

# Heterogeneity of plant mitochondrial responses underpinning respiratory acclimation to the cold in *Arabidopsis thaliana* leaves

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## ABSTRACT

**In this study, we investigated whether changes in mitochondrial abundance, ultrastructure and activity are involved in the respiratory cold acclimation response in leaves of the cold-hardy plant *Arabidopsis thaliana*. Confocal microscopy [using plants with green fluorescence protein (GFP) targeted to the mitochondria] and transmission electron microscopy (TEM) were used to visualize changes in mitochondrial morphology, abundance and ultrastructure. Measurements of respiratory flux in isolated mitochondria and intact leaf tissue were also made. Warm-grown (WG, 25/20 °C day/night), 3-week cold-treated (CT) and cold-developed (CD) leaves were sampled. Although CT leaves exhibited some evidence of acclimation (as evidenced by higher rates of respiration at moderate measurement temperatures), it was only the CD leaves that were able to re-establish respiratory flux within the cold. Associated with the recovery of respiratory flux in the CD leaves were: (1) an increase in the total volume of mitochondria per unit volume of tissue in epidermal cells; (2) an increase in the ratio of cristae to matrix within mesophyll cell mitochondria; and (3) an increase in the capacity of the energy-producing cytochrome pathway in mitochondria isolated from whole leaf homogenates. Regardless of growth temperature, we found that contrasting cell types exhibited distinct differences in mitochondrial ultrastructure, morphology and abundance. Collectively, our data demonstrated the diversity and tissue-specific nature of mitochondrial responses that underpin respiratory acclimation to the cold, and revealed the heterogeneity of mitochondrial structure and abundance that exists within leaves.**

*Key-words:* confocal microscopy; cytochrome oxidase; epidermic; mesophyll; respiration; temperature; ultrastructure.

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## INTRODUCTION

Over 95% of the Earth's surface experiences low, chilling temperatures every year (Larcher 2004). Exposure to low temperatures slows down critical metabolic processes, such as those involved in leaf respiration (Forward 1960; Berry & Raison 1981), whilst simultaneously increasing the likelihood of oxidative stress (Purvis & Shewfelt 1993). If a plant is to withstand sustained exposure to low temperature (which in many areas is unavoidable given the pervasive and inescapable nature of cold), then it must be capable of altering its metabolic activity to cope with the limitations that cold imposes upon metabolism. Many plants are capable of up-regulating the rate of key physiological processes in order to compensate for sustained exposure to cold (Chabot & Billings 1972). This compensatory adjustment in physiological activity is referred to as 'cold acclimation'.

Leaf respiration is a vital component of plant metabolism (Hurry *et al.* 1995; Krömer 1995; Hoefnagel, Atkin & Wiskich 1998; Atkin *et al.* 2000b) and a major determinant of plant productivity (Amthor 1989). There exists an abundance of physiological data demonstrating that leaf respiration is able to acclimatize to the cold (Billings *et al.* 1971; Collier 1996; Xiong, Mueller & Day 2000; Atkin & Tjoelker 2003; Bolstad, Reich & Lee 2003; Talts *et al.* 2004; Atkin *et al.* 2005). In this context, acclimation can be defined as 'a change in respiratory activity that compensates for a change in temperature'. Perfect compensation will result in a phenomenon known as 'respiratory homeostasis', in which plants grown at contrasting temperatures exhibit identical rates of respiration when measured at their respective growth temperatures. Although thermal acclimation invariably results in cold-grown plants exhibiting higher rates of respiration compared to warm-grown (WG) plants when measured at a common moderate set-temperature (Billings *et al.* 1971; Collier 1996; Atkin, Holy & Ball 2000a; Xiong *et al.* 2000; Bolstad *et al.* 2003), the occurrence of homeostasis (Collier 1996; Xiong *et al.* 2000; Atkin *et al.* 2005) is less common. In recent years, it has become apparent that in some plant species, the degree of homeostasis exhibited by a tissue depends on whether or not the tissue has developed in the new temperature environment (Loveys *et al.* 2003). In

spite of the abundance of information describing the acclimatory response at the physiological level, our understanding of the mechanisms underpinning thermal acclimation, and whether these mechanisms differ depending on whether the tissue has developed in or has been shifted to a new temperature regime, remains limited. In particular, we know little about the extent to which the acclimatory response we observe at the flux level reflects plasticity occurring at the organelle level. Intuitively, one might expect that changes in mitochondrial activity and abundance are involved.

There is some evidence to suggest that an increase in mitochondrial capacity (potential rates of O<sub>2</sub> uptake per unit mitochondrial protein) might be involved in the cold-acclimation response. Klikoff (1966, 1968) found that mitochondrial capacity was greater in plants originating from higher elevations than in those from lower elevations. Whether these differences were the result of phenotypic acclimation or genetic adaptation to the plant's native environment remains unclear, as measurements of all but one species were carried out on plants developed in their native environment and then shifted to a common environment. The extent to which changes in mitochondrial abundance are involved in the cold-acclimation response is not known. Miroslavov & Kravkina (1991) found that plants originating from higher elevations have a greater density of mitochondria in their leaf tissue, although it is not known whether these differences are the result of acclimation or adaptation. Nevertheless, there is some evidence to suggest that short-term cold treatment results in an increase in mitochondrial number, and that this increase supports an increase in respiration rate (Kislyuk, Miroslavov & Paleeva 1995). Whether such a response can be maintained in the long term, however, is not known. Furthermore, an increase in mitochondrial abundance is not always associated with an increase in the rate of respiration. Griffin *et al.* (2001) observed an increase in leaf mitochondrial number in response to plant growth in elevated CO<sub>2</sub> [an observation also made earlier (Robertson *et al.* 1995)], but a decrease in the rate of respiration within these leaves relative to those grown in ambient-CO<sub>2</sub> environments. Further work is needed to assess whether the mitochondrial populations within a leaf exhibit plasticity, with respect to mitochondrial structure and abundance, following long-term exposure to the cold.

