

## ON THE DEVELOPMENTAL DEPENDENCE OF LEAF RESPIRATION: RESPONSES TO SHORT- AND LONG-TERM CHANGES IN GROWTH TEMPERATURE<sup>1</sup>

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Using measurements of leaf respiratory O<sub>2</sub> uptake ( $R$ ), we investigated whether immature and mature *Arabidopsis thaliana* (ecotype Columbia) leaves differed in their response to temperature. Confocal microscopy (using plants with mitochondrially targeted green fluorescent protein [GFP]) was used to determine whether ontogenetic changes in  $R$  are associated with concomitant changes in mitochondrial morphology/abundance. Comparisons were made of warm-grown (25/20°C) leaves, warm-grown leaves shifted to cold (5°C) for 10 days, and cold-developed leaves. Short-term  $Q_{10}$  values and the ability to cold-acclimate were determined. In warm-grown plants, rates of  $R$  per mass were highest in immature leaves, decreasing as leaves developed. Moreover, although mitochondrial size (5.6–6.5  $\mu\text{m}^3$ ) remained constant during development, mitochondrial number per  $\mu\text{m}^3$  declined from 0.01 to 0.003 as leaves expanded (i.e., mitochondrial density decreased). Immature and mature leaves did not differ in  $Q_{10}$  values but did differ in their ability to cold-acclimate. Whereas mature leaves had clear evidence of cold acclimation (e.g., when measured at 25°C,  $R$  was highest in cold-developed leaves), young leaves had none. Collectively, the results highlight the changes in rates of  $R$ , mitochondrial density, and biomass allocation associated with leaf development and that changes in respiratory flux associated with acclimation only take place within mature tissues.

**Key words:** acclimation; development; leaf respiration; mitochondria; ontogeny;  $Q_{10}$ ; temperature.

Mitochondrial respiration ( $R$ ) plays a pivotal role in determining the growth and survival of plants (Hurry et al., 1995; Krömer, 1995; Hoefnagel et al., 1998; Atkin et al., 2000a) and has a profound influence on net ecosystem exchange and atmospheric CO<sub>2</sub> concentrations (Gifford, 2003). Plant  $R$  couples the production of energy and carbon skeletons (necessary for biosynthesis and cellular maintenance) to the release of large amounts of atmospheric CO<sub>2</sub>; 30–80% of daily photosynthetic carbon gain is released into the atmosphere by plant  $R$ , with approximately half of whole-plant  $R$  taking place in leaves (Poorter et al., 1990; Van Der Werf et al., 1994; Atkin et al., 1996; Loveys et al., 2002). Plant  $R$  contributes up to 65% of total CO<sub>2</sub> released into the atmosphere from terrestrial ecosystems (Janssens et al., 2001; Xu et al., 2001; Reichstein et al., 2002) with the remaining CO<sub>2</sub> coming from heterotrophic soil  $R$ . Understanding the impact of environmental change on  $R$  of plant tissues is therefore a prerequisite for predicting how future changes in climate will affect plant productivity and terrestrial ecosystem CO<sub>2</sub> release.

In plants, the rate of  $R$  is dependent upon the developmental stage of the tissue, with expanding immature tissues having higher rates of  $R$  compared to fully expanded, mature tissues (Azcón-Bieto et al., 1983b; McDonnell and Farrar, 1993; Atkin and Cummins, 1994; Winkler et al., 1994; Millar et al., 1998; Evans et al., 2000). This decrease in respiration associated with tissue expansion reflects a decrease in the demand for ATP for growth (whereas subsequent decreases in respiration occurring in mature, fully expanded tissue will likely reflect a decrease in

the demand for ATP by maintenance processes). Various factors may be responsible for the decrease in specific  $R$  rates as tissues expand, including changes in protein abundance and alterations in the density of mitochondria. Although information on changes occurring in leaves is lacking, Millar et al. (1998) found that decreases in the rate of  $R$  in soybean roots (during aging) were not associated with decreases in the amount of mitochondrial protein per unit root dry mass. Intuitively, this might suggest that the density of mitochondria in plant tissues remains constant during aging; however, definitive data on the abundance (and size) of mitochondria in immature and mature plant tissues is currently lacking. Moreover, it is not known whether immature and mature tissues have differential respiratory responses to changes in the environment (e.g., temperature).

