

Dynamic changes in the mitochondrial electron transport chain underpinning cold acclimation of leaf respiration

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ABSTRACT

We examined the effect of short- and long-term changes in temperature on gene expression, protein abundance, and the activity of the alternative oxidase and cytochrome oxidase pathways (AOP and COP, respectively) in *Arabidopsis thaliana*. The AOP was more sensitive to short-term changes in temperature than the COP, with partitioning to the AOP decreasing significantly below a threshold temperature of 20 °C. AOP activity was increased in leaves, which had been shifted to the cold for several days, but this response was transient, with AOP activity subsiding (and COP activity increasing) following the development of leaves in the cold. The transient increase in AOP activity in 10-d cold-shifted leaves was not associated with an increase in alternative oxidase (AOX) protein or *AOX1a* transcript abundance. By contrast, the amount of uncoupling protein was significantly increased in cold-developed leaves. In conjunction with this, transcript levels of the uncoupling protein-encoding gene *UCPI* and the external NAD(P)H dehydrogenase-encoding gene *NDB2* exhibited sustained increases following growth in the cold. The data suggest a role for each of these alternative non-phosphorylating bypasses of mitochondrial electron transport at different points in time following exposure to cold, with increased AOP activity being important only in the early stages of cold treatment.

Key-words: *Arabidopsis*; alternative oxidase; cytochrome oxidase; gene expression; temperature; uncoupling protein.

INTRODUCTION

Mitochondrial respiration is a vital component of plant metabolism, generating the energy and carbon skeletons necessary for the proper functioning of the plant (Lambers, Szaniawski & de Visser 1983). Respiratory energy transduction takes place in the inner mitochondrial membrane,

where high energy electrons generated during glycolysis and the TCA cycle pass via a series of electron transfer proteins to the terminal electron acceptor oxygen. Importantly, plant mitochondria possess two pathways of mitochondrial electron transport: the cytochrome oxidase pathway (COP), in which electrons in the ubiquinone (UQ) pool are transferred via the ubiquinol-cytochrome *c* oxidoreductase to cytochrome oxidase (COX), and the alternative oxidase pathway (AOP), in which electrons are transferred from the UQ pool directly to the alternative oxidase (AOX). Electron flow via the COP is coupled to the movement of protons across the inner mitochondrial membrane and the subsequent generation of ATP via the ATP synthase. In contrast, electron flow via the AOP is not coupled to proton translocation, and thus electron flow via this pathway does not result in the generation of ATP (Moore & Siedow 1991; Millenaar & Lambers 2003). A number of studies have proposed that the AOP functions to minimize the generation of reactive oxygen species (ROS) during periods of stress, or when the COP is restricted, by stabilizing UQ reduction levels (Møller 2001). However, the extent to which the AOP is engaged will have a significant impact on the efficiency of respiratory energy production, with increased engagement resulting in a decrease in the amount of ATP produced per unit CO₂ released and/or O₂ consumed. Given the significance of the AOP in determining plant energy production, it is important that we understand how changes in the environment affect the activity of this pathway. One of the most influential environmental variables in this respect is temperature (James 1953; Forward 1960; Berry & Raison 1981).

Respiration is a temperature-sensitive process, with a change in temperature resulting in an immediate alteration in the rate of respiration. The temperature sensitivity of respiration is measured and referred to as the Q_{10} of respiration (defined as the proportional increase in rate for every 10 °C rise in temperature). Q_{10} values are known to vary between species (Larigauderie & Körner 1995; Atkin *et al.* 2005), tissue types (Loveys *et al.* 2003; Atkin *et al.* 2005) and physiological conditions (Fitter *et al.* 1998; Atkin

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et al. 2000b, 2005), and typically lie between 1.4 and 4 (Azcón-Bieto 1992; Larigauderie & Körner 1995; Atkin, Edwards & Loveys 2000a). However, while the temperature sensitivity of respiration has been intensively studied at the total flux level (Atkin & Tjoelker 2003; Atkin *et al.* 2005), less is known about the underlying sensitivities of the alternative and cytochrome pathways to short-term changes in temperature. Earlier work suggested that the AOP might be less sensitive to cold than the COP in isolated cells and mitochondria (Yoshida & Tagawa 1979; Stewart *et al.* 1990a) and intact tissues (Kiener & Bramlage 1981; McNulty & Cummins 1987; Collier & Cummins 1990; Popov *et al.* 1997; Maxwell, Wang & McIntosh 1999), fueling the proposition that the AOP functions to prevent the over-reduction of the UQ pool under adverse environmental conditions (Purvis & Shewfelt 1993). However, more recent work has challenged these findings. Both Atkin, Zhang & Wiskich (2002) and Ribas-Carbó *et al.* (2005) observed little difference in the temperature sensitivity of the AOP and COP in soybean cotyledons and pea leaves. Furthermore, while González-Meler *et al.* (1999) also found little difference in the temperature sensitivity of the two pathways in soybean cotyledons, they found that the AOP was actually more sensitive to short-term changes in temperature than the COP in mung bean leaves and hypocotyls, with the AOP exhibiting Q_{10} values close to 3, and the COP exhibiting values close to 2.

Respiration is often able to acclimate to long-term changes in temperature, such that cold-grown plants often exhibit higher rates of respiration than their warm-grown (WG) counterparts when measured at their respective growth temperatures (Billings *et al.* 1971; Collier 1996; Atkin, Holly & Ball 2000b; Xiong, Mueller & Day 2000; Bolstad, Reich & Lee 2003; Talts *et al.* 2004). Previous work examining the impact of sustained exposure to cold on respiration has suggested that the AOP might play a critical role in the cold acclimation response. AOX transcript abundance (Ito *et al.* 1997; Takumi *et al.* 2002), protein abundance (Stewart *et al.* 1990a,b; Vanlerberghe & McIntosh 1992; González-Meler *et al.* 1999), and capacity (Elthon *et al.* 1986; McNulty & Cummins 1987; Vanlerberghe & McIntosh 1992) have all been shown to increase following growth in the cold. In addition, *in vivo* partitioning of electrons to the AOP has been shown to increase following sustained exposure to cold (González-Meler *et al.* 1999; Ribas-Carbó *et al.* 2000). It has been proposed that cold-induced increases in AOP activity function to prevent the over-reduction of the mitochondrial electron transport chain, and thus the accumulation of ROS, at low temperatures (Purvis & Shewfelt 1993). However, the response of the AOP to cold is not entirely clear cut. Ribas-Carbó *et al.* (2000) found that AOP activity is increased only in chilling-sensitive cultivars of maize, and more recently, Kurimoto *et al.* (2004) and Armstrong *et al.* (2006) found that re-establishment of respiratory flux in the cold is associated not with an increase in AOP capacity, but rather with an increase in COP capacity. Such variability in the AOP response suggests that different plant species employ

different strategies for coping with cold. We might predict that in a cold-tolerant species, such as *Arabidopsis thaliana*, cold acclimation involves a transitory increase in AOP activity, which subsides once the plant is able to re-establish flux via the energy-conserving COP.

In addition to the AOX, plant mitochondria possess two additional non-phosphorylating bypasses of the mitochondrial electron transport chain: the alternative NAD(P)H dehydrogenases (NDHs) and the uncoupling proteins (UCPs). Both of these proteins function to reduce the extent to which mitochondrial electron transport is coupled to the production of ATP. The NDHs oxidize matrix and cytosolic NAD(P)H, but do not contribute to proton pumping, while the UCPs facilitate proton flux back through the inner mitochondrial membrane, thereby partially dissipating the proton gradient across this membrane (Sluse & Jarmuszkiewicz 2002). Both the NDHs and the UCPs are thought to play a role in preventing oxidative stress; the UCP protein is activated by superoxides (Smith, Ratcliffe & Sweetlove 2004), UCP transcripts have been shown to increase in response to cold in *Arabidopsis* (Maia *et al.* 1998), and NDH activity and transcript levels have been shown to increase in tobacco leaves, red beet roots and *Arabidopsis* cell cultures following exposure to a range of abiotic stresses (Gutierrez *et al.* 1997; Clifton *et al.* 2005). Previous work on CMS tobacco lines (Gutierrez *et al.* 1997) and *Arabidopsis* cell cultures (Clifton *et al.* 2005) suggests that AOX and NDH activity and transcript levels might be co-regulated; indeed, an analysis of over 200 microarray experiments indicates a strong pattern of co-expression between *AOX1a* and *NDB2* in *Arabidopsis* (Clifton, Millar & Whelan 2006). Whether transcript levels of these alternative respiratory bypasses exhibit coordinated increases in abundance following sustained (as opposed to short-term) exposure to cold remains to be established.

