles of their genes for similar responses across all of the treatments. We again analysed the quantitative RT-PCR expression data using GeneCluster 2.0, but this time looked for similarity between treatments rather than similarity between genes. Treatments that induced common responses were grouped into treatment sets. It is important to note that within a treatment set the responses of the individual genes may have been very different – some may be up- and others down-regulated – but the pattern of responses of each individual gene

Figure 3. The transcript response profiles of the alternative pathway components form treatment sets. (A) GeneCluster 2.0 was used to analyse the quantitative RT-PCR generated expression profiles of the alternative pathway components for similarity in response across treatments; four treatment sets were identified. For each treatment set the mean ratio to $t = 0$ data obtained by quantitative RT-PCR is depicted with a different colour, broken into single gene responses, and the variation in ratios within each set is indicated by error bars. A compilation of three GeneCluster 2.0 analyses, involving the expression responses of the three *AOX* genes, or the six *NDH* genes, or the nine genes representing both the *AOX* and *NDH* genes to all treatments are shown. *Treatment set 1* (indicated in red) represents the expression responses of all nine alternative pathway genes to treatments likely to increase ROS production, 10 mM H_2O_2, 40 μM rotenone, 100 μM salicylic acid, and for the alternative *NDH* genes 0.125 μM oligomycin is also placed in this class. *Treatment set 2* (indicated in green) represents the expression responses of all nine alternative pathway genes to treatment with 3% ((w/v)) glucose, 200 μM chloramphenicol, 200 μM erythromycin, 100 μM norflurazon, with expression responses to 0.04 g/l paraquat included for the alternative *NDH* genes and to 3% (w/v) mannitol and 5 mM malonate included for the *AOX* family genes. *Treatment set 3* (indicated in purple) represents the expression responses of the three *AOX* genes in response to 1 mM cysteine and 0.04 g/l paraquat. *Treatment set 4* (indicated in dark blue) represents the expression responses of the six alternative *NDH* genes to 3% (w/v) mannitol and 5 mM malonate. (B) Hierarchical clustering of the signal ratio data derived from ATH1 GeneChip® experiments of Arabidopsis suspension cells after 3 h under 10 separate treatment conditions (100 and 10 μM salicylic acid (SA 100, SA 10), 40 μM rotenone (Rot), 1.25 μM and 0.125 μM oligomycin (O 1.25, O 0.125), anoxia (Anoxia), 200 μM chloramphenicol (Chl), 10 °C cold (Cold), 3% (w/v) mannitol (Man), and 10 mM H_2O_2 (H_2O_2). (I) TMeV analysis of GeneChip® data, under the 10 treatment conditions for 16 genes encoding mitochondrial proteins; two AOX genes (*AOX1a* and *AOX1c*), two UCP genes (*UCP1* and *UCP2*) and six members of the *NDH* gene family (*NDA1*, *NDA2*, *NDB1*, *NDB2*, *NDB4*, and *NDC1*), three aconitase genes (*ACON1*, *ACON2* and *ACON3*), two isocitrate dehydrogenase genes (*IDH1* and *IDH2*), and a cytochrome c gene (Cyt *c*). Data entered as $\log_2$ signal ratios, clustering performed using complete linkage and Euclidean distance. (II) Representation of colour scale used in I to indicate magnitude of fold changes, based on $\log_2$ signal ratio values. (III) Dendrogram illustrating the treatment clusters obtained with the average linkage hierarchical clustering tool in the TMeV analysis package when the whole transcriptome data is used.



across the treatments was consistent. Such coordination of response may point to a common step in the pathways involved in the regulation of the set of genes.