Temperature is a major determinant of respiratory activity. In the short-term, a change in temperature will result in an immediate alteration in the rate of  $R$ , with the extent of that alteration determined by the respiratory  $Q_{10}$  (the proportional increase in  $R$  for every 10°C rise in temperature). Long-term exposure to a change in temperature can result in respiratory acclimation (Billings et al., 1971; Collier, 1996; Atkin et al., 2000b; Xiong et al., 2000; Covey-Crump et al., 2002; Bolstad et al., 2003; Talts et al., 2004; Armstrong et al., 2006; Atkin et al., 2006). Acclimation is the subsequent adjustment in the rate of  $R$  to compensate for the initial change in temperature (Atkin and Tjoelker, 2003). Thermal acclimation results in cold-grown plants with higher rates of  $R$  than warm-grown plants when measured at a moderate set temperature. Perfect acclimation results in a phenomenon known as respiratory homeostasis, in which plants grown at contrasting temperatures have identical rates of  $R$  when measured at their respective growth temperatures (Collier, 1996; Xiong et al., 2000; Armstrong et al., 2006; Atkin et al., 2006). Substantial interspecific variations in  $Q_{10}$  values and degrees of acclimation have been reported (see Atkin et al., 2005, for a recent review); however,

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it is not known whether mature and immature tissues differ in their short- and long-term temperature sensitivities. Developmentally driven changes in the temperature dependence of  $R$  could significantly impact respiratory energy production as well as daily  $\text{CO}_2$  release (Atkin et al., 2000a). Such variations need to be accounted for in coupled climate and carbon-cycle models if future atmospheric  $\text{CO}_2$  concentrations, and their consequences for the energy balance of the globe, are to be accurately predicted (Wythers et al., 2005; King et al., 2006).

Our study had two objectives. First, we sought to establish whether decreases in the rate of leaf  $R$  during development are associated with a decrease in mitochondrial abundance. Using confocal microscopy and stably transformed *Arabidopsis thaliana* lines expressing green fluorescent protein (GFP) targeted to mitochondria (Logan and Leaver, 2000), we quantified mitochondrial size and density in epidermal cells of immature and mature leaves. Using these lines, we recently found that recovery of respiratory flux in mature, cold-developed leaves was partly the result of an increase in the total volume of mitochondria per unit volume of tissue in epidermal cells (Armstrong et al., 2006). Second, we sought to establish whether immature and mature leaves differ in their response to both short- and long-term changes in temperature. Our results demonstrate that the density of mitochondria per unit cell volume decreases as leaves develop and that immature and mature leaves differ in their ability to acclimate  $R$  to a sustained decrease in growth temperature.

## MATERIALS AND METHODS

**Plant material and growth conditions**—Leaf respiration and leaf anatomy measurements were carried out on wild-type *Arabidopsis thaliana* plants (ecotype Columbia) grown hydroponically after their initial establishment in compost (Levingtons F2; Scott's Professional, Ipswich, UK). Once roots had reached approximately 3 cm in length, seedlings were removed from the compost, and the roots were washed with water. Seedlings were then transferred to 16-L hydroponics tanks filled with a fully aerated modified Hoaglands nutrient solution. The pH of the solution was adjusted daily to 5.8, and the solution was changed weekly. Mitochondrial mean volume, density, and sum volume were calculated for transgenic *Arabidopsis thaliana* plants (ecotype Columbia) with GFP targeted to their mitochondria [mito-GFP (Logan and Leaver, 2000)]; plants were grown in compost (Levingtons F2). Previous work has shown that the labelling of mitochondria with GFP has no effect on the phenotype of *Arabidopsis thaliana* (Logan and Leaver, 2000). We thus assumed that any changes in the size and density of mitochondria in mito-GFP plants would parallel those occurring in wild-type plants.

Plants were initially grown in controlled environment growth chambers (Fitotron 600H, Gallenkamp Loughborough, Leicestershire, UK) maintaining a 25/20°C day/night temperature regime, a photon flux density of 150  $\mu\text{moles photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , and an 8-h photoperiod. After approximately 2 months, measurements were conducted on warm-grown (WG) leaves. The WG plants were then shifted to chilled, controlled environment chambers (Microclima 1750, Snijders Scientific, Tilburg, The Netherlands) maintaining a 5/5°C day/night temperature regime (150  $\mu\text{moles photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , 8-h photoperiod). Measurements were conducted on detached leaves from plants that were cold treated (CT) for 10 d and detached leaves developed in the cold (sampled after 50 days 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, 1999, 2003; Stitt and Hurry, 2002; Talts et al., 2004; Armstrong et al., 2006).

**Leaf respiration measurements**—The respiratory characteristics of WG, CT, and CD leaves of different developmental stages were characterized in the wild-type plants. Leaf dark respiration ( $\text{nmol O}_2 \cdot \text{g}^{-1} \cdot \text{s}^{-1}$ ) was measured in warm-grown plants in the most recently emerged leaves of the inner rosette,

referred to as immature leaves; in partially expanded leaves of the middle rosette, referred to as intermediate leaves; and in fully expanded leaves of the outer rosette, referred to as mature leaves. Following the shift to 5°C, respiration was measured in immature and mature 10-day CT and CD leaves.