Here, we utilize a novel modification of the ^{18}O discrimination technique (that permits a continuous sampling of gas samples) to examine the effect of short- and long-term changes in temperature on *in vivo* AOP and COP activity in *A. thaliana*. We measured ^{18}O discrimination of respiration in WG leaves, in WG leaves shifted to the cold for 1, 2, 4, 7 and 10 d, and in leaves that were developed in the cold. Measurements of ^{18}O discrimination were combined with measurements of AOX and COX protein and transcript abundance in order to gain a process-based understanding of the response. In addition, levels of the UCP and transcript levels of additional COP complexes and non-phosphorylating bypasses of mitochondrial electron transport were measured to gain a wider understanding of the changes involved in the respiratory cold acclimation response.

MATERIALS AND METHODS

Plant material and growth conditions

Measurements were conducted on wild-type *A. thaliana* (ecotype Columbia). Plants were grown on compost in a controlled environment growth chamber maintaining a

21/20 °C day/night temperature regime, a photon flux density of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and a 10 h photoperiod. After 5 weeks growth, the WG plants were shifted to a cold room maintaining a 5/5 °C day/night temperature regime (with growth irradiance and photoperiod being the same as in the 21/20 °C chamber). The shift to cold took place at the end of a night period shortly after the lights came on in the cabinet.

Measurements were conducted on (1) WG leaves; (2) WG leaves shifted to the cold and maintained there for a period of 1, 2, 4, 7 or 10 d, hereafter referred to as cold-treated (CT) leaves; and (3) leaves that had developed in the cold (sampled after 35–45 d at 5 °C), hereafter referred to as cold-developed (CD) leaves. All measurements were conducted on mature, fully expanded leaves.

Respiration and ^{18}O discrimination

Mass spectrometer

The concentrations of $^{16}\text{O}_2$, $^{18}\text{O}^{16}\text{O}$ and $^{12}\text{CO}_2$ were measured using a purpose-built IsoPrime stable isotope gas mass spectrometer from Micromass Instruments (Manchester, UK). The instrument has eight Faraday collector buckets arranged in a configuration where, for one accelerating voltage and magnetic field setting, it is possible to simultaneously monitor eight separate masses including masses 32 ($^{16}\text{O}_2$), 34 ($^{18}\text{O}^{16}\text{O}$) and 44 ($^{12}\text{CO}_2$). Data collection was modified such that the collector outputs could be monitored independently of the computer software supplied with the instrument. A Visual Basic (Microsoft) program was created on a measurement computer for conducting assays, calculating the data in 'real time' and saving the data to file. The measurement system was set up to continuously monitor the frequency outputs of the head amplifier. Data were divided into 'time slices', with a typical measurement point representing a 5 s average of the frequency output of each collector. A reference frequency generator from the counter card used by the measurement computer provided the time base for measurements. Measurements on the mass 32 collector were made with 10^9 ohm resistors, while measurements of masses 34 and 44 were made with 10^{11} ohm resistors.

During the development of the analysis procedure, it became apparent that the greatest source of error was the establishment of accurate zero values for mass 32 and mass 34 signals that were to be subtracted from the measured signals during the experiment. These zeros arise both from the electrical zero of the machine as well as small oxygen leaks into the vacuum system during the experiment. These give a small constant O_2 background and are not related to any leaks associated with the reaction cuvette. As we were measuring the changes in part per thousand, an incorrect zero could have biased the discrimination measurement quite dramatically. We found that a number of methods could be used to establish an accurate and reproducible zero value. These included flushing continuously with oxygen-free nitrogen, or isolation of the cuvette from the mass spectrometer source by a valve located adjacent to the

reaction cuvette. As the last technique was the most convenient and subject to least error, we regularly used this to measure the mass 32 and mass 34 zero values. These zero values were subtracted from each mass measurement during the experiment. These values were very reproducible and remained constant for several hours of experiments.

Measurement protocol

Measurements were carried out in the gas-phase, in a stainless steel, water-jacketed cuvette (Supplementary Fig. S1). A piece of filter paper moistened with 7 N NaOH was placed at the bottom of the cuvette to absorb the CO_2 released from the leaves (this was done to prevent CO_2 building up to high levels and potentially interfering with leaf metabolism); 10 leaf discs (totaling 130 mm^2 if using WG or CT leaves, and 60 mm^2 if using CD leaves) were placed above this. The cuvette was then sealed using a perspex lid and was covered with a dark cloth. Rates of O_2 uptake were recorded following a minimum of 30 min dark adaptation. Average net discrimination was calculated once; approximately one-fifth of the initial O_2 present in the cuvette had been consumed (Supplementary Fig. S2). During this reaction period, the consumption of O_2 by the permeable membrane at the base of the cuvette, because of the mass spectrometer vacuum, was less than 5% of the amount consumed by the leaf tissue. Leak rates for the cuvette were tested by flushing the cuvette with nitrogen and measuring the subsequent influx of O_2 . No influx of O_2 was observed under these conditions, indicating that atmospheric leaks were negligible in this system.

Discrimination by the COP and AOP was determined by exposing the leaf discs to separate inhibitor treatments prior to measurement. Discrimination by the COP was determined by soaking the leaf discs in 50 mM SHAM dissolved in 10% ethanol for 45 min prior to measurement; the leaves were blotted dry before use. Although lower concentrations of SHAM are often used in studies where leaves are sliced into 2 mm sections (e.g. Azcón-Bieto, Day & Lambers 1983; Atkin & Day 1990), we found that it was necessary to use 50 mM SHAM when working with unsliced leaf discs. A 50 mM SHAM was also used by Florez-Sarasa *et al.* (2007) in their study of AOP and COP activity in *Arabidopsis* leaves. In control experiments, exposure of the leaves to 10% ethanol reduced the discrimination value, but to a much lesser extent than SHAM; incubation with ethanol had no effect on the rate of respiration (data not shown). Discrimination by the AOP was determined by incubating leaf discs in a 15 mL sealed tube containing a piece of filter paper saturated with 1 M KCN (in 100 mM TES, pH 7) for 30 min prior to measurement. All stocks were freshly prepared on the day of use. Discrimination by the COP was measured in WG leaf discs. However, when discrimination by the AOP was measured in the WG leaves, rates were too low to establish an accurate value. Thus, discrimination by the AOP was measured in 10 d CT leaf discs, because of the higher rates of AOP respiration in these tissues.