Up to this date, studies examining the response of mitochondrial populations to a change in the environment have examined the response of one cell type (Kislyuk *et al.* 1995; Robertson *et al.* 1995; Griffin *et al.* 2001), the implied assumption being that this response represents the entire mitochondrial population within a tissue. However, an *in vivo* analysis of mitochondrial dynamics, using stable-transformed *Arabidopsis* lines expressing green fluorescence protein (GFP) targeted to mitochondria, has revealed that the mitochondrial population within individual plant cells is highly heterogeneous, with mitochondria varying in shape, size and mobility (Logan & Leaver 2000; Logan 2003). Whether such heterogeneity exists beyond the level of the cell remains unknown. Although spatial heterogeneity

of individual mitochondrial proteins has been observed (Rawsthorne *et al.* 1988; Tobin *et al.* 1989), it is not known whether this extends to heterogeneity of mitochondrial ultrastructure, gross morphology and abundance. However, it is plausible that the different cell types within the leaf, which have different functions and thus in all likelihood different metabolic demands, possess distinct mitochondrial populations, and that these populations exhibit differences in their response to low temperature.

In this study, we investigated the extent to which cold acclimation of respiratory flux in intact leaves is underpinned by changes in the abundance, ultrastructure and activity of mitochondria within those leaves. Our data highlight the diversity of mitochondrial responses that underpin the respiratory cold acclimation response and demonstrate the tissue-specific nature of those responses.

## MATERIALS AND METHODS

### Plant material and growth conditions

Wild-type *Arabidopsis thaliana* plants (ecotype Columbia), and transgenic *A. thaliana* plants (ecotype Columbia) with GFP targeted to their mitochondria [mito-GFP (Logan & Leaver 2000)], were used in this study. The plants were grown on compost (Levingtons, F2, Fisons Ltd, Ipswich, UK) in controlled environment chambers (Fi-totron 600H, Gallenkamp Loughborough, Leicestershire, UK) and (Microclima 1750, Snijders Scientific, Tilburg, The Netherlands); 25/20 °C day/night temperature regime, 150 μmol photons m<sup>-2</sup> s<sup>-1</sup>, 8 h photoperiod. After approximately 2 months, some of the WG plants were shifted to chilled controlled environment chambers; 5/5 °C day/night temperature regime, 150 μmol photons m<sup>-2</sup> s<sup>-1</sup>, 8 h photoperiod. Measurements were conducted on WG leaves. The WG leaves were shifted to the cold and maintained there for a period of 17–22 d, hereon referred to as 3-week cold-treated (CT) leaves; and those that had developed in the cold (sampled after 50 d at 5 °C), hereon referred to as cold-developed (CD) leaves. Comparisons of WG, CT and CD leaves have previously been used in numerous studies investigating cold acclimation of photosynthesis and respiration in *Arabidopsis* (e.g. Strand *et al.* 1997, 2003; Stitt & Hurry 2002; Talts *et al.* 2004).

### Leaf respiration measurements

Leaf dark respiration (nmol O<sub>2</sub> g fresh mass<sup>-1</sup> s<sup>-1</sup>) was measured in mature, fully expanded WG, CT and CD wild-type leaves using Clark-type oxygen electrodes (Rank Brothers, Cambridge, UK) coupled to a computer-based data acquisition system (NI-DAQ for Windows 2000, National Instruments, Berkshire, UK). The electrodes were calibrated with deionized water equilibrated to the appropriate temperature and saturated with air. Respiration was measured at 5, 10, 15, 20, 25 and 30 °C. At each measurement temperature respiration was measured in leaves from four replicate plants. The leaves were weighed, sliced into sections and

incubated (in darkness) in leaf respiration buffer containing 10 mM Hepes, 10 mM MES and 0.2 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (pH 7.2) for 30 min prior to measurement. Respiration was measured in darkened airtight cuvettes containing a known volume of fully aerated leaf respiration buffer.

### Mitochondrial isolation

Mitochondria were isolated from WG, CT and CD fully expanded wild-type leaves using a procedure adapted from Purvis (1997), using a 2-step discontinuous Percoll gradient. The gradient consisted of 24 mL of 23% Percoll in wash buffer, underlaid with 4 mL of 45% Percoll in wash buffer. Three to five mitochondrial extractions were carried out per treatment. In all plants used for mitochondrial isolations, the majority of leaves were fully expanded and thus no longer actively growing.

### Mitochondrial respiration measurements

Following extraction, mitochondrial respiration was measured at 5 and 25 °C using Clark-type oxygen electrodes (as described previously); activities of both cytochrome and alternative oxidase pathways were measured ( $\text{nmol O}_2 \text{ mg mitochondrial protein}^{-1} \text{ min}^{-1}$ ). Between 50 and 200  $\mu\text{L}$  of mitochondrial suspension (with a protein concentration ranging between 3.4 and 5.5  $\text{mg mL}^{-1}$ ) were used per run; two replicate runs were carried out per pathway at each measurement temperature. Respiration was measured in a known volume of fully aerated respiration medium, containing 0.3 M sucrose, 5 mM  $\text{KH}_2\text{PO}_4$ , 10 mM TES, 10 mM NaCl, 2 mM  $\text{MgSO}_4$  and 0.1% bovine serum albumin (w/v) (pH 7.0). Cytochrome pathway respiration was measured in the presence of  $\text{CaCl}_2$  (1.0 mM), ATP (0.1 mM), pyruvate (5.0 mM), octyl gallate (0.002 mM), saturating concentrations of succinate (10 mM), NADH (2.0 mM), ADP (0.8 mM), and in the presence and absence of the uncoupler CCCP (0.001 mM). Potassium cyanide (KCN, 1.0 mM) and antimycin A (0.005 mM) were used to inhibit respiration. Alternative pathway respiration was measured in the presence of  $\text{CaCl}_2$  (1.0 mM), ATP (0.1 mM), pyruvate (5.0 mM), dithiothreitol (5.0 mM), KCN (1.0 mM), antimycin A (0.005 mM), and saturating concentrations of succinate (10 mM) and NADH (2.0 mM). Octyl gallate (0.002 mM) was used to inhibit respiration. The remaining mitochondria were placed in a  $-80$  °C freezer for storage. Mitochondrial protein was subsequently measured (Bradford 1976).