A GeneCluster 2.0 matrix was generated where the expression data for all genes at four time points was entered, each treatment representing a single row. Thus the 16 genes analysed by quantitative RT-PCR yielded 64 data points in each treatment row, forming a matrix composed of 64 data points with 10 treatment rows to give 640 data points in total. The unsupervised SOM function of GeneCluster 2.0 was then used to find sets of treatments representing common response trends. Because the large changes observed in expression of *AOX* and *NDH* gene families dominated the clustering process and the resulting output, we analysed the expression data for treatment sets in a batch manner. Firstly we analysed the *AOX* and *NDH* gene families separately and then we analysed the

two families together. We also analysed the remainder of the genes in supplementary Table 2 together. The trends in gene expression produced from GeneCluster 2.0 were expressed as the actual fold changes obtained by quantitative RT-PCR, with the range of fold changes within each set indicated by error bars. The analysis of the *AOX* and *NDH* gene families together or separately, yielded two treatment sets (sets 1 and 2). Additional treatment sets appeared when the *AOX* and *NDH* gene families were analysed separately (sets 3 and 4). For clarity, the gene expression patterns that define each treatment set are shown in Figure 3A as four different colours. The sets identified were:

- Set 1: The components of this group of treatments (H_2O_2, rotenone and 100 μM salicylic acid) are all likely to increase ROS – either directly or by inhibiting the electron transport chain – and the set was characterized by a dramatic induction of *AOX1a*, with maximum expression occurring between 3 and 12 h after commencement, and repression of *AOX2* expression (Figure 3A, red). For *NDH* gene expression, set 1 was characterized by a 3 h peak in *NDA2* transcript abundance and a 12 h peak in transcript levels of *NDB2* and *NDC1*, although the response of *NDB2* was by far the greatest. When the data for the *AOX* gene family was analysed separately, the oligomycin treatment also grouped with this set (data not shown).
- Set 2: This group of treatments (erythromycin, norflurazon, paraquat, glucose and chloramphenicol) targets plastid functions and was characterized by a constant increase in *NDB2* and *NDB4* expression over the entire time course of treatment, with stable expression of the other family members (Figure 3A, green – *NDH* gene family). Again, when the data for the *AOX* gene family was analysed separately, the same treatment set was seen but paraquat was replaced by malonate and mannitol (Figure 3A, green – *AOX* gene family). This is characterized by an increase in *AOX1a* expression over 24 h and a decrease in *AOX2* expression at 3 h.
- Set 3: This treatment set was only seen when the *AOX* gene family data was analysed alone; it contained cysteine and paraquat treatments and was characterized by a sharp increase in *AOX1a* transcript abundance over the first 3 h, followed by an equally sharp increase in *AOX2* transcript abundance between 3–12 h (Figure 3A, purple).
- Set 4: This treatment set was only seen when the *NDH* gene family was analysed alone; it contained malonate and mannitol and was characterized by a gradual increase in transcripts for all of the *NDH* genes over the entire time course (Figure 3A, blue).The distinction between these treatment sets suggests that multiple pathways regulate expression of alternative pathway components, which is not necessarily evident from single time point analysis. For instance, *AOX1a*, *AOX1c* and *AOX2* were all induced at 12 h, but analysis over the complete time course showed that the kinetics of induction were quite distinct for the three genes. Interestingly, antimycin A was not included in any of the treatment sets identified. This suggests that none of the other treatments restricted the cytochrome pathway in the same manner as antimycin A.

To further investigate the pathways regulating expression of alternative respiratory chain components, we utilised our Arabidopsis ATH1 GeneChip® data. The 3 h time point data for a number of treatments (10 and 100 μM salicylic acid, rotenone, H_2O_2, chloramphenicol, cold, oligomycin at 1.25 and 0.125 μM, anoxia and mannitol) was used to identify initial responses. To enable a comparison between the two approaches at this time point, the array data were initially analysed only for the genes for which quantitative RT-PCR data were obtained using the hierarchical cluster function in TMeV (Figure 3B, panel I). When the whole transcriptome response was examined with the TMeV software, again using hierarchical clustering, these clusters were maintained (Figure 3B, panel III).

A possible role for AOX2 in inter-organelle signalling involving plastid-dependent mitochondrial functions?