$R$  was measured 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). Electrodes were calibrated with deionized water equilibrated to the appropriate temperature and saturated with air.  $R$  was measured at 5, 10, 15, 20, 25, and 30°C. At each temperature,  $R$  was measured in leaves of 3–5 replicate plants (with each replicate representing a leaf from a different plant). Leaves were weighed and passed through a leaf area meter (Li-Cor 3000C Portable Leaf Area Meter). Leaf pieces were 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 (to overcome postillumination transients [Azcón-Bieto et al., 1983a; Atkin et al., 1998]).  $R$  was measured in darkened, airtight cuvettes containing a known volume of fully aerated leaf  $R$  buffer, equilibrated to the appropriate temperature for 30 min prior to usage. Following measurement, the leaves were dipped in liquid nitrogen and placed in a –80°C freezer. Leaves were subsequently freeze dried under vacuum, and dry mass for each was recorded. The fresh and dry mass data together with the leaf area data were used to calculate gram fresh mass per meter squared (which has been shown to correlate closely with leaf thickness; Vile et al., 2005), specific leaf area (SLA, meter-squared per gram dry mass) and dry mass per unit fresh mass (referred to as dry matter content [DMC] per gram) in the 3–5 replicate leaves.

**Confocal microscopy and image analysis**—Immature, intermediate, and mature WG leaves of mito-GFP *Arabidopsis* plants were measured, as recently described in Armstrong et al. (2006). Confocal microscopy was carried out using a Zeiss (Thornwood, NY, USA) LSM 510 Meta on an Axioplan 2M fitted with a 63× Plan Apo lens (NA 1.4). The sample was excited with a 488 nm Argon laser and 633 nm HeNe laser, and light was collected with the Zeiss Meta detector at approximately 10.5-nm intervals centered from 488 nm through to 730 nm via a multiple band dichroic mirror (UV/488/543/633). GFP fluorescence was detected and separated from autofluorescent components, chlorophyll, and light scatter, with the use of postacquisition spectral unmixing. Images were acquired as 20–50- $\mu\text{m}$  stacks (0.44  $\mu\text{m}$  slice interval, 146  $\mu\text{m}^2$ ).

Armstrong et al. (2006) found that mitochondrial characteristics were similar in abaxial and adaxial epidermal cells; moreover, environment-induced changes in respiratory flux (after plant growth in the cold) were matched by concomitant changes in mitochondrial density in epidermal cells. Based on such observations, we limited our analysis to ontogenetic changes in mitochondria in adaxial epidermal cells. Images of the mitochondrial populations present in adaxial epidermal tissue were recorded in immature, intermediate, and mature WG leaves from six replicate plants. Once the images had been collected, three-dimensional reconstructions of the mitochondrial populations within adaxial epidermal tissue were generated using Velocity image processing software (Velocity 3.1, Improvise, Coventry, UK). Mean volume of mitochondria, density per unit volume of tissue, and sum volume per unit volume of tissue, were calculated. Nontransfected cells were imaged under identical conditions as positive controls to ensure that no false assignment was given to the GFP channel.

**Statistical analysis**—Data were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov test and one-way analyses of variance in SPSS v10 (SPSS Science, Birmingham, UK). If the data were suitable for parametric testing, a one-way analysis of variance was carried out with least significant difference (LSD) 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 per unit fresh mass**—The short-term temperature response characteristics of immature, intermediate, and mature WG *Arabidopsis* leaves were measured (Fig. 1; data for intermediate leaves not shown). The rate of respiration per unit fresh mass declined with age, such that immature leaves had the highest rates of  $R$ , and mature leaves had the lowest (comparison of Figs. 1A and B). A one-way ANOVA