Calculation of oxygen isotope discrimination

Discrimination against ^{18}O during dark respiration was calculated as the slope of the linear regression of $\ln(R/R_o) \cdot 1000$ versus $-\ln f$ (Supplementary Fig. S2), according to the method of Guy *et al.* (1989); regressions were not forced through the origin (Henry *et al.* 1999). Electron partitioning was calculated as described by Guy *et al.* (1989). Flux of electrons through the two pathways was calculated by multiplying total respiration in the absence of inhibitors by the respective partitioning factors.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting

Two hundred and fifty milligrams of fully expanded leaf tissue was snap frozen in liquid N_2 and was stored at -80°C . The frozen tissue was ground to a fine powder using a pestle and mortar, and was solubilized in 1 mL double-strength SDS sample buffer (20% glycerol, 282 mM Tris base, 212 mM Tris-HCl, 4% SDS, 1.02 mM EDTA, 0.44 mM bromophenol blue and 0.35 mM phenol red) with 50 mM DTT. The sample was then heated for 10 min at 70°C , and was centrifuged for 5 min at 10 000 g. The supernatant was collected, and 5 μL was loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) for separation by SDS–PAGE. Proteins from the gel were blotted onto either PVDF (for detection of AOX, porin and UCP) or nitrocellulose (for detection of COXII). The blots were then blocked in Tris-buffered saline plus Tween (TBST) (25 mM Tris-HCl, 125 mM NaCl and 0.1% Tween 20) with 5% skim milk powder. To probe for AOX and porin, blots were incubated overnight in antibody buffer (TBST with 5% skim milk powder) with either purified IgG against AOX isolated from a mouse hybridoma line that was originally produced by Dr Tom Elthon (University of Nebraska, Lincoln, NE, USA), at a dilution of 1:500, or purified IgG against porin isolated from a mouse hybridoma line (originally produced by Dr Tom Elthon, University of Nebraska), at a dilution of 1:5000. The blots were then washed in TBST and were incubated for 1 h in antibody buffer with alkaline phosphatase-conjugated anti-mouse antibody at a dilution of 1:3000. The blots were then washed in TBST, and the proteins were visualized using alkaline phosphatase (Promega, Madison, WI, USA); the blots were quantitated using ImageQuant (Molecular Dynamics, Sunnyvale, CA, USA). To probe for COXII, blots were incubated for 1 h in Western Blot Signal Enhancer (Vigene, Mountain View, CA, USA), plus 5% skim milk powder, with COXII antibody (Dr Vaughan Hurry, Umeå University, Umeå, Sweden; Campbell *et al.* 2007) at a dilution of 1:10 000. The blots were then washed in TBST and were incubated for 1 h in Western Blot Signal Enhancer (plus 5% skim milk powder) with alkaline phosphatase-conjugated anti-rabbit antibody at a dilution of 1:3000. Proteins were detected using the AttoPhos AOP Fluorescent Substrate System (Promega); fluorescence was visualized using a fluorimeter, and the proteins were quantitated using Image J

(NIH, URL: <http://rsb.info.nih.gov/ij/>). To probe for the UCP, the membrane was blocked in Roche's (Roche Diagnostics Corporation, Indianapolis, IN, USA) blocking reagent (Chemiluminescence Western Blotting Kit) for 1 h. The membrane was then incubated for 1 h in antibody buffer [TBS plus 0.1% (v/v) tween-2] with antibody raised against soybean UCP (Considine, Daley & Whelan 2001), at a dilution of 1:2000. The membrane was then washed in TBS and was incubated for 1 h in antibody buffer [0.1% (v/v) tween-2] with anti-rabbit antibody at a dilution of 1:10 000. The proteins were detected using the BM Chemiluminescence Western Blotting Kit, visualized using an LAS-1000 (Fuji-Film, Tokyo, Japan), and quantitated using the Image Guage v4.22 software (Fujifilm).

Isolation of total RNA and cDNA synthesis

Total RNA was extracted from WG, 10 d CT and CD leaves using the RNeasy plant mini protocol (Qiagen, Doncaster, Vic., Australia); three separate extractions, using one to two leaves from three separate plants, were carried out per treatment. Each RNA sample was treated with TURBO DNase (Ambion, Scoresby, Vic., Australia) to remove the contaminating DNA. DNase Inactivation Reagent (Ambion, Australia) was added after the DNase treatment to terminate the reaction. One microgram of RNA was reverse transcribed into cDNA using Expand Reverse-transcriptase (Roche) according to the manufacturer's instructions.

Quantitative RT-PCR

Transcript levels were analysed using the ABI PRISM 7700 Sequence Detection or the ABI 7500 Real-Time PCR system (Applied Biosystems, Scoresby, Vic., Australia) using iTaq SYBR Supermix ROX (Bio-Rad, Regents Park, New South Wales, Australia). The nuclear gene protodermal factor 2 (*PDF2*, AT1G13320) was used as a reference for determining the relative transcript abundance; this gene has previously been determined to have a stable transcription throughout development and under a wide range of environmental conditions (Czechowski *et al.* 2005). cDNA samples from each biological replicate were assayed in duplicate for quantification. The reaction mix contained 2.5 μL of a 10-fold dilution of cDNA, 0.008% (w/v) BSA, 0.7 μM of each primer (see Supplementary Table S1) and 12.5 μL iTaq SYBR Supermix ROX (Bio-Rad) in a 25 μL reaction volume.

The relative transcript abundance was quantified using the $2^{(-\Delta\Delta\text{Ct})}$ comparative Ct method according to the manufacturer's instructions. Using this method, data acquired from each sample were normalized to the endogenous reference gene (*PDF2*), and were expressed relative to WG controls (gene expression levels in the WG leaves were set to 1, and levels in CT and CD leaves were expressed relative to this).

Statistics

Data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov test and a one-way

ANOVA in SPSS v10 (SPSS Science, Birmingham, UK). If the data were suitable for parametric testing, a one- or two-way ANOVA was performed with LSD post hoc testing using SPSS v10. Protein data were analysed using paired *t*-tests. If the data were not suitable for parametric testing, Kruskal–Wallis and Mann–Whitney *U*-tests were carried out using SPSS v10. As Q_{10} values were calculated using mean rates at each temperature, it was not possible to statistically analyse the differences in Q_{10} values.

RESULTS

End-point ^{18}O discrimination values

To assess how temperature influenced partitioning between the cytochrome and alternative pathways, it was first necessary to establish the end-point ^{18}O discrimination values in the presence of SHAM and KCN, respectively. Respiration was significantly reduced in the presence of SHAM (one-way ANOVA, $P < 0.001$) and KCN ($P < 0.001$); percentage reduction was 38% in the presence of SHAM, and 72% in the presence of KCN. In the presence of KCN, fractionation against ^{18}O by the AOP alone was found to be $23.7 \pm 1.1\text{‰}$ ($\pm\text{SE}$, $n = 5\text{--}6$), whereas in the presence of SHAM, ^{18}O discrimination by the COP alone was $16.2 \pm 0.6\text{‰}$ ($\pm\text{SE}$, $n = 5\text{--}6$). These discrimination values were then used to calculate partitioning of electrons between the AOP and COP.

Effect of initial changes in temperature on AOP and COP activity

In the absence of inhibitors, discrimination against ^{18}O by WG *Arabidopsis* leaves was highly dependent upon measurement temperature (one-way ANOVA, $P < 0.001$); lowering the temperature below 20 °C resulted in a marked decrease in discrimination (Fig. 1a), whereas increasing the temperature above 20 °C had little effect. This apparent temperature sensitivity of ^{18}O discrimination is indicative of differential changes in AOP and COP activity in response to short-term changes in temperature, with the decrease in discrimination below 20 °C suggesting a decreased partitioning of electrons to the AOP at lower temperatures. Using the discrimination values for the individual pathways (see previous discussion) and associated total rates of O_2 uptake, we were able to calculate the contribution each pathway made to total respiratory flux (Fig. 1b) over the $5\text{--}30\text{ °C}$ temperature range. At 5 °C , the contribution of the AOP was minimal, with flux via this pathway comprising just 5% of overall flux (Fig. 1b, Table 1); thus, flux via the COP dominates overall O_2 uptake in chilled, WG leaves. Between 20 and 30 °C , however, flux via the AOP constituted approximately 39% of total respiratory flux (Fig. 1b, Table 1). This marked increase in the partitioning of electrons to the AOP at higher measurement temperatures resulted in the AOP having a much higher average Q_{10} value (over the $5\text{--}21\text{ °C}$ temperature range) compared with the COP (Table 2).