### Confocal microscopy and image analysis

Measurements were carried out on mature WG, CT and CD leaves of mito-GFP *A. thaliana* plants. Confocal microscopy was performed using a Zeiss LSM 510 Meta (Carl Zeiss, Inc., Thornwood, NY) on an Axioplan 2 M (Carl Zeiss, Inc., Thornwood, NY), fitted with a  $63 \times$  PlanApo lens (NA 1.4). The sample was excited with a 488 nm Argon laser and a 633 nm HeNe laser, and light was collected using the Zeiss Meta detector at approxi-

mately 10.5 nm intervals centred from 488 to 730 nm using a multiple band dichroic (UV/488/543/633). GFP was detected and separated from autofluorescent components, chlorophyll and light scatter, using post-acquisition spectral unmixing. The images were acquired as 4–14  $\mu\text{m}$  stacks (0.44  $\mu\text{m}$  slice interval, 146  $\mu\text{m}^2$ ).

The images of the mitochondrial populations present in epidermal tissue were recorded in WG, CT and CD leaves from three to 12 replicate plants. In some leaves, the measurement extended into the adjacent mesophyll-cell layer. The images of the mitochondrial populations in mesophyll tissue were recorded in WG and CD leaves from three to 6 replicate plants. One to five images were recorded per replicate leaf. Three-dimensional reconstructions of the mitochondrial populations within adaxial and abaxial epidermal tissues, and palisade and spongy mesophyll tissues, were generated using Volocity image processing software (Volocity 3.1, Improvision, Coventry, UK). Mean volume of mitochondria, number of mitochondria per unit volume of tissue and sum volume of mitochondria per unit volume of tissue were calculated. The number and mean volume of mitochondria were calculated per unit volume of leaf tissue (including cell walls and air spaces) as it was not possible to calculate mitochondrial characteristics per unit cell volume.

Non-transfected cells were imaged under identical conditions as positive controls to ensure that no false assignment was given to the GFP channel. The non-transfected samples were also used to collect the various autofluorescent, chlorophyll and scatter component profiles.

### Transmission electron microscopy (TEM)

Ultrastructural images of adaxial and abaxial epidermal cell mitochondria, and palisade and spongy mesophyll cell mitochondria were recorded in WG and CD leaves from three replicate plants (five mitochondria, each from a separate thin section, were examined per cell type in each replicate leaf) using TEM. The leaf sections were vacuum-fixed and embedded in Spurr resin (see light microscopy section). Random sections of 70 nm were cut and stained with saturated uranyl acetate in 50% ethanol and Reynolds lead citrate. The images were recorded using a TEM (FEI Tecnai 12 BioTwin at 120 kV) with a digital camera attached. The amount of cristae and matrix and the ratio of cristae to matrix components were estimated on an area basis according to the following method. Two images of each mitochondrion were traced out on clear acetate sheets. In one, the mitochondrial matrix was darkened, whereas in the other, the entire mitochondrial area was darkened. Then, the area of the darkened regions was measured using a LiCor 3100 Area Meter (Lincoln, NE, USA). The area of cristae was yielded by subtracting the matrix area from the total area.

### Light microscopy

Transverse sections of WG and CD leaves from three wild-type replicate plants were examined using light microscopy.

The leaf sections were vacuum-fixed, washed in 10 mM phosphate buffer, dehydrated in acetone, and infiltrated and embedded in Spurr resin. Sections of 0.5  $\mu\text{m}$  were cut and stained with 0.6% toluidine blue. The Images were recorded using a light microscope (Nikon FXA, Melville, NY, USA) with a digital camera attached.

## Statistical analyses

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-way ANOVA was performed with least significant difference *post hoc* testing using SPSS v10. If the data were not suitable for parametric testing, Mann–Whitney *U*-tests were carried out using SPSS v10.

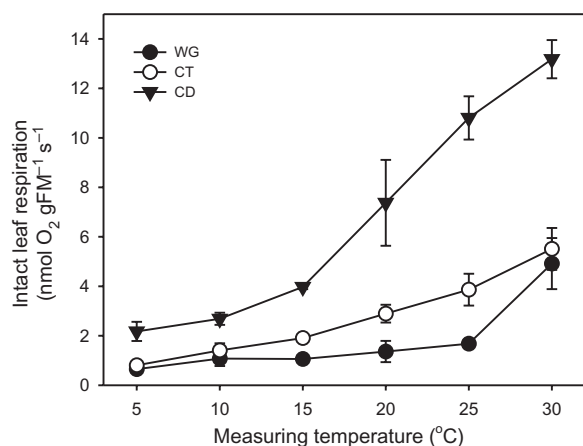
## RESULTS

### Leaf respiration

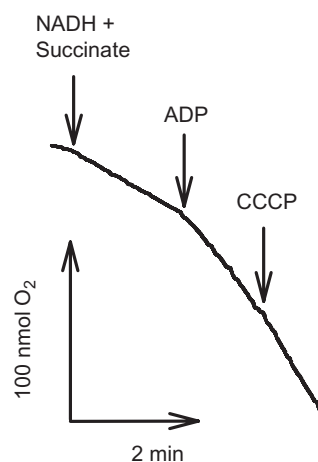
Figure 1 shows the respiratory temperature response curves for intact WG, CT and CD leaves. CT leaves showed some degree of acclimation; rates were significantly higher compared to WG leaves between the measurement temperatures of 15 and 25  $^{\circ}\text{C}$  ( $P < 0.001$ ). However, there were no significant differences in rate at the very lowest temperatures, or at the very highest temperature of 30  $^{\circ}\text{C}$ . CD leaves exhibited a far greater degree of acclimation; rates were significantly higher, compared to both WG ( $P < 0.01$ ) and CT ( $P < 0.05$ ) leaves, across the entire temperature range.

