New Phytologist

Research

Acclimation of photosynthesis and respiration is asynchronous in response to changes in temperature regardless of plant functional group

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Received: *23 March 2007*
Accepted: *13 June 2007*

Summary

• Gas exchange, fluorescence, western blot and chemical composition analyses were combined to assess if three functional groups (forbs, grasses and evergreen trees/shrubs) differed in acclimation of leaf respiration (*R*) and photosynthesis (*A*) to a range of growth temperatures (7, 14, 21 and 28°C).

• When measured at a common temperature, acclimation was greater for *R* than for *A* and differed between leaves experiencing a 10-d change in growth temperature (PE) and leaves newly developed at each temperature (ND). As a result, the *R* : *A* ratio was temperature dependent, increasing in cold-acclimated plants. The balance was largely restored in ND leaves. Acclimation responses were similar among functional groups.

• Across the functional groups, cold acclimation was associated with increases in nonstructural carbohydrates and nitrogen. Cold acclimation of *R* was associated with an increase in abundance of alternative and/or cytochrome oxidases in a species-dependent manner. Cold acclimation of *A* was consistent with an initial decrease and subsequent recovery of thylakoid membrane proteins and increased abundance of proteins involved in the Calvin cycle.

• Overall, the results point to striking similarities in the extent and the biochemical underpinning of acclimation of *R* and *A* among contrasting functional groups differing in overall rates of metabolism, chemical composition and leaf structure.

Key words: acclimation, functional group, global antibodies, photosynthesis, protein abundance, respiration, temperature.

New Phytologist (2007) **176**: 375–389

doi: 10.1111/j.1469-8137.2007.02183.x

Introduction

The balance between plant respiration (*R*) and photosynthetic carbon assimilation (*A*) (i.e. the *R* : *A* ratio) is important in processes ranging from plant productivity to ecosystem balance and the flux of carbon through the biosphere. Anywhere from 30 to 80% of the carbon taken up through *A* can later be released by whole-plant *R* (Loveys *et al.*, 2002). The extent of respiratory CO_2 release is globally significant because plant *R* releases approx. 60 Gt of carbon into the atmosphere each year, approx. 10 times that currently released by the burning of fossil fuels (Amthor, 1997; Field, 2001). In addition, both *R* and *A* are temperature sensitive, as is the *R* : *A* ratio (Berry & Björkman, 1980; Loveys *et al.*, 2003; Atkin *et al.*, 2006). Short-term decreases to low positive temperature, or increases to very high temperature, typically result in an increase in *R* : *A*, because the response of *R* to temperature is

This was an equal collaboration between research groups based at the University of York and Umeå University.

exponential, whereas the response of *A* has a relatively broad temperature optimum (Atwell *et al.*, 1999). Temperature-dependent changes in *R*: *A* could have important consequences for the global carbon balance (Gifford, 2003) and, because of this, it is vital that we develop a processed-based understanding of the factors contributing to variations in *R*: *A* in a wide range of plant species representative of different functional groups.

The impacts of long-term changes in growth temperature on *R*: *A* depend on the extent to which *R* and *A* differ in the degree of thermal acclimation. Acclimation is defined as the adjustment in metabolism such that plant performance is improved at the new growth temperature (Lambers *et al.*, 1998). Two developmentally dependent stages of acclimation are recognized: (1) changes in respiratory and photosynthetic metabolism of pre-existing (PE) tissues following a sustained temperature shift; and (2) changes in metabolism that occur when tissues develop at the new growth temperature (ND) (Strand *et al.*, 1997; Atkin & Tjoelker, 2003). In PE tissues (i.e. stage 1), acclimation relies on changes to the cellular machinery already in place within the cell (e.g. changes in protein abundance within existing organelles and/or changes in substrates/energy demand (Stitt & Hurry, 2002; Atkin *et al.*, 2005). For example, after 1–2 d at a lower temperature, soluble sugars accumulate in source leaves (Strand *et al.*, 2003; Lundmark *et al.*, 2006) and this increase may be directly responsible for the subsequent recovery of *R* associated with cold acclimation, through increased substrate availability (Atkin & Tjoelker, 2003). By contrast, this rapid increase in soluble sugars may inhibit *A* through feedback inhibition (Hurry *et al.*, 1993; Strand *et al.*, 1997) and down-regulation of nuclear-encoded photosynthetic gene expression (Strand *et al.*, 1997; Hurry *et al.*, 2000). Extended cold-treatment of PE leaves can also result in photodamage; low temperature slows the consumption of ATP and NADPH by the Calvin cycle and the resulting over-reduction of the photosynthetic electron transport chain may cause oxidative damage to the light-harvesting machinery (Somersalo & Krause, 1988; Hurry & Huner, 1992). Ribas-Carbó *et al.* (2000) also found that the flux through the alternative oxidase (AOX) pathway increased when PE leaves were cold-treated for several days. Collectively, such changes can result in the *R*: *A* ratio increasing in PE leaves during acclimation to low positive temperatures (Atkin *et al.*, 2005).