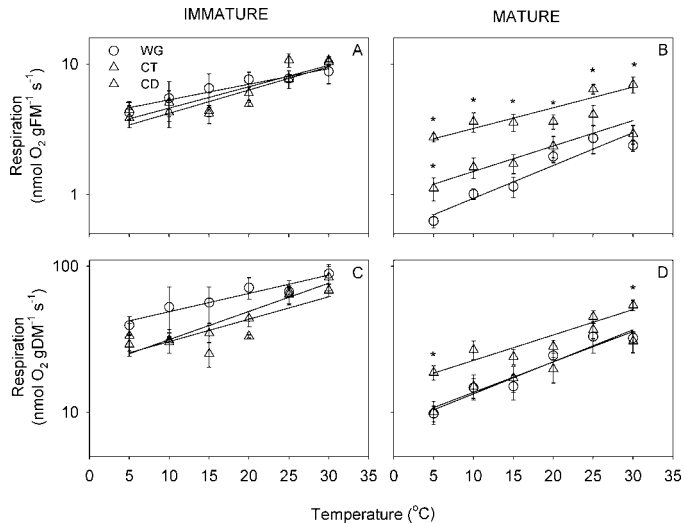


Fig. 1.  $\log_{10}$  respiration of immature (A and C) and mature (B and D) wild-type *Arabidopsis thaliana* leaves plotted against temperature ( $^{\circ}\text{C}$ ) for warm-grown (WG), 10-day cold-treated (CT), and cold-developed (CD) leaves expressed per unit fresh mass,  $\text{nmol O}_2 \cdot \text{g FM}^{-1} \cdot \text{s}^{-1}$  (A and B) and per unit dry mass,  $\text{nmol O}_2 \cdot \text{g DM}^{-1} \cdot \text{s}^{-1}$  (C and D). Values represent the mean of 3–5 replicate plants ( $\pm$  SE). Linear regression fit through each treatment. Asterisks have been used to denote when the CT and CD means differed significantly from the WG means at each temperature ( $P < 0.05$ ).

confirmed that immature leaves (Fig. 1A) had significantly higher rates of  $R$  per unit fresh mass when compared to both intermediate ( $P < 0.001$ ; data not shown) and mature leaves ( $P < 0.001$ ; Fig. 1B). Similarly, intermediate leaves had higher rates of  $R$  compared to mature leaves ( $P < 0.001$ ). The temperature-sensitivity of  $R$  was independent of the developmental stage of the tissue; there were no statistical differences in the  $Q_{10}$  values of immature and mature leaves (Table 1).

Immature and mature leaves did, however, differ in their response to long-term changes in temperature when data expressed on a fresh mass basis was considered. Immature leaves had little evidence of acclimation (Fig. 1A), with no significant differences in the rate of  $R$  in WG, 10-day CT or CD leaves. Mature leaves, however, had clear evidence of acclimation, with the extent of that response being dependent upon whether the leaves were shifted to or developed in the cold (Fig. 1B). There was a slight increase in the rate of  $R$  in the mature 10-day CT leaves (Fig. 1B), although this increase was only significant at the measurement temperature of  $5^{\circ}\text{C}$  ( $P < 0.05$ ). There was a further increase in the rate of  $R$  in the

TABLE 1.  $Q_{10}$  values for immature and mature warm-grown (WG), 10-day cold-treated (CT), and cold-developed (CD) leaves of wild-type *Arabidopsis thaliana*.  $Q_{10}$  values were calculated across the temperature range of  $5$ – $30^{\circ}\text{C}$ , using the data in Figs. 1A and B (i.e., respiration data expressed on a fresh mass basis), according to the equation  $Q_{10} = 10^{[10 \times \text{regression slope}]}$ . Values represent the mean of 3–5 replicate plants ( $\pm$  SE). Asterisks denote  $Q_{10}$  values significantly different from those found in WG leaves of the equivalent developmental stage ( $P < 0.05$ ).

Developmental stage	WG	CT	CD
Immature	$1.36 \pm 0.16$	$1.54 \pm 0.06$	$1.51 \pm 0.08$
Mature	$1.78 \pm 0.11$	$1.59 \pm 0.08$	$1.44 \pm 0.05^*$

mature CD leaves (Fig. 1B). CD leaves had significantly higher rates of  $R$  across the entire temperature range when compared to WG leaves ( $P < 0.05$ ), and at the measurement temperatures of 5, 10, 15, and  $30^{\circ}\text{C}$  when compared to 10-day CT leaves. Mature CD leaves were significantly less temperature sensitive (i.e., lower  $Q_{10}$ ) than their WG counterparts ( $P < 0.05$ ; Table 1).

To obtain a measure of the degree of acclimation in mature 10-day CT and CD leaves, the degree of respiratory homeostasis was calculated. The rate of respiration in the WG, 10-day CT, and CD leaves measured at  $5^{\circ}\text{C}$  was divided by the rate of respiration in the WG plants measured at  $25^{\circ}\text{C}$ , and multiplied by 100 to give percentage homeostasis. Whereas the rate at  $5^{\circ}\text{C}$  was only 20% of the rate at  $25^{\circ}\text{C}$  in WG leaves, this increased to 41% in 10-day CT leaves, and to 101% in CD leaves. Thus, whereas 10 days of cold-treatment resulted in only a partial recovery of flux at  $5^{\circ}\text{C}$ , development of leaves in the cold resulted in a complete recovery of flux at  $5^{\circ}\text{C}$ .