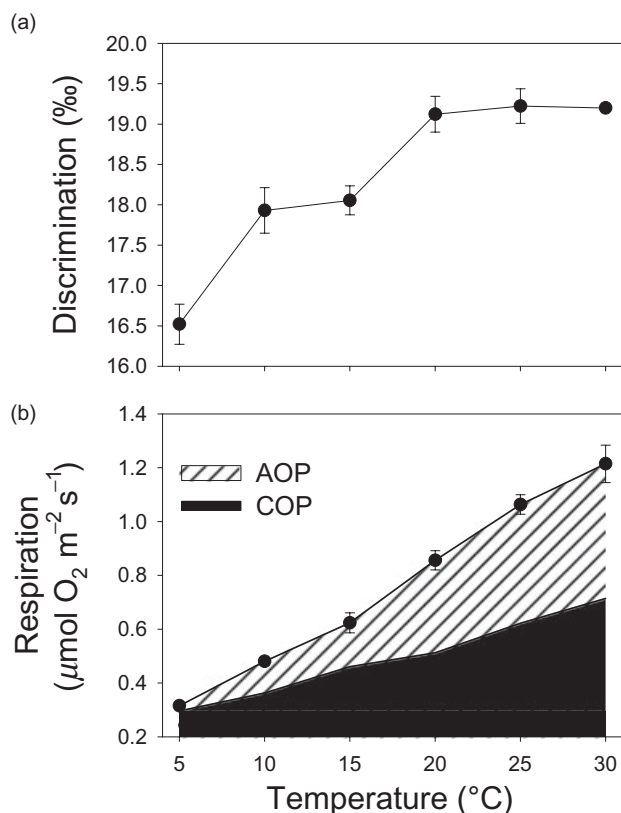


Figure 1. Discrimination (‰) (a) and total O_2 uptake ($\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$) (b) plotted against measuring temperature ($^{\circ}\text{C}$) in warm-grown leaves. In (b), the black area represents the proportion of flux by the cytochrome pathway (COP), and the diagonal area represents the proportion of flux via the alternative pathway (AOP). Values represent the mean of 5–6 replicate taken $\pm\text{SE}$. (with each sample comprising leaf discs taken from one to two replicate plants) from one experiment.

Sustained cold: impacts on electron partitioning

Our results suggest that AOP respiration is more sensitive to initial exposure to cold than COP respiration, reflecting the dynamic shifts in partitioning to the COP following cold treatment. In order to establish whether this response is maintained following sustained exposure to cold (5 °C), respiration and ^{18}O discrimination were measured in leaves developed in the warm and then shifted to the cold for 1, 2, 4, 7 and 10 d, and in leaves that subsequently developed in the cold. Measurements were carried out at 5 and 21 °C . When measured at 21 °C , respiration was higher in pre-existing leaves that were cold treated for 2 d, when compared with rates exhibited by the WG leaves (Fig. 2; $P < 0.05$). The difference between 21 °C measured rates of respiration exhibited by the CT and WG leaves continued to increase following further exposure to the cold ($P < 0.05$). As expected, the highest 21 °C measured rates were exhibited by CD leaves (Fig. 2), which exhibited significantly higher rates compared with both WG and CT leaves ($P < 0.001$). At 5 °C , total respiration was unchanged

Measuring temperature (°C)	Treatment	% AOP	% COP	AOP	COP
5	WG	5 ± 3	95 ± 3	0.01 ± 0.01	0.29 ± 0.01
	CT1	14 ± 2	86 ± 2	0.06 ± 0.01	0.37 ± 0.02
	CT2	17 ± 7	83 ± 7	0.07 ± 0.03	0.35 ± 0.04
	CT4	47 ± 4	53 ± 4	0.18 ± 0.02	0.20 ± 0.02
	CT7	30 ± 1	70 ± 1	0.12 ± 0.00	0.27 ± 0.01
	CT10	24 ± 4	76 ± 4	0.10 ± 0.02	0.31 ± 0.01
	CD	17 ± 5	83 ± 5	0.11 ± 0.03	0.57 ± 0.09
21	WG	39 ± 4	61 ± 4	0.31 ± 0.04	0.47 ± 0.03
	CT1	41 ± 2	59 ± 2	0.39 ± 0.02	0.58 ± 0.04
	CT2	42 ± 1	58 ± 1	0.43 ± 0.02	0.58 ± 0.02
	CT4	43 ± 2	57 ± 2	0.48 ± 0.04	0.62 ± 0.02
	CT7	46 ± 2	54 ± 2	0.63 ± 0.03	0.76 ± 0.04
	CT10	50 ± 3	50 ± 3	0.94 ± 0.11	0.93 ± 0.06
	CD	39 ± 3	61 ± 3	0.87 ± 0.07	1.37 ± 0.07

Table 1. Percentage total respiratory flux attributable to the alternative pathway (% AOP) and the cytochrome pathway (% COP), and rates of alternative pathway (AOP) and cytochrome pathway (COP) respiration ($\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$) in warm-grown (WG), 1, 2, 4, 7 and 10 d cold-treated (CT) and cold-developed (CD) leaves (see Fig. 2 for total respiration data). Discrimination values obtained in the absence of inhibitors were used to calculate the percentage flux and rates of respiration according to Guy *et al.* (1989). Measurements were carried out at 5 and 21 °C. Values represent the mean of 3–6 replicate runs \pm SE

in CT leaves (even after 10 d cold treatment), but was significantly higher in CD leaves (Fig. 2; $P < 0.001$). The net result of these differential responses to cold at 5 and 21 °C was an increase in the Q_{10} of total respiration in CT leaves, which increased from 1.8 in WG leaves to 2.6 in 10 d CT leaves (Table 2). Relative to 10 d CT leaves, the Q_{10} fell following the development of leaves in the cold (Table 2).

A two-way ANOVA revealed that long-term cold treatment and measurement temperature had a significant and interactive effect upon ^{18}O discrimination (Fig. 3; $P < 0.001$). Separate one-way ANOVAs showed that discrimination was unchanged following cold treatment at the measurement temperature of 21 °C (Fig. 3), but was increased significantly at the measurement temperature of 5 °C (Fig. 3; $P < 0.001$). However, this increase in discrimination was not incremental with time; instead, discrimination, which was unchanged in 1 and 2 d CT leaves, peaked following 4 d exposure to cold (AOP respiration represented 47% of total respiratory flux in 4 d CT leaves compared with 5% in CT WG leaves; Fig. 3, Table 1), and then declined following further exposure to cold (AOP respiration represented 24% of total flux in the 10 d CT leaves; Fig. 3, Table 1). There were no significant differences in discrimination values between WG and CD leaves (Fig. 3), suggesting that

increased AOP activity is only a requirement in pre-existing tissue experiencing cold for several days.

Sustained cold: impacts on protein abundance

AOX, COXII and UCP protein levels were measured in WG, 4 and 10 d CT and CD leaves. Very low levels of the AOX protein were detectable in WG leaves, and although the intensity of the band was increased in CD leaves (Fig. 4a,c), analysis of the data revealed that, on a fresh mass basis, the amount of AOX was statistically no different in WG, CT and CD leaves (Fig. 4a). Similarly, there was little difference in COXII and UCP protein content, when measured on a fresh mass basis, in WG and CT leaves (Fig. 4a,c). However, there was a significant increase in COXII and UCP protein levels in CD leaves ($P < 0.05$ and 0.001 respectively), which had approximately two times more COXII and approximately 10 times more UCP, compared with their WG counterparts (Fig. 4a,c).