Unlike the dominantly expressed *AOX1a* isoform, which responds to most treatments examined with an

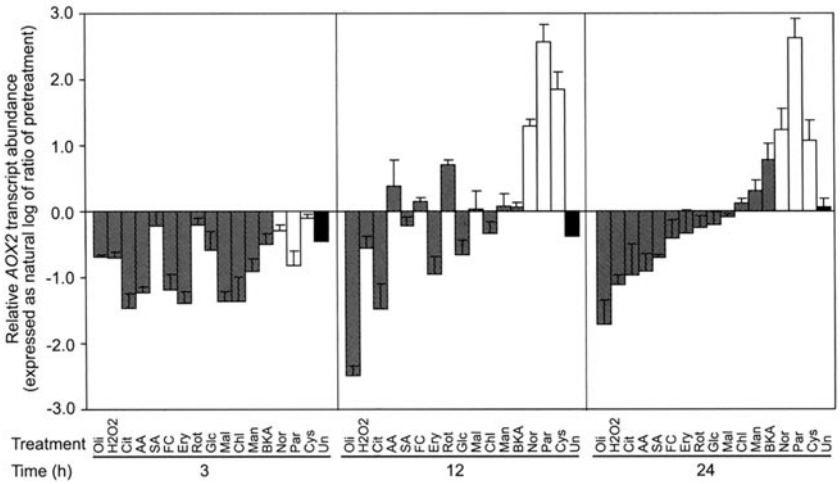


Figure 4. Transcript response profile of *AOX2* in response to mitochondrial and chloroplast dysfunction. Relative transcript abundance of *AOX2*, detected by quantitative RT-PCR in Arabidopsis cell cultures over a 24 h time course following treatment with chemicals, from left to right, 0.125 μM oligomycin (Oli), 10 mM H_2O_2 (H_2O_2), 10 mM citrate (Cit), 5 μM antimycin A (AA), 100 μM salicylic acid (SA), 2.5 μM FCCP (FC), 200 μM erythromycin (Ery), 40 μM rotenone (Rot), 3% (w/v) glucose (Glc), 5 mM malonate (Mal), 200 μM chloramphenicol (Chl), 3% (w/v) mannitol (Man), 50 μM bongkrekic acid (BKA), 100 μM norflurazon (Nor), 0.04 g/l paraquat (Par) and 1 mM cysteine (Cys), and in untreated cells (Un). Transcript abundance is expressed as the natural log of the ratio to the pre-treated levels for each treatment (time = 0), error bars represent SE.

increase in transcript levels, *AOX2* expression was repressed by many of these treatments (Figure 4). Interestingly, repression of *AOX2* was observed following treatments likely to increase the flux of substrates to the TCA cycle, such as addition of glucose or citrate, or to disrupt mitochondrial function, such as antimycin A and malonate, as well as in response to more direct inducers of ROS such as H_2O_2 and salicylic acid. In contrast, a distinct group of treatments, all of which potentially perturb chloroplast function (paraquat, cysteine, and norflurazon), resulted in dramatic induction of *AOX2* (Figure 4 – indicated in white).

Discussion

The use of whole genome arrays coupled with quantitative RT-PCR enables analysis of the impacts of treatments to be assessed broadly, providing a wealth of candidate genes and responses that can then be investigated in a more specific and detailed manner using single gene analysis. Whilst the signal response for array data is linear over three to four orders of magnitude, low abundance transcripts may not be detected, or potentially removed from analysis as their signals may not be sufficiently above background to ensure a robust quantitation (Yue *et al.*, 2001; Yang *et al.*, 2002; Lyne *et al.*, 2003). Thus, quantitative RT-PCR with a typical detection range of five to six orders of magnitude, high accuracy and high reproducibility is the method of choice to detect subtle changes in gene expression (Chuaqui *et al.*, 2002; Yang *et al.*, 2002; Stanley Kim *et al.*, 2005). This technique is particularly relevant for this study, due to the low abundance of certain alternative family genes, such as *AOX2* (Thirkettle-Watts *et al.* 2003).