**Leaf respiration per unit dry mass**—Figures 1C and D show the respiratory temperature response curve data presented in Figs. 1A and B plotted on a dry mass (DM) basis. In line with the data expressed on a fresh mass basis, immature leaves had no evidence of cold acclimation when data was expressed on a dry mass basis (Fig. 1C) (i.e., rates were not significantly higher in the 10-day CT and CD leaves compared to their WG counterparts). When  $R$  was expressed per unit dry mass, the acclimation response observed in mature leaves was depressed (Fig. 1D) relative to that observed when  $R$  was expressed per unit fresh mass (Fig. 1B). There was no longer any increase in rate in the 10-day CT leaves, and although  $R$  remained higher in the CD leaves when compared to both WG ( $P < 0.05$ ) and 10-day CT ( $P < 0.05$ ) leaves at the measurement temperatures of 5 and  $30^{\circ}\text{C}$ , there were no longer any differences in rate at the measurement temperatures of 10, 15, 20, or  $25^{\circ}\text{C}$ . Furthermore, the degree of respiratory homeostasis in the mature CD leaves decreased from 101% when  $R$  was expressed per unit fresh mass, to 57% when respiration was expressed per unit dry mass.

**Leaf respiration per unit area**—To provide a means for comparison with other findings in the literature, the SLA values were used to convert mean rates of respiration expressed per unit dry mass basis to rates of respiration expressed per unit area. On an area basis, respiration increased from  $0.57 \mu\text{mol O}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  in the mature WG leaves, to  $0.88 \mu\text{mol O}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  in the mature 10-day CT leaves, and to  $2.05 \mu\text{mol O}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  in the mature CD leaves when measured at  $25^{\circ}\text{C}$ .

**Mitochondrial size, abundance, and leaf development**—Figure 2 shows the mitochondrial mean volume (i.e., size), density, and sum volume characteristics of the mitochondrial populations within epidermal cells of immature, intermediate, and mature WG leaves. Mitochondrial population dynamics were dependent upon the developmental stage of the leaf. Although mitochondrial size was independent of developmental stage (Fig. 2A), mitochondrial density (Fig. 2B), and thus sum volume (Fig. 2C) declined with age. Immature leaves had a significantly higher density of mitochondria compared to both intermediate ( $P < 0.001$ ) and mature ( $P < 0.001$ ) leaves, and intermediate leaves had a significantly higher density when compared to mature leaves ( $P < 0.01$ ; Fig. 2B). The result of