Table 2. Q_{10} values for total leaf respiration (total), alternative pathway respiration (AOP) and cytochrome pathway respiration (COP) in warm-grown (WG), 1, 2, 4, 7 and 10 d cold-treated (CT) and cold-developed (CD) leaves. Q_{10} values were calculated using the 5 and 21 °C respiration data presented in Fig. 2, according to the equation $Q_{10} = (\text{Resp}_{21}/\text{Resp}_5)^{10/(21-5)}$

Treatment	Total Q_{10}	AOP Q_{10}	COP Q_{10}
WG	1.8	5.9	1.4
CT1	1.7	3.1	1.3
CT2	1.7	3.0	1.4
CT4	1.9	1.8	2.0
CT7	2.3	2.9	1.9
CT10	2.6	4.0	2.0
CD	2.0	3.3	1.7

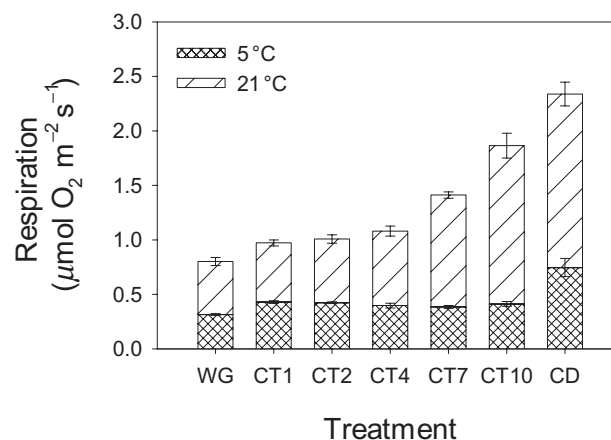


Figure 2. O_2 uptake ($\mu\text{mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$) measured at 5 °C (hatching) and 21 °C (diagonals) in warm-grown (WG), 1, 2, 4, 7 and 10 d cold-treated (CT) and cold-developed (CD) leaves. Values represent the mean of 5–6 replicate samples \pm SE (with each sample comprising leaf discs taken from one to two replicate plants) from one experiment.

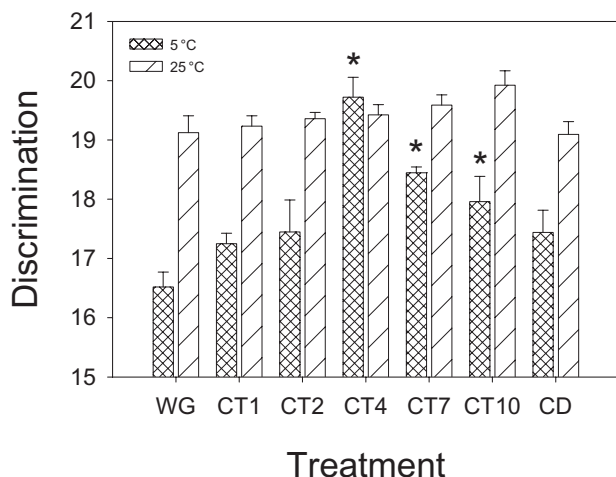


Figure 3. Discrimination against ^{18}O (‰) during dark respiration measured at 5 (hatching) and 21 °C (diagonals) in warm-grown (WG), 1, 2, 4, 7 and 10 d cold-treated (CT) and cold-developed (CD) leaves. Values represent the mean of 3–6 replicate samples \pm SE (with each sample comprising leaf discs taken from one to two replicate plants) from one experiment. Discrimination was calculated as the slope of the linear regression of $\ln(R/R_0)_{1000}$ versus $-\ln f$. *Indicates discrimination values significantly different from those in WG leaves, $P < 0.05$.

The amount of porin (the outer membrane voltage-dependent anion channel) was also measured in the WG, CT and CD leaf samples. Porin is used as a marker of mitochondrial protein because of its stability under a range of environmental conditions (Shane *et al.* 2004; Noguchi *et al.* 2005). Thus, normalizing COXII and UCP protein levels to those of porin allowed us to assess whether the observed changes in COXII and UCP protein content were simply the result of an increase in the total amount of mitochondrial protein. When COXII protein levels were normalized to those of porin, there were no longer any differences in COXII protein content in WG and CD leaves (Fig. 4b), suggesting that the increase in COXII observed in the CD leaves (Fig. 4a) was the result of a non-specific increase in the amount of mitochondrial protein per gram fresh mass, rather than a specific increase in the amount of COXII per unit mitochondrial protein. Conversely, UCP protein levels remained higher in the CD leaves following normalization to porin (Fig. 4b), suggesting that this increase was the result of a specific up-regulation of UCP protein in these leaves.

Sustained cold: respiratory transcript abundance

Low temperature influences components of the respiratory apparatus other than the terminal oxidases. Thus, in addition to examining transcript levels of the cytochrome oxidase gene, *COX6b*, and the alternative oxidase gene, *AOX1a*, we also analysed transcript levels of several other

selected respiratory genes. In particular, we examined transcript levels of the TCA cycle gene *IDH2*, one representative from each of the additional COP complexes (I, II, III and V), and representatives from two additional non-phosphorylating bypasses of the respiratory electron transport chain; the alternative NAD(P)H dehydrogenase gene, *NDB2*, and the plant uncoupling protein gene, *UCP1*. In agreement with the protein data (Fig. 4b), the transcript levels of the terminal oxidase genes *COX6b* and *AOX1a* were unchanged following treatment and development of leaves in the cold (Fig. 5). Similarly, the transcript levels of *IDH2* and the additional COP complex genes were also unchanged (Fig. 5). Indeed, the only transcripts to change in abundance following long-term exposure to the cold were *NDB2* and *UCP1* (Fig. 5). Both of these transcripts were up-regulated in the cold, with *NDB2* displaying a more than fivefold increase in abundance in 10 d CT and CD leaves, and *UCP1* undergoing a more than threefold increase in abundance in CD leaves.

DISCUSSION

Our results challenge prevailing notions concerning the impact of temperature on engagement of the alternative pathway. While our results (Fig. 5) support previous findings that AOP activity increases following sustained chilling (McNulty *et al.* 1987; Stewart *et al.* 1990a,b; Vanlerberghe & McIntosh 1992; Wagner & Krab 1995; González-Meler *et al.* 1999; Ribas-Carbó *et al.* 2000), we have shown that in *A. thaliana*, the increase in AOP activity is a short-lived response that subsides once the plant is able to re-establish flux via the COP. Moreover, by integrating gas exchange and ^{18}O discrimination studies with measurements of protein abundance and transcript levels in CT and CD leaves, we found that the transitory increase in AOP activity following cold stress is independent of changes in protein and transcript abundance. This suggests that although an increase in AOP activity might be required during the early stages of cold treatment, it does not play a substantive role when a new equilibrium has been established.

^{18}O discrimination values using the continual sampling procedure

We used a new development in the ^{18}O discrimination technique, in which gas samples are monitored continuously, in order to examine the effect of short- and long-term changes in temperature on *in vivo* AOP activity in mature *Arabidopsis* leaves. This method decreases experimental time and increases accuracy because of the high frequency of sampling. Using this procedure, we found that the end-point ^{18}O discrimination values for the AOP and COP were lower than those reported previously for green tissues (Robinson *et al.* 1995) including *Arabidopsis* (Florez-Sarasa *et al.* 2007). The cause for such differences in end-point ^{18}O discrimination values is not clear. We have tested the cuvette