### Mitochondrial respiratory capacity

The capacities of the cytochrome and alternative pathways of mitochondrial electron transport were measured in mitochondria that were extracted from mature, fully expanded

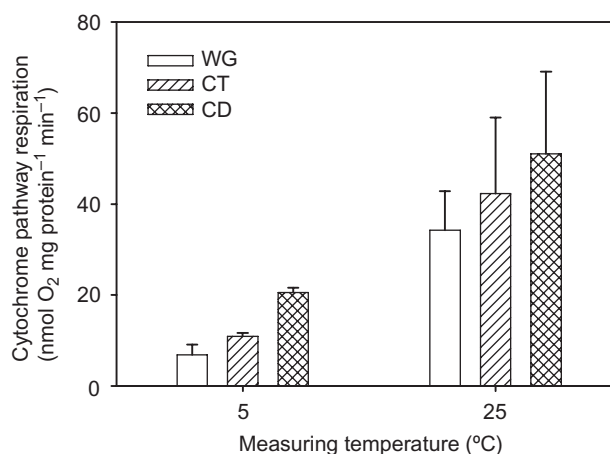


**Figure 1.** Leaf respiration ( $\text{nmol O}_2 \text{ g FM}^{-1} \text{ s}^{-1}$ ) plotted against temperature ( $^{\circ}\text{C}$ ) for warm-grown (WG), 3-week cold-treated (CT) and cold-developed (CD) leaves of wild-type *Arabidopsis thaliana*. Values represent the mean of four replicate plants ( $\pm$  SE).



**Figure 2.** Representative trace of mitochondrial  $\text{O}_2$  uptake (measured at 25  $^{\circ}\text{C}$ ) through the cytochrome pathway for mitochondria isolated from mature, fully expanded leaves of 10-day cold-treated (CT) plants. Respiration was measured in the presence of 0.002 mM octyl gallate, saturating concentrations of substrate (10 mM succinate and 2 mM NADH), and following the addition of 0.8 mM ADP and 0.001 mM CCCP. For this replicate, the  $\text{O}_2$  uptake in presence of substrates (ADP and CCCP) was 44  $\text{nmol O}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$ , with a respiratory coupling value of 2.0 (i.e. the ratio of rates in the absence and presence of ADP).

leaves of WG, CT and CD plants. Figure 2 shows a representative trace of mitochondrial  $\text{O}_2$  uptake through the cytochrome pathway measured at 25  $^{\circ}\text{C}$  (for CT leaves). Rates of  $\text{O}_2$  uptake in mitochondria extracted from WG leaves (Fig. 3), as well as the degree of respiratory coupling in mitochondria extracted from WG, CT and CD leaves (ranging from 1.5 to 2.1 when measured at 25  $^{\circ}\text{C}$ ), were similar to those values reported by Keech, Dizengremel & Gardeström (2005) for mitochondria extracted from 23/



**Figure 3.** Cytochrome pathway respiration in isolated mitochondria ( $\text{nmol O}_2 \text{ mg protein}^{-1} \text{ min}^{-1}$ ) measured in the presence of saturating concentrations of substrate, ADP, and CCCP plotted against measuring temperature ( $^{\circ}\text{C}$ ) in warm-grown (WG), 3-week cold-treated (CT) and cold-developed (CD) leaves. Values represent the mean of three to five mitochondrial isolations ( $\pm$  SE).

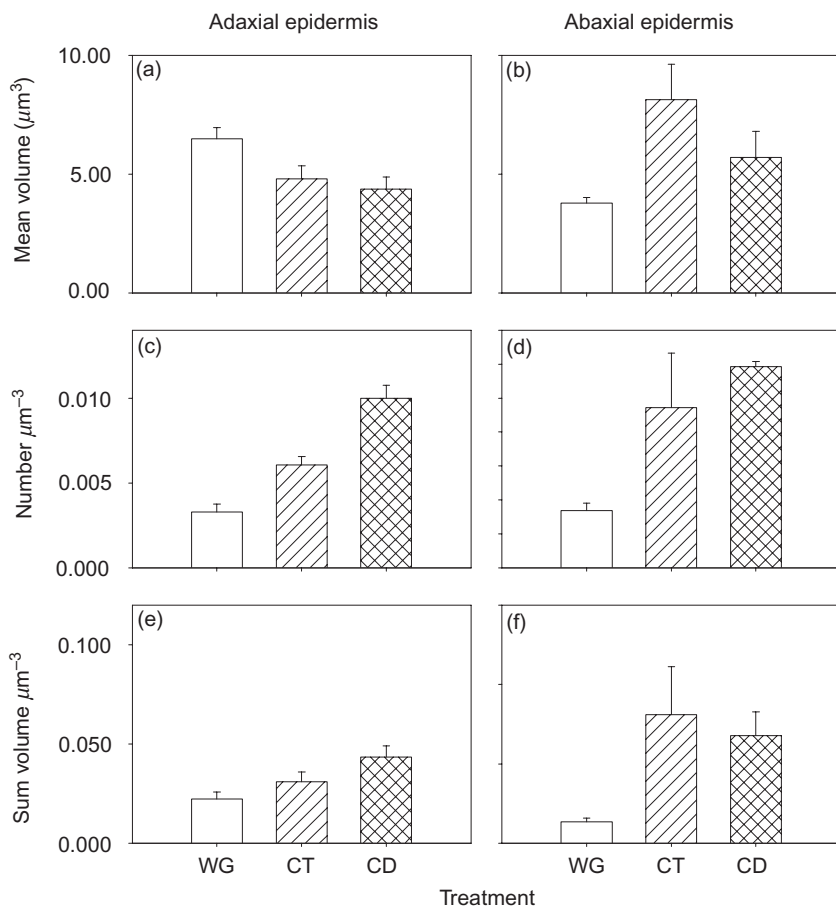
18 °C grown *Arabidopsis* leaves grown and measured under similar conditions to those used in our study. There was a slight, although statistically insignificant, increase in the maximal rate of cytochrome pathway respiration in mitochondria isolated from the CT leaves at the measurement temperatures of 5 and 25 °C (Fig. 3). There was a further increase in rate, at both 5 and 25 °C, in mitochondria isolated from the CD leaves (Fig. 3). At 5 °C, this increase was significant when compared with both WG ( $P < 0.001$ ) and CT ( $P < 0.005$ ) leaves. There was no change in the rate of alternative pathway respiration (the rate of which remained low, ranging between 5 and 20 nmol O<sub>2</sub> mg protein<sup>-1</sup> min<sup>-1</sup>) at 5 or 25 °C, in either the CT or CD leaves (data not shown).