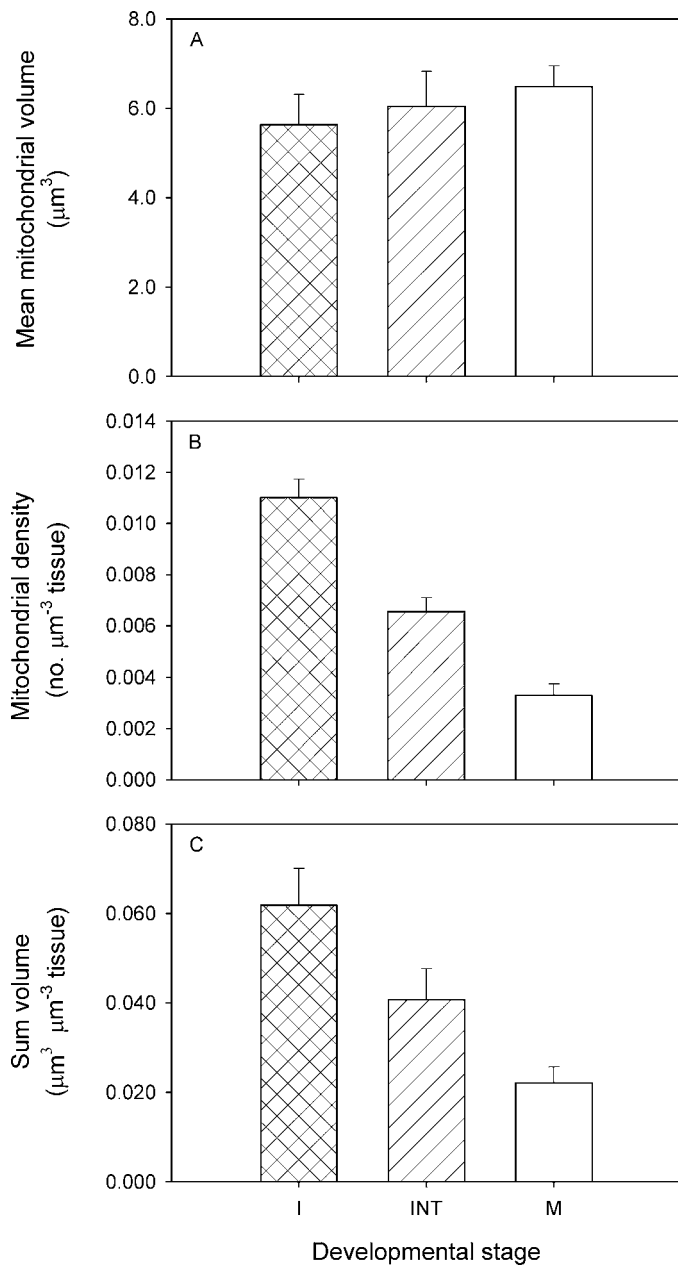


Fig. 2. Mitochondrial mean volume ( $\mu\text{m}^3$ ) (A), density of mitochondria per  $\mu\text{m}^{-3}$  tissue (B), and sum volume ( $\mu\text{m}^3$  mitochondria  $\cdot \mu\text{m}^{-3}$  tissue) (C) in adaxial epidermal tissue of immature (I) (hatched), intermediate (INT) (diagonals), and mature (M) (clear) warm-grown (WG) leaves of mito-*Arabidopsis thaliana*. Values represent the mean of six replicate plants ( $\pm$ SE).

this decrease in density was a marked decline in the total volume of mitochondria, per unit volume of tissue, as the leaf aged (Fig. 2C). Consequently, immature leaves had a significantly higher sum volume of mitochondria when compared to both intermediate ( $P < 0.05$ ) and mature ( $P < 0.01$ ) leaves.

**Leaf biomass allocation**—Developmental stage had a clear impact on apparent leaf thickness (as estimated from the ratio of leaf fresh mass to leaf area) and dry mass per fresh mass (or leaf dry matter content, DMC); mature WG leaves were thicker

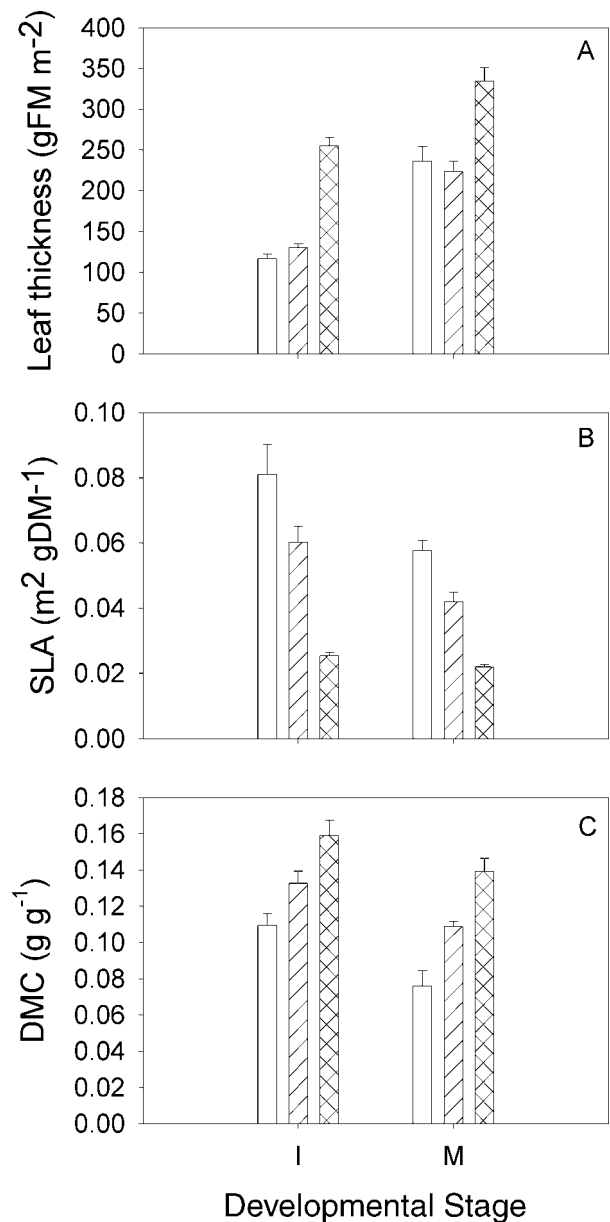


Fig. 3. Leaf structural traits of immature (I) and mature (M) leaves of wild-type *Arabidopsis thaliana*. Data for warm-grown (WG) (clear), 10-day cold-treated (CT) (diagonals), and cold-developed (CD) (hatched) leaves are shown for: (A) leaf "thickness" as estimated from fresh mass ( $\text{g} \cdot \text{m}^{-2}$ ), (B) specific leaf area (SLA,  $\text{m}^2 \cdot \text{g dry mass}^{-1}$ ), and (C) leaf dry matter content (DMC,  $\text{g dry mass} \cdot \text{g fresh mass}^{-1}$ ). Values represent the mean of 3–5 replicate plants ( $\pm$ SE).

( $P < 0.01$ ; Fig. 3A) and had a lower DMC ( $P < 0.05$ ; Fig. 3C) compared to immature WG leaves.