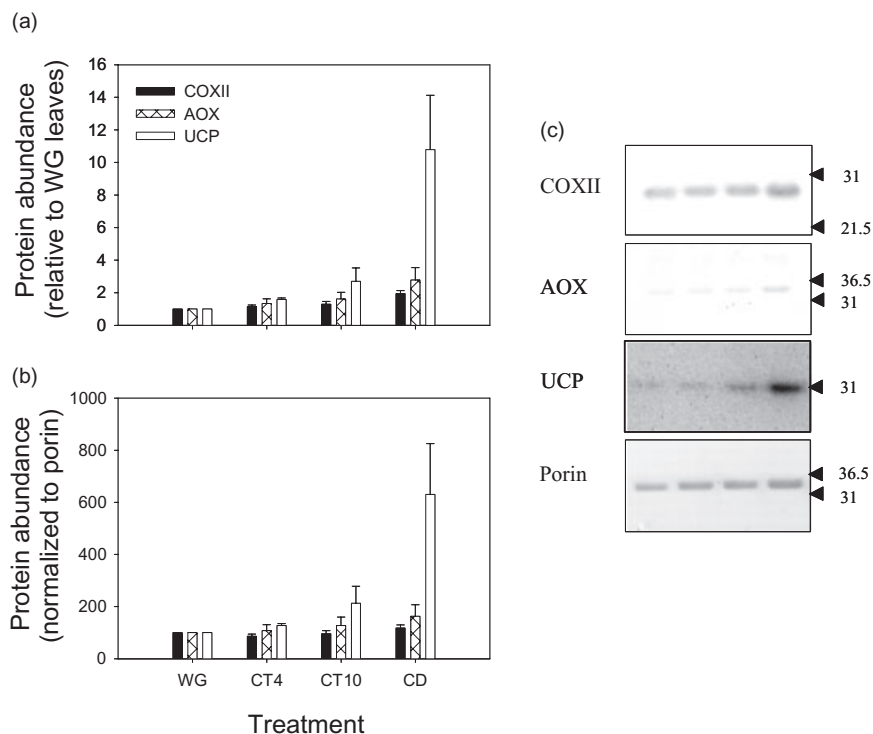


Figure 4. COXII (black), AOX (hatched) and UCP (white) protein abundance in warm-grown (WG), 4 and 10 d cold-treated (CT) and cold-developed (CD) leaves. Values are expressed relative to WG leaf values (a), and relative to WG leaf values and normalized to porin (b). Values represent the mean of 3–4 independent western blots, with each blot representing leaf tissue taken from separate plants (\pm SE) from one experiment. (c) Representative immunoblots for COXII, AOX, UCP and porin. Lanes run, from left to right, WG, 4 d CT, 10 d CT and CD. Location of molecular weight markers (in kDa) is shown on the right. COX, cytochrome oxidase; AOX, alternative oxidase; UCP, uncoupling protein.

assay system with soybean leaf discs using the same assay procedure. Uninhibited tissue gave values of $23.6 \pm 0.3\%$, while tissue inhibited with KCN gave values of $29.1 \pm 1\%$. These are in agreement with other measurements in the literature for similar tissues and treatments (Robinson *et al.* 1995). However, it is still conceivable that the lower values

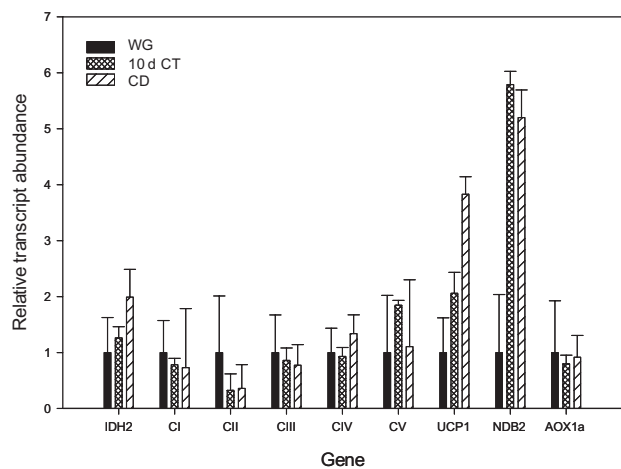


Figure 5. Relative transcript abundance of selected respiratory genes in warm-grown (WG, black), 10 d cold-treated (10 d CT, hatching) and cold-developed (CD, diagonals) leaves. From left to right, IDH2, NDUFV1 (CI), SDH2-2 (CII), QCR6-1 (CIII), Cox6b (CIV), ATP2a (CV), UCP1, NDB2 and AOX1a. For each gene transcript, relative abundance was calculated using the comparative Ct method. Each cDNA sample was amplified in duplicate. Values represent the mean of three replicate RNA extractions \pm SE.

may be related to consumption of some O_2 by the membrane of the mass spectrometer during the assay (this is not easily corrected for but is minimized by the use of low permeability membranes for the experiment, where membrane consumption is generally less than 5% of the tissue consumption). Nevertheless, irrespective of why the end points differ, our procedure provides a valuable tool to monitor *relative* changes in ^{18}O discrimination (and thus relative changes in AOP and COP activity) in plants experiencing short- and long-term changes in temperature. Moreover, the two approaches yield similar estimates of AOP activity in *Arabidopsis* leaves; 39 and 32% of total leaf respiratory flux occurred via the AOP in our study and in Florez-Sarasa *et al.* (2007), respectively. Our technique provides significant advantages in being able to sample discrimination values in many samples without the significant time delays involved in other time point sampling and separation procedures that have been previously used. It is also able to sample relatively small amounts of leaf tissue, enabling sampling of single *Arabidopsis* plants over a time course.

Initial changes in temperature: ^{18}O discrimination and electron partitioning

To the best of our knowledge, only two studies have examined the effect of short-term changes in temperature on ^{18}O discrimination (González-Meler *et al.* 1999; Guy & Vanlerberghe 2005), and these have yielded conflicting results, with the response depending on tissue type and species. In the present study, we show that discrimination in *Arabidopsis* is highly temperature sensitive in the short term (Fig. 1),

with a decrease in temperature below a threshold value (in this case, 20 °C), significantly reducing the proportion of flux directed through the AOP (Fig. 1, Table 1). This response is very similar to that observed in mung bean leaves (González-Meler *et al.* 1999), where ^{18}O discrimination was found to decrease below a threshold temperature of 19 °C (reaching the COP discrimination value at 10 °C), but remained stable across the 19–28 °C temperature range. The substantial decrease in discrimination below 20 °C observed in this study meant that the Q_{10} (over the 5–21 °C temperature range) of the AOP was substantially higher than that of the COP (5.9 and 1.4 respectively; Table 2), a phenomenon also observed in mung bean leaves (González-Meler *et al.* 1999).

The greater sensitivity of the AOP to cold (i.e. higher Q_{10}) than the COP is unlikely to reflect differences in the underlying temperature sensitivity of the two terminal oxidases, as the Q_{10} s of the two oxidases are similar when each is provided with saturating exogenous substrate (Atkin *et al.* 2002). Rather, temperature-mediated changes in adenylate control may provide an answer. Although adenylates can restrict flux through the COP complexes (Day & Lambers 1983; Amthor 1994; Atkin *et al.* 2000a), recent work on potato leaf protoplasts indicates that adenylate limitations have a greater inhibitory effect on the activity of the UQ-reducing pathways (glycolysis and complex I) than on the activity of the UQ-oxidizing pathways (complexes III and IV) (Covey-Crump *et al.* 2007); as a result, the redox poise of the UQ pool was lowest under conditions of high adenylate restriction (Covey-Crump *et al.* 2007). A cold-induced decrease in UQ redox poise could have important implications for AOX, the activity of which is positively correlated with the reduction status of the UQ pool (Hoefnagel & Wiskich 1998; Millenaar *et al.* 1998), and might explain why in WG leaves, the relative engagement of the AOP declined from 39% at 21 °C to 5% at 5 °C (Fig. 1, Table 1) and thus why the Q_{10} of the AOP is higher than that of the COP. Alternatively, allosteric activation of the AOX (Millar *et al.* 1993; Umbach & Siedow 1993, 1996) could potentially increase its temperature sensitivity, as shown by the higher Q_{10} values of the AOX of isolated mitochondria in the presence of pyruvate compared with measurements made in the absence of pyruvate (Atkin *et al.* 2002). Further work is needed to establish which of these regulatory mechanisms are responsible for the greater temperature sensitivity of the AOP. Whatever the underlying mechanism responsible for the reduced engagement of the AOP at low temperatures, our data (Fig. 1) indicate that AOX does not help to maintain flux through the mitochondrial electron transport chain during initial exposure to cold.