### Mitochondrial volume and abundance in WG leaves

Comparison of WG adaxial and abaxial epidermal tissues revealed that mitochondria were smaller in the abaxial tissue ( $P < 0.001$ ) (Fig. 4a & b). Although there was no difference in the number of mitochondria per unit volume of tissue between the two epidermal cell layers (Fig. 4c & d), the total volume of mitochondria was lower within the abaxial tissue ( $P < 0.05$ ) (Fig. 4e & f). Comparison of palisade and spongy mesophyll tissues revealed that mitochon-

drial size and density was slightly (although not significantly) lower in the spongy mesophyll cell layer. As a consequence, the total volume of mitochondria was lower in the spongy mesophyll cell layer ( $P < 0.05$ ) (Table 1).

In addition to the differences observed between the upper and lower surfaces of the leaf, marked differences appeared to exist in the mitochondrial populations within epidermal and mesophyll cell layers. In WG leaves, the mitochondria within the palisade mesophyll cells were significantly smaller compared to those within the adjacent adaxial epidermal cells ( $P < 0.001$ ) (Table 1 and Fig. 4a). A similar pattern was observed after comparing the spongy mesophyll cells and the adjacent abaxial epidermal cells ( $P < 0.001$ ) (Table 1 and Fig. 4b). The density of mitochondria within palisade mesophyll tissue was significantly higher than that within adaxial epidermal tissue ( $P < 0.05$ ) (Table 1 and Fig. 4c). A similar trend was observed after comparing the spongy mesophyll and abaxial epidermal tissues, although the difference was not significant (Table 1 and Fig. 4d). Collectively, there was no difference in the total volume of mitochondria in the adaxial epidermal and palisade mesophyll tissues (Fig. 4e and Table 1), and abaxial epidermal and spongy mesophyll tissues (Fig. 4f and Table 1), of WG leaves. Taken together, these results highlight the heterogeneity that exists within the mitochondrial population of a mature warm-grown *Arabidopsis* leaf.



**Figure 4.** Mean mitochondrial volume ( $\mu\text{m}^3$ ) (a & b), number of mitochondria  $\mu\text{m}^{-3}$  tissue (c & d) and sum volume of mitochondria  $\mu\text{m}^{-3}$  tissue (e & f) in adaxial and abaxial epidermal tissues of warm-grown (WG), 3-week cold-treated (CT) and cold-developed (CD) leaves. Values represent the mean of 3–12 replicate plants ( $\pm$  SE).

**Table 1.** Impact of growth in the cold on the mean volume, density and sum volume of mesophyll cell mitochondria

Tissue	Treatment	Mean volume ( $\mu\text{m}^3$ )	Density (number $\mu^{-1}\text{m}^3$ )	Sum volume ( $\mu\text{m}^3 \mu^{-1}\text{m}^3$ tissue)
Palisade mesophyll	WG	2.56 $\pm$ 0.65	0.010 $\pm$ 0.002	0.024 $\pm$ 0.001
	CD	2.40 $\pm$ 0.50	0.009 $\pm$ 0.002	0.025 $\pm$ 0.009
Spongy mesophyll	WG	1.77 $\pm$ 0.25	0.006 $\pm$ 0.002	0.012 $\pm$ 0.003
	CD	1.71 $\pm$ 0.26	0.007 $\pm$ 0.001	0.012 $\pm$ 0.002

Mean mitochondrial volume ( $\mu\text{m}^3$ ), density (number of mitochondria  $\mu\text{m}^{-3}$  tissue) and sum volume ( $\mu\text{m}^3$  mitochondria  $\mu\text{m}^{-3}$  tissue) in palisade and spongy mesophyll tissue of warm-grown (WG) and cold-developed (CD) leaves.

Values represent the mean of three to six replicate plants ( $\pm$  SE).