Alternative respiratory pathway responds rapidly to stress

In response to a range of conditions imposed on Arabidopsis cell cultures, microarray analysis provided a snapshot of the transcriptome revealing that most classical respiratory pathway and TCA cycle genes were stable under the majority of conditions, at the time points sampled. Transcript abundance of the alternative respiratory pathways, on the other hand, was much more dynamic. Quantitative RT-PCR was used to validate and extend the microarray data, examining the transcript response of all members of the alternative respiratory by-passes in addition to components of the classical respiratory chain and TCA cycle over

a 24 h time course, confirming the response of the alternative pathway components.

Gene families encoding alternative respiratory enzymes respond differently

AOX and *NDH* gene family members displayed dramatic and different responses to the treatments applied, suggesting that differential regulation occurs within the alternative gene families, which may be a reason for these component being encoded in small gene families in plants. Analyses of genetic lesions that disrupt mitochondrial respiration have shown that the locus of inhibition defines the response. Gene-specific induction has been demonstrated previously for the *AOX* gene family in maize, where different non-chromosomal stripe mutants (NCS) display up-regulation of different AOX isoforms; notably, NCS2 which has a defect in complex I induced *AOX2* whereas NCS6 which lacks cytochrome oxidase activity induced *AOX3* (Karpova *et al.*, 2002).

Both NDHs and AOX are encoded in two discrete gene subfamilies in higher plants. Members of the *AOX1* subfamily, represented by *AOX1a–d* in Arabidopsis, are induced by a range of stress stimuli and proposed to relate to a particular need under stress conditions. Members of the *AOX2* subfamily, on the other hand, display constitutive or developmental expression, and are proposed to play a 'housekeeping' role in respiratory metabolism (Considine *et al.*, 2002). However, the dramatic response of *AOX2* to a specific subset of treatment conditions in this study, indicates a role for *AOX2* in stress responses. Interestingly, all of the treatments inducing *AOX2* – norflurazon, paraquat and cysteine – disrupt plastid function or impair plastid regulation. For example, the *gun* mutant lines, with disrupted plastid to nucleus signalling, were identified via a screen looking for altered expression of light harvesting complex (*Lhcb1*) to norflurazon treatment (Susek *et al.*, 1993; Gray *et al.*, 2003; Rodermel and Park, 2003). Norflurazon induces chlorophyll photooxidation, and results in repression of largely chloroplast-targeted genes, but also genes for photorespiratory enzymes (Gray *et al.*, 2003). Likewise paraquat acts to disrupt chloroplast function as an electron acceptor from photosystem I (PSI) of the chloroplast thylakoid (Vicente *et al.*, 2001), whilst both paraquat and cysteine, as a redox modulators, may act by disrupting the balance between the redox retrograde plastid to nucleus signalling pathways (Peixoto *et al.*, 2004; Fey *et al.*, 2005). Several groups have suggested light, or chloroplast activity, may be involved in the regulation of *AOX2* transcript and/or protein expression in a number of species (Finnegan *et al.*, 1997; McCabe *et al.*, 1998; Thirkettle-Watts *et al.*, 2003; Escobar *et al.* 2004). Light regulation of nuclear genes encoding photosynthesis related proteins has been demonstrated to act via redox signals, and both cysteine and paraquat have the potential to act as pro-oxidants and modulate redox signals (Vanlerberghe *et al.*, 2002; Peixoto *et al.*, 2004). Based on these observations, we propose that *AOX2* may play a role in inter-organellar crosstalk, possibly involving a redox signal. Norflurazon induction of *AOX1* was observed in soybean and thus it appears that a role for *AOX* induction in response to plastid perturbation may exist in all species, but the specific gene family member may be different (Djajanegara *et al.*, 2002).