Long-term exposure to cold and AOX activity

At the measurement temperature of 5 °C, the proportion of total respiratory flux via the AOP was increased in 4, 7 and 10 d CT leaves, when compared with their WG counterparts, with the maximal increase in flux occurring just after 4 d exposure to cold (Fig. 3, Table 1). This response, which

subsided in 7 and 10 d CT leaves and was altogether absent in CD leaves, was transitory in nature (Fig. 3, Table 1). However, in contrast to the dynamic response observed at 5 °C, AOP activity was largely unaffected by growth in the cold when measured at 21 °C (Fig. 3, Table 1). The temperature-dependent, transient nature of this response suggests that we are dealing with a highly regulated, adaptive response, which is tailored to the growth regime of the plant and which is not simply the result of changes in protein abundance. Indeed, because the increase in AOP activity observed in 4 d CT leaves was independent of any increase in AOX protein (Fig. 4a,b), it is likely that we are observing an increase in the activation status of pre-existing AOX, a protein which is known to be under tight metabolic control (Millar *et al.* 1993; Umbach & Siedow 1993, 1996; Hoefnagel *et al.* 1995, 1997; Ribas-Carbó *et al.* 1995, 1997). Other studies have also shown that AOX protein levels can be poorly correlated with the partitioning of electrons to the AOP (Lennon *et al.* 1995; Millar *et al.* 1998; Guy & Vanlerberghe 2005; Ribas-Carbó *et al.* 2005). Taken together, these results indicate that the metabolic conditions within the cell can be more important in determining AOP activity than the level of AOX protein itself.

In contrast to our study, Fiorani, Umbach & Siedow (2005) reported lower AOX protein concentrations in WG leaves, and an increase in AOX protein levels in cold-grown *Arabidopsis*. Moreover, they found that AOX was required for normal growth at low temperatures, particularly during the early stages of development, whereas in our study, growth in the cold was associated with a decline in AOP activity (Fig. 3, Table 1). The differences in AOX protein levels observed in WG leaves might reflect differences in growth conditions [they grew their plants under much higher growth irradiance (600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), a longer photoperiod (14 h) and higher daytime temperatures (23 °C). The differences observed in CD leaves (between our results and those of Fiorani *et al.* 2005) might result from the differences in the starting level of AOX. In those instances where AOX is negligible or even absent in WG leaves (as seen by Fiorani *et al.* 2005), any increase in AOP engagement will be dependent upon an increase in AOX protein levels (via transcriptional up-regulation and/or post-translational processes). Conversely, if AOX protein is already present, as observed in our study in WG leaves (Fig. 4c), increased AP engagement might result from biochemical activation of pre-existing protein. Thus, the differences in the starting level of AOX may determine the mechanism via which AP activity is increased, and could account for the variability in response observed between species and experimental conditions. Alternatively, these differences might stem from the fact that Fiorani *et al.* (2005) grew their plants from seed in the cold. This represents a major difference in cold treatment and life cycle development from that in our study, and might indicate that the importance of AOP activity in maintaining plant growth in the cold depends on the developmental stage when the plant experiences the cold.

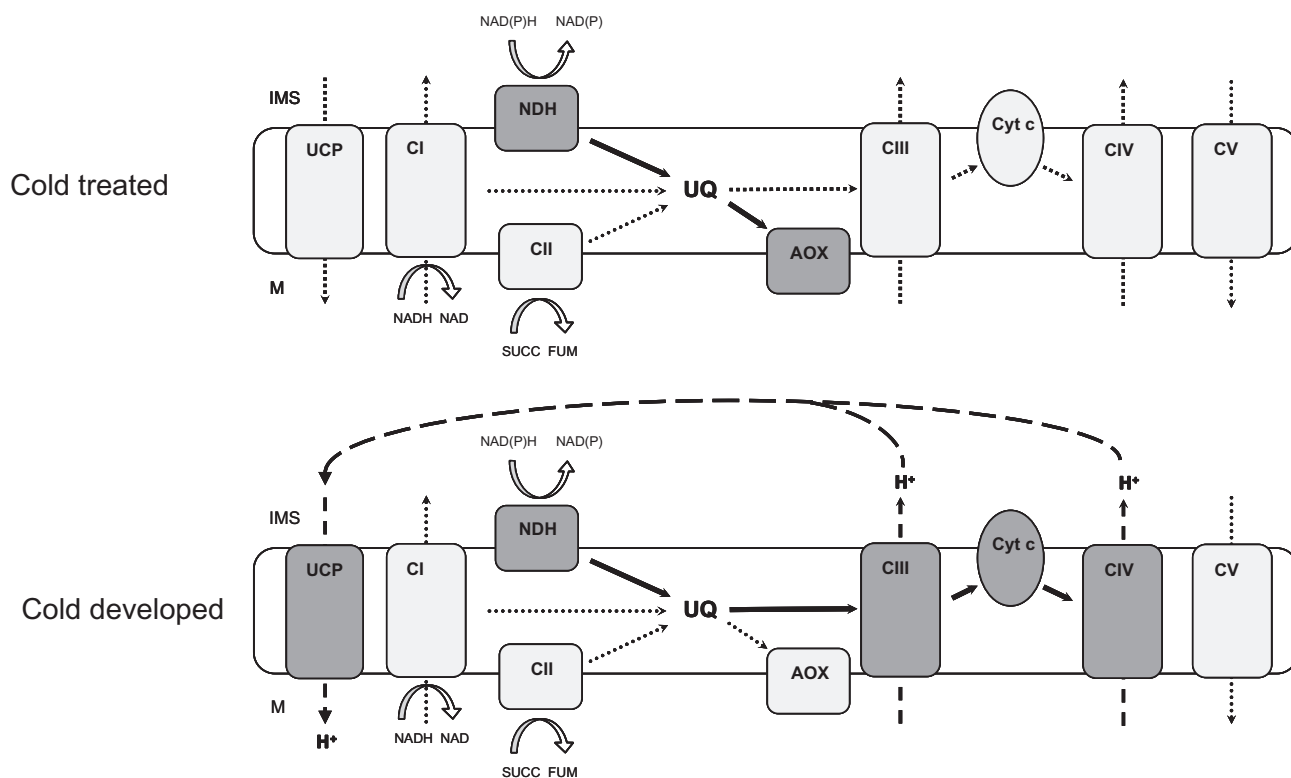


Figure 6. Diagrammatic representation of the plant mitochondrial electron transport chain following several days of exposure to the cold (cold treated) and development of leaves in the cold (cold developed). Proteins proposed to increase in activity are shown in dark grey, proteins which are not proposed to increase in activity are shown in light grey; the flow of electrons is indicated by solid arrows and the flow of protons by dashed arrows. Dotted lines indicate those electron and proton transport pathways not up-regulated in cold. AOX, alternative oxidase; NDH, NAD(P)H dehydrogenase; UCP, uncoupling protein; UQ, ubiquinone.

Adaptive benefits of a transient increase in AOP activity

Prior research has suggested that environmentally induced increases in AOP activity are part of a stress-response mechanism designed to prevent the over-reduction of the mitochondrial electron transport chain, and the concomitant accumulation of toxic ROS, under adverse environmental conditions (Stewart *et al.* 1990a,b; Purvis & Shewfelt 1993; Millar & Day 1997; González-Meler *et al.* 1999; Maxwell *et al.* 1999; Ribas-Carbó *et al.* 2000; Umbach, Fiorani & Siedow 2005). Thus, the rapid increase in AOP activity observed in 4 d CT leaves at 5 °C (Fig. 3, Table 1) might provide the plant with an energetically inexpensive means of oxidizing excess cellular reductant during the early stages of cold stress; past studies have shown that excess cellular reductant accumulates in *Arabidopsis* leaves when WG plants are chilled (Savitch *et al.* 2001), reflecting an imbalance between the production and consumption of NAD(P)H in the chloroplast, cytosol and mitochondria (Hoefnagel *et al.* 1998; Møller 2001). If so, acclimation in its initial stages might simply be functioning to prevent the over-reduction of the UQ pool. However, the progressive switchback to COP predominance in 10 d CT and CD leaves (Fig. 3, Table 1) suggests that acclimation in its later stages might function to re-establish energy production in

the cold and/or that the increased flux via the COP further helps oxidize excess reductant (see Fig. 6 and further associated text).