### Mitochondrial volume and abundance in 3-week CT and CD leaves

In adaxial epidermal tissue, the mean mitochondrial volume was unchanged in CT leaves relative to WG leaves, but was decreased in CD leaves ( $P < 0.05$ ) (Fig. 4a). The number of mitochondria per unit leaf volume was increased within CT leaves ( $P < 0.05$ ), and further increased in the CD leaves, such that the mitochondrial density within these leaves was significantly higher compared to both WG ( $P < 0.001$ ) and CT ( $P < 0.001$ ) leaves (Fig. 4c). The collective result of these changes was an increase in the total volume of mitochondria in the CD leaves ( $P < 0.05$ ) (Fig. 4e), which had on average two times more mitochondria per unit volume of tissue compared to WG leaves. There was no significant change in sum volume within CT tissue (Fig. 4e). Thus, the mitochondrial population dynamics within adaxial epidermal tissue were significantly altered in response to low temperature, with the greatest response occurring in CD leaves.

In abaxial epidermal tissue, the mean mitochondrial volume was increased in CT leaves when compared with WG leaves ( $P < 0.005$ ), but was not statistically different in CD leaves (Fig. 4b). Mitochondrial density was increased in CT leaves ( $P < 0.05$ ), and in CD leaves when compared with WG leaves ( $P < 0.005$ ; Fig. 4d). Collectively, these changes

resulted in an increase in the total volume of mitochondria within CT leaves ( $P < 0.05$ ), and in CD leaves when compared with WG leaves ( $P < 0.01$ ), but not when compared with CT leaves (Fig. 4f). The magnitude of response was greater in abaxial epidermal tissue, which had on average five times more mitochondria per unit tissue volume compared to WG leaves. There was little difference in the degree of response observed in CT and CD leaves.

Whilst the mitochondrial populations within epidermal tissue exhibited a high degree of plasticity in response to low temperature, those within the mesophyll tissue exhibited none. Growth temperature had no effect on the mean volume, density or sum volume of mitochondria within the palisade and spongy mesophyll tissues of CD leaves (Table 1).

### Mitochondrial ultrastructure

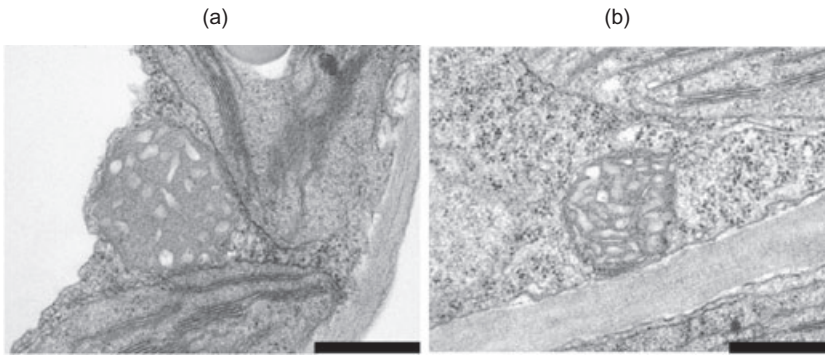
Comparison of epidermal and mesophyll cells of WG leaves revealed that the mitochondria within adaxial and abaxial epidermal cells exhibited a significantly higher ratio of cristae to matrix components compared to the mitochondria within palisade and spongy mesophyll cells ( $P < 0.01$ ; Table 2). Furthermore, these spatially heterogeneous populations appeared to exhibit differences in their response

**Table 2.** Ultrastructural characteristics of mitochondria in adaxial and abaxial epidermal cells, and palisade and spongy mesophyll cells, of warm-grown (WG) and cold-developed (CD) leaves

Tissue	Cell type	Growth treatment	Total area ( $\text{nm}^2$ )	Matrix ( $\text{nm}^2$ )	Cristae ( $\text{nm}^2$ )	Cristae to matrix ratio
Epidermis	Adaxial	WG	1633 $\pm$ 117	488 $\pm$ 21	1144 $\pm$ 135	2.37 $\pm$ 0.40
		CD	1626 $\pm$ 270	554 $\pm$ 143	1071 $\pm$ 167	2.05 $\pm$ 0.35
	Abaxial	WG	1989 $\pm$ 360	573 $\pm$ 106	1382 $\pm$ 255	2.33 $\pm$ 0.03
		CD	1373 $\pm$ 27	407 $\pm$ 56	965 $\pm$ 54	2.43 $\pm$ 0.40
Mesophyll	Palisade	WG	2259 $\pm$ 279	1216 $\pm$ 189	1043 $\pm$ 94	0.88 $\pm$ 0.07
		CD	1915 $\pm$ 340	761 $\pm$ 101	1154 $\pm$ 251	1.57 $\pm$ 0.23
	Spongy	WG	2403 $\pm$ 309	1313 $\pm$ 354	1089 $\pm$ 73	0.99 $\pm$ 0.25
		CD	1870 $\pm$ 134	788 $\pm$ 85	1082 $\pm$ 104	1.43 $\pm$ 0.19

Mean cross-sectional area of individual mitochondria ( $\text{nm}^2$ ), area of matrix and cristae in individual mitochondria ( $\text{nm}^2$ ) and ratio of cristae to matrix in individual mitochondria is shown.

Values represent the mean of three replicate leaves ( $\pm$  SE), with each replicate value being made up of the average of five sectioned mitochondria.



**Figure 5.** Representative mitochondria from spongy mesophyll cells of warm-grown (a) and cold-developed (b) leaves. Scale bars represent 500 nm.

to low temperature. Comparison of WG and CD leaves revealed that whereas there was no difference in the ratio of cristae to matrix components in mitochondria within adaxial and abaxial epidermal cells of WG and CD leaves, there was an increase in the ratio of cristae to matrix components in the mitochondria within both the palisade and spongy mesophyll cells of CD leaves, although this increase was only significant in the palisade cell mitochondria ( $P < 0.05$ ; Table 2). This increase in the ratio of cristae to matrix components appeared to result from a decrease in the area of matrix, rather than an increase in the area of cristae (Table 2, Fig. 5).