Specific genes display co-expression

Co-expression of specific *AOX*s and *NDH*s was observed in several treatments, possibly indicating co-ordinated regulation between alternative component gene families. Genes displaying similar expression patterns may share regulatory pathways and have common binding sites for the relevant transcription factors (DeRisi *et al.*, 1997; Spellman *et al.*, 1998; Park *et al.*, 2002). In plant mitochondria this has been illustrated for three complex I subunits in Arabidopsis, which show anther specific co-expression and which are all regulated by the transcription factor ARR2 (Lohrmann *et al.*, 2001). We propose that the observed co-expression of *AOX1a* and *NDB2* may be due, at least in part, to co-regulation, supported by the presence of common sequence elements with similar organization within the sequence upstream of the coding region of both these genes (Figure 2B). Whilst some of the shared sequence elements identified are prevalent in the upstream regions of a number of Arabidopsis genes, others occur very infrequently. This does not imply that the 'rarer' elements are necessarily more significant in directing the co-expression of *AOX1a* and *NDB2*, as limited correlations between individual

transcription factor binding sites and gene expression patterns have been observed (Brazma *et al.*, 1998). As has been demonstrated in mammalian systems, transcriptional specificity often depends on combinations of at least two transcription factor binding sites (Yuh *et al.*, 1998; Fessele *et al.*, 2002) and in yeast it has been shown that different functionalities can be conferred on one transcription factor by its association with different co-factors (Pilpel *et al.*, 2001). In complex organisms, transcription factor binding site organization shows a functional hierarchy, which dramatically increases the potential specificity and selectivity available for gene regulation (Werner *et al.*, 2003). Thus it is the multiplicity of common sequence elements with similar organization that gives weight to the proposal that *AOX1a* and *NDB2* may be co-regulated. *In vivo* assessment of the functionality of these elements needs to be determined to prove this idea.

It has been proposed that the difference in response observed between the cytochrome pathway and alternative pathway in response to light may be related to the necessity of quick adaptation to changes in energy demand and reductant levels that can arise under certain environmental conditions (van Lis and Atteia, 2004). The proteins involved in the alternative pathways, AOXs and NDHs, are thought to be active as single subunit or homodimeric enzymes (Siedow and Umbach, 2000). In contrast the respiratory complexes of the cytochrome pathway require the assembly of multiple subunits into complexes up to 1000 kDa, which may then form supercomplexes (Millar *et al.*, 2004; Dudkina *et al.*, 2005). We propose that the products of *AOX* and *NDB*, a terminal oxidase and external NAD(P)H dehydrogenases, are capable of forming a simple but functional alternative respiratory chain, allowing oxidation of cytosolic NAD(P)H in a manner which is uncoupled from oxidative phosphorylation (and the attendant adenylate regulation) (Figure 5). The co-ordinated induction of these two activities enables formation of a distinct and self-contained biochemical pathway that may help maintain cellular redox balance of the cell and turnover of carbon pathways. Such co-ordinated induction of specific mitochondrial activities and of genes encoding mitochondrial proteins has been demonstrated before in plant systems. For example rotenone-insensitive malate oxidation, external NDH activity and AOX activity, all responded to phosphate limitation in a co-ordinated manner in tobacco cells (Rasmusson *et al.*, 2004). AOX and external rotenone-insensitive NADH dehydrogenase activity were also co-ordinately induced in tobacco mutants carrying a deletion of mitochondrial DNA encoding the *NAD7* subunit of complex I (Gutierres *et al.*, 1997).

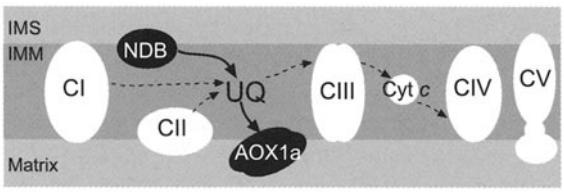


Figure 5. Diagrammatic representation of the plant mitochondrial electron transport chain. The multi subunit complexes I, II, III and IV and cytochrome c which comprise the cytochrome electron transport chain are shown in white. The stress inducible alternative external NADH dehydrogenases (*NDB*) and alternative oxidase (*AOX1a*) are shown in black. The flow of electrons is indicated by arrows with the proposed stress inducible pathway indicated by solid arrows. IMS = intermembrane space, IMM = inner mitochondrial membrane, UQ = ubiquinone.