Cold-induced changes in transcript abundance and their functional significance

Previous work on *Arabidopsis* cell cultures has revealed that the expression of COP genes is unchanged following several hours exposure to a range of abiotic stress treatments (Clifton *et al.* 2005). Similarly, we found that the expression of the classical respiratory pathway genes (encoding complexes I–V) was unchanged following treatment and development of leaves in the cold at the time points sampled (Fig. 5). The fact that we also observed no change in COP transcript abundance following much more sustained exposure to stress further testifies to the inherent stability of COP gene expression under stressful conditions in *Arabidopsis* (Clifton *et al.* 2005). By contrast, *IDH2* has been shown to exhibit changes in expression following several hours exposure to a range of abiotic stress treatments (Clifton *et al.* 2005), but was not induced here by 10 d of cold treatment. Importantly, while components of the TCA cycle and COP exhibited little change in expression following sustained exposure to cold, components of the non-phosphorylating bypasses of mitochondrial

electron transport exhibited much more dynamic gene expression responses to cold (Fig. 5). However, this response did not involve any increase in *AOX1a* transcript (Fig. 5). Rather, transcript levels of *NDB2*, one of the type II NAD(P)H dehydrogenase (NDH)-encoding genes, and *UCPI*, one of the uncoupling protein-encoding genes, were increased.

NDB2 has been shown to be strongly co-expressed with *AOX1a* under a range of abiotic stress treatments (Gutiérrez *et al.* 1997; Rasmusson, Soole & Elthon 2004; Clifton *et al.* 2005), and because of several common sequence elements with similar organization in the upstream region of these two genes, it has been proposed that co-expression of these transcripts reflects co-regulation of the genes (Clifton *et al.* 2005). The fact that the increase in *NDB2* transcript levels in 10 d CT and CD leaves occurred in the absence of any increase in *AOX1a* suggests that while co-expression of these genes might occur, the type of abiotic stress encountered, the duration of that stress and the degradation rate of specific transcripts can likely lead to very different steady-state transcript levels in longer-term experiments. Consistent with this notion, the data from Clifton *et al.* (2005) reveal that *AOX1a* transcript abundance is often maximal 3 h after the imposition of stress, but is significantly reduced 24 h after stress, whereas *NDB2* transcript levels continue to increase 24 h after the addition of stress. Furthermore, while analysis of the co-expression of *AOX1a* and *NDB2* in public microarray data through the *Arabidopsis* coexpression tool (ACT) shows they are highly co-expressed (Supplementary Fig. S3a), analysis of these transcripts in stress arrays in the Genevestigator database shows that there are exceptions; notably under cold treatment (Supplementary Fig. S3b).

While *UCPI* has previously been reported to be induced after 24 h at 4 °C in both *Arabidopsis* (Maia *et al.* 1998) and potato (Laloi *et al.* 1997), it has not been reported to increase in concert with *NDB2* (Clifton *et al.* 2005; Fiorani *et al.* 2005). Thus, the increase in *NDB2* and *UCPI* transcript levels observed after prolonged cold treatment, in the absence of any increase in *AOX1a*, represents an interesting finding and suggests a biochemical role for the external NDHs and UCPs during long-term exposure to cold in *Arabidopsis*.

The external NDHs function to oxidize cytosolic NAD(P)H. Thus, the increase in *NDB2*, if accompanied by an increase in NDH protein and activity (as is the case in CT seed mitochondria, even when experiencing temperatures as low as -3.5 °C; Stupnikova *et al.* 2006), suggests that there is an increased demand for the oxidation of cytosolic reductant in 10 d CT and CD leaves. UCP on the other hand functions to mildly uncouple mitochondrial electron transport from oxidative phosphorylation when ROS levels increase. Thus, the increase in *UCPI* (Fig. 5), which we know to be coupled to an increase in UCP protein levels (Fig. 4a,b), suggests an increase in the adenylate restriction of respiration in CD leaves. We propose that these two proteins act synergistically in CD leaves, allowing electrons to be delivered to O₂ via the COP without the need for all

protons translocated by the COP to be used in the generation of ATP (Fig. 6).

Coupling this longer-term scenario observed in CD leaves with the transient shift to AOP activity observed in 4 d CT leaves, the initial stress scenario proposed by Clifton *et al.* (2005), and the observations by Stupnikova *et al.* (2006) of cold-tolerant NDH activity, we can now develop a more in-depth understanding of non-phosphorylating pathway flexibility in the respiratory chain. Clifton *et al.* (2005) proposed that during short-term (several hours) exposure to stress, an increased proportion of electrons enter the UQ pool via the NDHs, and exit via the AOX. This proposal is consistent with transcriptional co-regulation of *AOX* and *NDH* transcripts following several hours of exposure to a range of stresses (Rasmusson *et al.* 2004; Clifton *et al.* 2005), and with our data on a transient shift to AOP activity in early-stage CT leaves (Fig. 3). Although these data sets suggest that control of AOP induction may reside at different levels (transcriptional versus post-translational), they are consistent in their intent and functional effect on respiration (Fig. 6). This short-term scenario is quite distinct from that which appears to take place in the longer term, at least during cold. In this scenario, there is no longer any attempt to up-regulate *AOX1a* transcript, AOX protein or AOX activity, but *NDB2* and *UCPI* transcript levels (Fig. 5) and UCP protein content (Fig. 4) are increased. We can therefore conceive of a longer-term scenario in CD leaves, where an increasing proportion of electrons enter the UQ pool via the NDHs, and exit via the COP (as was proposed by Stupnikova *et al.* 2006 for chilled seed mitochondria), and where elevated levels of UCP facilitate a controlled dissipation of the mitochondrial proton gradient, allowing a partial uncoupling of electron flow via the COP (Fig. 6). Thus, while AOX is still present and indeed active (Table 1) in CD tissue, its biochemical importance might be superseded by that of UCP. However, given that UCP activity depends on the level of ROS (Considine *et al.* 2003; Smith *et al.* 2004), and that increased levels of *NDB2* do not necessarily indicate increased levels of NDH, this hypothesis needs to be tested. Nevertheless, the data indicate that the increased rates of COP respiration observed in CD leaves (Table 1) do not necessarily reflect a concomitant increase in plant energy production in the cold.

Taken together, our data suggest that plants may have more than one strategy to cope with environmental stress at the mitochondrial level, and that in order to understand these strategies and their functional significance, both short- and long-term experiments under a variety of conditions are needed.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Figure S1. Gaseous assay chamber used to measure respiration and ^{18}O discrimination. Volume of the leaf chamber was 2 mL.

Figure S2. (a) Representative plot of raw voltage signals for masses 32 ($^{16}\text{O}_2$) and 34 ($^{18}\text{O}^{16}\text{O}$) during dark respiration in *Arabidopsis* leaf discs. Data were used to calculate discrimination against ^{18}O ; arrow indicates the time from which discrimination was calculated. (b) Representative plot of discrimination against ^{18}O during dark respiration based on data in (a). Discrimination was obtained from the regression of $\ln(R/R_0) \cdot 1000$ versus $-\ln f$, where R is the isotopic ratio of O_2 in the cuvette at any given time, R_0 is the initial isotopic ratio of O_2 in the cuvette, and f is the fraction of O_2 remaining in the cuvette.

Figure S3. (a) Co-correlation plot of Pearson's correlation coefficients for all *Arabidopsis* genes represented on ATH1 microarray chip with respect to *AOX1a* and *NDB2*. The plot was generated using the *Arabidopsis* Co-expression Tool (ACT) available online at <http://www.arabidopsis.leeds.ac.uk/act/>. (b) Heat mAOP of responses to a variety of stresses for *AOX1a* (258452_at) and *NDB2* (255259_at) obtained from Genevestigator V3, available online at <https://www.genevestigator.ethz.ch/index.php>. Fold changes are shown in linear scale with green representing a decrease and red indicating an increase in transcript abundance. Only high quality ATH1 arrays were included.

Table S1. List of genes targeted and primers used in the RT-PCR investigations.

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