Growth at  $5^\circ\text{C}$  resulted in marked alterations in leaf morphology, particularly when leaves were developed in the cold. Shifting preexisting leaves to the cold for 10 days had little effect on leaf "thickness" (as estimated from the ratio of leaf fresh mass to leaf area) in immature or mature leaves (Fig. 3A). However, development of leaves in the cold resulted in a substantial increase in leaf thickness in both immature and mature leaves when compared to WG ( $P < 0.01$ ) and 10-day CT ( $P < 0.01$ ) leaves (Fig. 3A). Specific leaf area (SLA) was

significantly reduced in immature 10-day CT leaves when compared to immature WG leaves ( $P < 0.05$ ) and was further reduced in immature CD leaves when compared to immature WG ( $P < 0.001$ ) and 10-day CT ( $P < 0.001$ ) leaves (Fig. 3B). Similarly, SLA was significantly reduced in mature CT leaves compared to mature WG leaves ( $P < 0.01$ ) and in mature CD leaves compared to mature WG ( $P < 0.001$ ) and 10-day CT ( $P < 0.001$ ) leaves (Fig. 3B).

Dry matter content (DMC) was significantly higher in immature CD leaves compared to immature WG ( $P < 0.01$ ) and CT ( $P < 0.05$ ) leaves (Fig. 3C). DMC was also higher in mature 10-day CT leaves than in mature WG leaves ( $P < 0.01$ ) and in mature CD leaves than in mature WG ( $P < 0.001$ ) and CT ( $P < 0.01$ ) leaves (Fig. 3C). Such changes, which likely reflect accumulation of nonstructural carbohydrates in the cold (Strand et al., 1999), need to be considered when assessing the impact of growth temperature on temperature responses of leaf  $R$  (see above).

## DISCUSSION

Respiratory activity is closely related to the developmental stage of a tissue, with immature tissues often having higher rates of respiration per unit mass compared to mature tissues (Azcón-Bieto et al., 1983b; McDonnell and Farrar, 1993; Atkin and Cummins, 1994; Winkler et al., 1994; Millar et al., 1998). Indeed, in our study developmental stage and respiratory activity were markedly correlated in warm-grown leaves, such that immature, partially expanded leaves had significantly higher rates of respiration compared to mature fully expanded leaves (Fig. 1). Data derived from analysis of mito-GFP *Arabidopsis* by confocal microscopy revealed that associated with this decrease in respiratory activity with developmental stage was a decline in mitochondrial density in epidermal cells (Fig. 2B), with no change in the size of individual mitochondria, suggesting that the ontogenetic decline in specific rates of  $R$  may have been the result of an increasing dilution of mitochondria as the leaves expanded. Whether this decline in density is the sole cause of the decrease in  $R$  in mature tissues or whether it is accompanied by a decrease in capacity per unit mitochondrial protein is uncertain. However, Millar et al. (1998) found that the decrease in soybean root  $R$  with age could be explained by a decrease in the capacity of the cytochrome pathway, with the amount of mitochondrial protein per unit dry mass changing minimally. It is possible that similar changes in respiratory capacity are taking place within the leaves examined in our study; further work is needed to determine whether the decline in mitochondrial density with age is accompanied by a decrease in the capacity for mitochondrial electron transport.

In addition to differences in gross respiratory activity, immature and mature leaves differed in their respiratory response to changes in temperature. Whilst the developmental stage of the leaf had little effect upon the short-term temperature sensitivity of respiration, it had a marked effect upon the respiratory response to long-term changes in temperature (Fig. 1). In an abundance of literature,  $R$  can acclimate to long-term changes in temperature in mature plant tissues (e.g., Billings et al., 1971; Collier, 1996; Atkin et al., 2000b; Xiong et al., 2000; Bolstad et al., 2003; Talts et al., 2004; Armstrong et al., 2006; Atkin et al., 2006), and there is emerging evidence to suggest that complete acclimation

requires development of new tissue in the new temperature regime (Loveys et al., 2003; Talts et al., 2004; Armstrong et al., 2006). Such studies have not, however, established whether the developmental stage of the tissue has an impact on the acclimation response. Here we reveal for the first time that the developmental stage of a leaf actually has a profound impact on its ability to acclimate. Whereas immature leaves had no evidence of acclimation in response to the 10-day exposure to the cold and/or development in the cold (Fig. 1A), mature leaves partially acclimated following the 10-day exposure to the cold, and completely acclimated, that is, reached homeostasis of  $R$ , following development in the cold (Fig. 1B).