AOX is regulated both at a transcriptional and a post-transcriptional level. The gene is induced in response to a range of stress and environmental stimuli, whilst reduction of the enzyme to its active form can be stimulated by certain α-keto acids, enabling it to respond to the redox state of the mitochondrial matrix (Vanlerberghe *et al.*, 1999; Gray *et al.*, 2004). Likewise, it has been suggested that the primary mode of UCP regulation is biochemical activation (Considine *et al.*, 2003). Since the proposed role of UCPs is to mildly uncouple the proton-pumping ETC from ATP synthesis, it might be expected that their expression is regulated coordinatedly with that of the cytochrome chain itself. Indeed, the stable expression of the majority of cytochrome chain subunits in the face of the stresses imposed here, was mirrored by UCP1 expression, whilst UCP2 responded to treatments in a similar manner to cytochrome *c*. In addition, cluster analysis did not reveal similarities in the transcript responses of either of the *UCP* genes with any of the *AOX* or *NDH* genes. This is consistent with the non-responsive nature of UCPs in maize non-chromosomal stripe (NCS) mutants exhibiting altered AOX

expression (Karpova *et al.*, 2002). Accordingly, we propose that the UCPs are regulated and function in a manner distinct from that of AOX and NDHs in plants.

Treatment sets indicate multiple signalling pathways

In a manner analogous to the expression of the Arabidopsis glutathione peroxidase gene family (Rodriguez Milla *et al.*, 2003), our results indicate that the responses of the alternative respiratory gene families are mediated by multiple signalling pathways. The fact that not all stress conditions affected the expression of components of both the alternative and classical respiratory genes in the same way suggests that specific signals, not just general stress, are needed for the responses observed. Previous studies have begun to discriminate between the multitude of conditions able to stimulate AOX expression at a protein, activity, or transcript level (Vanlerberghe and McLntosh, 1996; Vanlerberghe *et al.*, 1998). This study extends this process, providing support for the existence of both ROS dependent and ROS independent TCA cycle linked redox associated signalling pathways in the regulation of the alternative pathway components.

The qRT-PCR derived set 1 (Figure 2) is also seen in cluster analysis of the array data, which makes an additional distinction between treatments targeting the entry points to the classical respiratory pathway and treatments targeting the energy production stages viz., rotenone versus oligomycin. Whilst all the treatments in set 1 have been associated with ROS, the absence of antimycin A – long associated with ROS production – from this set may be due to the magnitude of the response it induced. The idea that different type of signals arise depending on the magnitude of the stimulus has been proposed for the light intensity derived redox signalling pathways of photosynthetic electron transfer (Pfannschmidt *et al.*, 2001). In this model, responses to low light intensity are controlled by redox signalling from the plastoquinone pool, responses to moderate light conditions are regulated by redox signalling from thioredoxin, and extreme light intensities induce a stress response mediated by glutathione and ROS. It remains to be determined if the signals of these three redox signalling chains converge at a single element in the promoters of responsive genes or if they affect different regulatory steps in gene expression (Gray *et al.*, 2003). The magnitude of *AOX1a* induction in response to salicylic acid, rotenone and hydrogen peroxide treatments was not seen with other treatments which also disrupt mitochondrial function and induce ROS, supporting the proposal that the manner in which the alternative pathway components respond may be controlled in part by the intensity of the stimulus. This provides a mechanism allowing the integration of the ROS and the citrate associated AOX induction pathways. The ability of some compounds to produce ROS and cause an accumulation of citrate, e.g. H_2O_2 (Vanlerberghe, 1996, p. 46), provides a mechanism to amplify the intensity of some signals by crossing over of signalling pathways that lead to the induction of AOX.

The fact that treatments affecting chloroplast function caused changes in expression of genes encoding mitochondrial proteins, supports the idea of inter-organellar signalling. Treatments constituting set 2 (Figure 2) can all be linked to plastid function, yet these treatments vary significantly in their proposed mode of action. For example, erythromycin inhibits plastid protein synthesis, which is required for production of transcripts of nuclear encoded photosynthetic genes, effectively disrupting the plastid signal (Sullivan and Gray, 1999). In contrast, elevated levels of cellular sugars such as glucose, upregulate genes involved in synthesis of polysaccharides, storage proteins, pigments, as well as genes associated with defence responses and respiration (Price *et al.*, 2004). Further investigation is needed to produce a coherent explanation of how these apparently diverse treatments converge at some point in a signalling pathway controlling the expression of the alternative respiratory pathways.