### Leaf anatomy

Figure 6 shows transverse sections of representative WG and CD leaves. Development of leaves in the cold resulted in an apparent reduction in the size of both epidermal and mesophyll cells, and an apparent increase in the number of palisade mesophyll cell layers.

### DISCUSSION

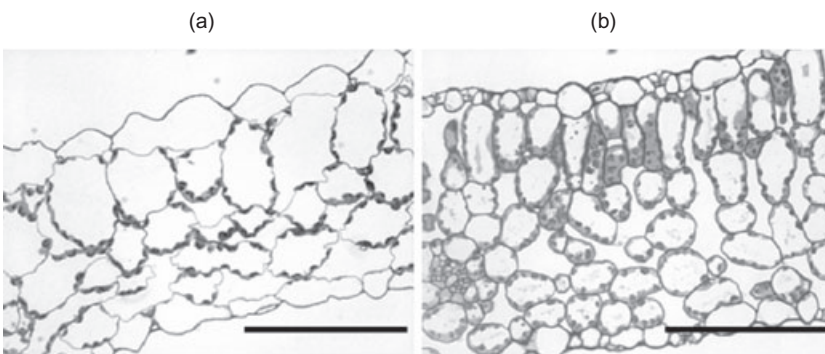
There exists an abundance of evidence demonstrating the ability of leaf respiration to acclimatize to low temperatures (Billings *et al.* 1971; Collier 1996; Xiong *et al.* 2000; Atkin & Tjoelker 2003; Bolstad *et al.* 2003; Talts *et al.* 2004; Atkin *et al.* 2005). Little is known, however, about the mechanistic changes that underpin this response. In this study, we investigated whether changes in mitochondrial physiology and biochemistry are involved in the respira-

tory cold acclimation response in leaves of the cold-hardy plant *A. thaliana*.

In recent years, it has become apparent that, in some plant species, CD tissues acclimatize to a greater extent than WG tissues that are shifted to the cold (Loveys *et al.* 2003). Our results corroborate these findings. We found that although 3-week CT leaves exhibited some degree of acclimation (as evidenced by the higher rates of intact leaf respiration at moderate measuring temperatures) (Fig. 1), it was only the CD leaves that were able to re-establish respiratory flux within the cold, achieving near-perfect compensation (i.e. homeostasis) of respiratory flux (Fig. 1). Associated with the increased rates of  $O_2$  uptake in CD leaves (Fig. 1) were: (1) an increase in the total volume of mitochondria per unit volume of tissue in epidermal cell layers (Fig. 4); (2) an increase in the ratio of cristae to matrix components within mesophyll cell mitochondria (Table 2); and (3) an increase in the capacity of the energy-producing cytochrome pathway in mitochondria isolated from whole leaf homogenates (Fig. 3).

### Cold-induced changes in sum volume of mitochondria

Based on past studies (Miroslavov & Kravkina 1991; Kislyuk *et al.* 1995), we hypothesized that CD leaves would exhibit a greater sum volume of mitochondria (per unit tissue volume) compared to their WG counterparts. Our results demonstrate that the sum volume of mitochondria was indeed greater in certain cell types within CD leaves. Despite contrary changes in mitochondrial size, the



**Figure 6.** Transverse section through representative warm-grown (a) and cold-developed (b) leaves. Scale bars represent 100  $\mu\text{m}$ .

total volume of mitochondria per unit volume of tissue was increased in both the adaxial epidermal tissue of CD leaves, and the abaxial epidermal tissue of CT and CD leaves (Fig. 4). This increase in sum volume in CT and CD leaves was underpinned by an increase in the number of mitochondria per unit volume of tissue, which could have resulted from an increase in mitochondrial number per cell and/or a decrease in cell size. The apparent decrease in epidermal cell size in CD leaves (Fig. 6) suggests that the increase in mitochondrial density was the result, at least in part, of a reduction in cell size. Regardless of the factors responsible for the increase in density (Fig. 4), the net result was an increase in the sum volume of mitochondria (per unit volume of tissue) in the epidermal cell layers of CT and CD leaves. We suggest that this increase contributed to the increased rates of respiration observed *in vivo* in the CT and CD leaves (Fig. 1). Importantly, however, this increase was limited to the epidermal cell layers. Despite concurrent decreases in mesophyll cell size (Fig. 6) the mitochondrial populations within mesophyll cells exhibited little change in sum volume or number in response to growth in the cold (Table 1). Given the apparent decrease in size of mesophyll cells in CD leaves (Fig. 6), the absence of any change in density suggests that, contrary to epidermal cells, CD mesophyll cells actually contained fewer mitochondria per cell than their WG counterparts. Such contrasting responses to growth temperature suggest that the changes occurring in mitochondrial density in epidermal cell layers following growth in the cold were not solely the dictate of developmental changes, but were also the result of control exerted from within the cell itself.

In spite of the fact that mitochondrial density was increased in epidermal tissue in CD leaves, the apparent absence of any significant change in mitochondrial density in mesophyll tissue of CD leaves [which in all likelihood will have dominated the respiratory signal because of the greater abundance of this tissue within the leaf (Fig. 6)] indicates that changes in mitochondrial density alone cannot fully account for the increase in flux observed following acclimation to the cold, at least in the CD leaves (Fig. 1).