In previous work we have shown that respiratory acclimation to the cold is underpinned by an increase in mitochondrial density (Armstrong et al., 2006). It is possible that the cold acclimation response observed in mature CD leaves in this study is the consequence of an increase in mitochondrial density, which results from a suppression of cellular expansion in the cold. If acclimation were simply an expression of a suppression of leaf expansion this might explain why an acclimation response is not evident in immature leaves, which have yet to undergo the majority of their expansion. However, whether the increase in respiratory flux observed in mature leaves is coupled to a cold-induced suppression of leaf expansion is questionable, as one would expect cold to have an equivalent effect on the expansion processes of both immature and mature leaves. Indeed, the immature CD leaves, which had no evidence of acclimation (Fig. 1A), had similar changes in leaf morphology when compared to the mature CD leaves (Fig. 3), suggesting that cold has a uniform effect on leaf morphology, but a non-uniform effect on respiratory flux. Furthermore, leaf expansion results largely from the accumulation of water (Taiz and Zeiger, 2002). Thus, if the increase in respiratory flux observed in mature CD leaves were simply the result of a suppression of leaf expansion, then we would expect to see little increase in rate when differences in leaf water content are removed, i.e., when  $R$  is expressed per unit dry mass (although it should be noted that any suppression of the acclimation response when  $R$  is expressed per unit dry mass might also reflect an accumulation of starch and soluble sugars in the cold [Strand et al., 1999]). However, when differences in leaf water content were removed,  $R$  remained higher, although the difference was not so pronounced, in the CD leaves when compared to both WG and CT leaves (Fig. 1D), despite any potential build up of soluble sugars. Thus, whilst the increase in flux observed in mature CD leaves might be dependent upon an increase in mitochondrial density (Armstrong et al., 2006), it is unlikely that this increase in density results from a suppression of cellular expansion in the cold. Thus it is unlikely that a suppression of leaf expansion can explain the developmentally dependent acclimation response. It is perhaps more likely that immature leaves do not possess the capacity for change. A particularly significant consequence of this inability of immature leaves to acclimate will be that the proportion of immature leaves on the plant will determine the whole-shoot acclimation response and the capacity for respiratory homeostasis in response to long-term changes in temperature.

Our finding that immature and mature leaves differ in their responses to long-term changes in temperature could have important implications for estimates of how much respiratory  $\text{CO}_2$  is released into the atmosphere over large spatial and temporal scales. Each year, plant  $R$  releases approximately 60

gigatonnes (Gt) of carbon into the atmosphere, which is a large flux compared with the relatively small release of CO<sub>2</sub> from the combustion of fossil fuels (<6 Gt C·year<sup>-1</sup>) (Schimel, 1995; Houghton et al., 2001). At present, most global circulation models (GCMs) such as the HadCM3 model (Cox et al., 2000) assume that *R* of all tissues increases exponentially with temperature and that the *Q*<sub>10</sub> of *R* will remain constant over time (i.e., *R* will not acclimate to future changes in temperature; Rustad, 2001). Our results demonstrate that such assumptions are clearly incorrect. Long-term changes in temperature (e.g., week-to-week or month-to-month) are unlikely to result in acclimation-based shifts in the temperature response curve of *R* in immature leaves; in contrast, large shifts do occur in mature leaves (Fig. 1) (Tjoelker et al., 1999; Bolstad et al., 2003; Atkin et al., 2005). Such developmentally linked differences in the acclimation response could potentially impact predictions made by coupled climate and carbon cycle models on future atmospheric CO<sub>2</sub> concentrations and its consequences for the energy balance of the globe. Luo et al. (2001) reported that failure to account for acclimation potential can result in an over-estimate of the effects of global warming on respiratory CO<sub>2</sub> release over long periods, particularly in models that assume a positive feedback of global warming on respiration rates. Similarly, when running the ecosystem model PnET with a temperature-driven algorithm that accounted for thermal acclimation (and variations in *Q*<sub>10</sub>), Wythers et al. (2005) found that incorporation of these algorithms resulted in large decreases in predicted annual foliar *R* and increases in predicted net primary productivity, especially in the context of respiratory acclimation to temperature. Finally, King et al. (2006) recently reported that incorporation of acclimation of leaf *R* into a global ecosystem model, GTEC 2.0, resulted in lower predicted rates of leaf *R* at higher temperatures at the end of the 21<sup>st</sup> century and more carbon stored in plants and soils. Development-linked changes in acclimation of *R* could, therefore, have significant impacts on our predictions of future climates and the extent of carbon storage in terrestrial biospheres.

In conclusion, we have shown that the specific rate of *R* decreases as leaves expand and that this decrease is linked to a concomitant decrease in the number of mitochondria per unit cell volume. Such results point to a decrease in respiratory capacity as leaves develop. Importantly, the change in respiratory flux associated with acclimation to long-term changes in temperature only occurs in mature leaves. If the differential ability of immature and mature leaves to acclimate following growth in the cold is also manifest following growth in the warm, then, if more widespread, this could have important implications for the impact of seasonal and longer-term changes in climate on rates of ecosystem *R* and net primary productivity.

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