In support for a TCA cycle (citrate) associated regulation of alternative and classical respiratory pathways, we found that citrate treatment was able to stimulate *NDB*s, and *IDH2* was induced under many conditions which also stimulated *AOX1a*. Of the TCA cycle genes examined, the response of *IDH2* was unique. *IDH2*, also referred to as *mtIDH*, has been proposed as a regulatory switch involved in TCA cycle flux and the reductive modulation of AOX (Gray *et al.*, 2004). Correlation between the oxidation of specific TCA cycle substrates (citrate, isocitrate, and malate) whose oxidation can produce matrix NADPH and an

abundance of reduced AOX, has been clearly demonstrated (Vanlerberghe and McIntosh, 1996; Vanlerberghe *et al.*, 1999). Gray *et al.* (2004) proposed that intramitochondrial reducing power (NADPH) generated by the activity of mtICDH in tobacco promotes the reduction of AOX to its more active form, possibly mediated by a thioredoxin/thioredoxin reductase system. Under conditions of restricted electron transport through the cytochrome pathway, accumulation of pyruvate and TCA cycle intermediates would result in increased NADPH, which could potentially activate the alternative oxidase. While this would decrease the ATP yield of respiration, it would concomitantly act as a 'clutch', whose engagement would permit increased glycolytic and TCA cycle turnover for the production of biosynthetic carbon skeletons. Thus a redox-modulated regulation of AOX would couple AOX activity to TCA cycle metabolism and the redox state of the mitochondrial matrix (Vanlerberghe *et al.*, 1998). This coupling allows effective balancing of cellular NAD(P)H/NAD(P)+ and ATP/ADP ratios, thus linking respiratory energy coupling with demands for carbon skeletons (Gray *et al.*, 2004 and refs. within). Support for the proposal that flux through mtICDH may be part of a biochemical framework for the post-translational reductive regulation of AOX also comes from tobacco, where citrate feeding results in increased concentrations of both NADPH and the reduced form of AOX (Gray *et al.*, 2004).

Cysteine is a dramatic repressor of the cytochrome chain capacity (Vanlerberghe *et al.*, 2002). Our data reveal that both classical and alternative respiratory genes, in addition to genes of the TCA cycle, were affected by cysteine treatment. As described by Vanlerberghe *et al.* (2002), elucidating the mechanism through which cysteine treatment has induced the majority of genes examined is complicated by the variety of ways cysteine can act on the cell, which include: acting as a reducing agent, possibly capable of reducing regulatory disulfide bonds in target proteins; acting as a substrate, cysteine is the primary precursor of all organic molecules containing reduced sulfur, hence addition of cysteine may result in an increase in a downstream metabolite; and acting via a ROS, thiols, since cysteine readily undergoes auto-oxidation, generating superoxide anion, and this pro-oxidant effect is greatly enhanced in the presence of redox-active metal ion Fe. Interestingly, cluster analysis revealed that paraquat induced similar responses in the alternative pathway genes. Chloroplast and mitochondrial targets for both treatments have been described (Vanlerberghe *et al.*, 2002; Peixoto *et al.*, 2004).

Conclusions

In this study we demonstrate the stability of the transcript levels of the classical respiratory chain components under a range of stress treatments over a 24 h period. In contrast, transcript levels of components of the alternative respiratory pathways, namely members of the *AOX* and *NDH* gene families, display dramatic and co-ordinated responses to stress treatments. Analysis of transcript responses, in terms of genes that cluster together and treatments that induce common responses, has provided insight into the potential mechanisms regulating these two gene families, enabling further dissection of the pathways leading to induction of the individual genes and aiding understanding of their functions. We propose that the presence of multiple members of the alternative components in gene families provides response flexibility, enabling the cell to more finely control its response to various stresses.

Acknowledgements

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