### Cold-induced changes in mitochondrial ultrastructure

Ultrastructural examination of mesophyll cell mitochondria (Table 2 and Fig. 5) provides compelling evidence that respiratory activity within the mesophyll cells might have been significantly altered following growth in the cold. The decrease in the amount of matrix in mesophyll cell mitochondria in CD leaves (Table 2 and Fig. 5) suggests that the capacity for matrix-localized processes such as the tricarboxylic acid cycle and/or photorespiration were decreased, relative to the capacity for mitochondrial electron transport, in CD leaves. No change was observed in the ratio of cristae to matrix components in the epidermal cell mitochondria of CD leaves (Table 2). The differential

responses of these two cell types could be explained if the decrease in matrix volume was the result of a decrease in the activity of the photorespiratory cycle in mesophyll cells (mesophyll cells, contrary to their epidermal counterparts, contain chloroplasts) of CD leaves. Flux through the photorespiratory pathway is decreased at low temperatures because of a decreased affinity of Rubisco for O<sub>2</sub> (Jordan & Ogren 1984). Thus, the requirement for photorespiratory enzymes is likely to be lower in mesophyll cells of CD leaves. Tobin *et al.* (1989) demonstrated that mesophyll cells are capable of regulating the amount of the matrix-localized photorespiratory enzyme glycine decarboxylase according to photorespiratory activity. Thus, a decrease in photorespiratory activity, if accompanied by a decrease in the concentration of photorespiratory enzymes, could account for the mesophyll-specific decrease in matrix volume within the CD leaves. A decrease in the amount of mitochondrial matrix could have had repercussions for the activity of non-matrix-localized processes. In particular, it is conceivable that an increase in the amount of cristae relative to matrix might have increased the capacity for ion and metabolite diffusion from the matrix to the inner mitochondrial membrane, and thus enhanced the capacity for mitochondrial electron transport within these mitochondria. Previous studies have highlighted the dependence of mitochondrial electron transport chain activity, particularly the process of oxidative phosphorylation, upon rapid diffusion of ion and metabolites from the matrix to the inner mitochondrial membrane (Frey & Mannella 2000). This raises the possibility that alterations in mitochondrial ultrastructure might have contributed to the increased rates of O<sub>2</sub> uptake observed *in vivo* in the CD leaves.

### Cold-induced changes in mitochondrial capacity

Results derived from the measurement of whole tissue homogenized mitochondrial extracts from mature, fully expanded leaves revealed that the ability of the CD leaves to re-establish flux at 5 °C was associated with an increase in the capacity for mitochondrial O<sub>2</sub> uptake, per unit mitochondrial protein, through the energy-producing cytochrome pathway (Fig. 3). Although the CT leaves (which were unable to increase flux at 5 °C) showed no evidence of an increase in capacity, CD leaves (which were able to fully re-establish flux at 5 °C) exhibited a significant increase in cytochrome pathway capacity per unit mitochondrial protein at 5 °C (Fig. 3). The data therefore suggest that an increase in mitochondrial capacity per unit mitochondrial protein might be required for the re-establishment of respiratory flux in the cold, and that this increase in capacity requires the development of new tissue in the cold. The fact that the rate increase in the CD leaves was only significant at 5 °C suggests that there may have been a downward shift in the temperature optimum of respiration, perhaps reflecting an accumulation of unsaturated fatty acids in the inner mitochondrial membrane, following growth in the cold (Berry & Raison 1981).

Interestingly, previous studies have shown that the amount and/or activity of the alternative oxidase protein is increased in response to plant growth in the cold (Stewart *et al.* 1990; Vanlerberghe & McIntosh 1992; González-Meler *et al.* 1999; Ribas Carbó *et al.* 2000). It has been proposed that an increase in alternative oxidase helps protect plants against oxidative stress at low temperatures by preventing over-reduction of the mitochondrial electron transport chain, and thus minimizing the production of damaging reactive oxygen species in the cold (Purvis & Shewfelt 1993). However, in this study, we found no evidence for an increase in the capacity of the alternative pathway in either the CT or CD leaves (data not shown). Similarly, Kurimoto *et al.* (2004) reported an increase in cytochrome pathway capacity, but no increase in alternative pathway capacity, in roots of certain cultivars of wheat and rice that were developed in the cold. Given the differential roles of the cytochrome and alternative pathways of mitochondrial electron transport, it is conceivable that differences in the response of these two pathways reflect differences in the functional significance of the cold acclimation response in different plant species, or following differing periods of exposure to cold.

### Morphological heterogeneity of mitochondria

The analysis of ultrastructural and confocal micrographs of WG leaves revealed that the mitochondrial population within a WG leaf is highly heterogeneous, with different cell types exhibiting distinct differences in mitochondrial ultrastructure, gross morphology and abundance (Tables 1 & 2 and Fig. 4). Comparisons of the volume and density characteristics of epidermal and mesophyll cell mitochondria in the WG leaves need to be treated with caution, however, as results derived from GFP analysis suggest that mitochondria were smaller in mesophyll cells than in adjoining epidermal cells (comparison of data in Fig. 4 with those in Table 1), whereas those derived from ultrastructural analysis revealed the opposite trend, suggesting that mesophyll mitochondria were larger than their epidermal counterparts (Table 2). Although this discrepancy might well have arisen because of the limited number of mitochondria that were examined ultrastructurally, it might also have arisen because of a decrease in the GFP signal at increased focal depth (as a result of chromatic aberration and increased light scatter of both the excitation beam and emission), which would have resulted in the loss of the mitochondrial edges within the mesophyll cells. Despite these differences, any potential underestimation of mean mitochondrial volume in the mesophyll cells should have remained constant between treatments, and thus should not have affected relative changes in size and density. Indeed, both the GFP and ultrastructural data suggest that cold had little to no impact on mitochondrial size within the mesophyll cells, suggesting that any discrepancies arising from the methodology did not affect relative changes in the mean volume and density of mitochondria.

### CONCLUSIONS

Collectively, our results reveal the heterogeneity that exists within the mitochondrial population of a WG leaf, and demonstrate the diversity and cell-specific nature of mitochondrial responses that accompany cold acclimation, thus highlighting the care needed in interpreting results based on the response of one cell type. Together, the data suggest that changes in mitochondrial abundance, ultrastructure and capacity might play a pivotal role in the re-establishment of respiratory flux within the cold.

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