The second stage of acclimation that occurs in ND leaves has been studied most extensively in short-lived herbaceous plants (Hurry *et al.*, 1995a,b; Strand *et al.*, 1997; Loveys *et al.*, 2003; Atkin *et al.*, 2005). This form of acclimation includes physical changes to the leaves themselves; leaves developed in the cold tend to be thicker, with a higher leaf mass per area and higher nitrogen and protein concentrations than leaves developed at warmer temperatures (Ryan, 1995; Tjoelker *et al.*, 1999a; Hurry *et al.*, 2002). Although the impact of development in the cold on mitochondrial protein abundance is not known, it has been shown that cold-developed *Arabidopsis thaliana* leaves exhibit increased mitochondrial density and an increase in the density of cristae within mitochondria; this is accompanied by concomitant increases in respiratory capacity and respiratory rate (Armstrong *et al.*, 2006). Cold stress and acclimation also alter the transcript and protein abundance of most of the Calvin cycle enzymes (Stitt & Hurry, 2002; Goulas *et al.*, 2006). The abundance of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) increases, whereas the other Calvin cycle enzymes tend to decline with cold acclimation relative to Rubisco (Strand *et al.*, 1999; Goulas *et al.*, 2006). Recovery of sucrose synthesis further assists in the recovery from cold stress and photoinhibition (Stitt & Hurry, 2002; Strand *et al.*, 2003); sucrose-phosphate synthase (SPS) transcript abundance, protein and the activity of the enzyme increases relative to the Calvin cycle and starch synthesis during acclimation (Strand *et al.*, 1997, 1999; Stitt & Hurry, 2002). Collectively, such changes result in recovery of both *A* and *R* rates in leaves of herbaceous plants that develop at low growth temperatures.

Homeostasis is the most pronounced example of acclimation, where metabolic processes acclimate to the point that the rate at the new temperature is the same as it originally was at the initial temperature (Stitt & Hurry, 2002; Atkin & Tjoelker, 2003). Although there is growing evidence that the acclimation processes already described may lead to homeostasis of *R* and *A* in plants exposed to a range of moderate growth temperatures (e.g. 15–25°C), less is known about the extent to which homeostasis of *A* and *R* is achieved when individual plants experience a wider range of growth temperatures (e.g. < 10°C). Moreover, while there is evidence that the *R*: *A* ratio is restored, following acclimation, to a moderate temperature range (Gifford, 1995; Dewar *et al.*, 1999; Loveys *et al.*, 2002, 2003; Atkin *et al.*, 2005, 2006), it is not known whether constant *R*: *A* ratios are exhibited by leaves acclimated to a wider range of temperatures.

Several studies have shown that contrasting species differ in their capacity for acclimation; while some species acclimate strongly (particularly fast-growing herbaceous species) others seem incapable of acclimation (Berry & Björkman, 1980; Larigauderie & Körner, 1995; Tjoelker *et al.*, 1998; Xiong *et al.*, 2000). Tjoelker *et al.* (1999b) found that broad-leaved tree species exhibited a lower degree of acclimation of leaf *R* than selected conifer species, suggesting that acclimation might differ among functional groups. Moreover, a high degree of thermal acclimation is exhibited by herbaceous species, as reported in several studies (Hurry *et al.*, 1995a; Strand *et al.*, 1999; Bunce, 2000; Yamasaki *et al.*, 2002; Talts *et al.*, 2004; Yamori *et al.*, 2005). However, at present it is not known whether systematic differences in thermal acclimation potential occur among species representative of different functional groups. Moreover, it is not known whether the biochemical changes associated with cold acclimation that occur in a small number of herbaceous species (e.g. Arabidopsis and winter wheat) also occur in a wider range of plant species.

In the current study, leaf gas exchange, fluorescence and western blot analysis were combined to assess the extent to which contrasting plant species belonging to three different functional groups (forbs, grasses and evergreen trees/shrubs) differed in their ability to acclimate to a broad range of growth temperatures (7, 14, 21 and 28°C). The acclimation responses of PE and ND leaves were assessed. Our objectives were as follows: to determine whether acclimation results in the homeostasis of R : A ratios in all functional groups; and to explore the biochemical underpinnings of the acclimation response of R and A in these different functional groups. Previous studies assessing acclimation in contrasting species/functional groups have been limited to a narrow range of growth temperatures (e.g. 18–28°C in Loveys *et al.*, 2003); our study provides the first comprehensive analysis of thermal acclimation in wild plant species that includes responses to both low and high growth temperatures.

Materials and Methods

Plant material and treatments

Nineteen species, representing a range of growth forms, were chosen: grasses (*Bromus ramosus*, *B. erectus*, *Poa trivialis* L., *P. costiniana* J. Vickery); forbs (*Achillea millefolium*, *A. ptarmica*, *Plantago major*, *P. euryphylla*, *Silene dioica*, *S. uniflora*, and the single species *Arabidopsis thaliana* (ecotype Ost-0)); and evergreen shrubs and trees (*Acacia melanoxylon* R. Br., *A. aneura* R. Muell Ex Benth, *Cistus ladanifer* L., *C. laurifolius* L., *Eucalyptus dumosa*, *E. delegatensis*, *Quercus suber* and *Q. ilex* ssp. ballota). Details of the origin and natural distribution of some of these species can be found in Loveys *et al.* (2002): the rest can be found in Supplementary material Table S1. All species were placed in a glasshouse maintained at 25 ± 2°C/20 ± 2°C (day/night); a 16-h d was achieved using 400-W high-pressure sodium bulbs. Once roots had reached a sufficient length, plants were removed from the growth medium, the roots were washed and the plants were transferred to 17-l hydroponics tanks containing fully aerated modified Hoagland's nutrient solution (Poorter & Remkes, 1990). The tanks were placed in growth cabinets (Snijders Microclima 1750; Snijders Scientific BV, Tilberg, the Netherlands) at a constant 21°C, 16-h d, with 300 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (i.e. providing daily quanta input of 17.3 mol m^{-2} d^{-1}) provided by fluorescent tubes. After establishment at 21°C, some plants of each species were transferred to matched cabinets maintained at 7, 14 and 28°C. These conditions were maintained until new, fully expanded leaves had developed (30 or 60 d, depending on the growth rate of individual species). Some plants were transferred back to 21°C at this time. One cabinet was used for each temperature treatment, and plants were rotated through, with two to four species in each chamber at a time. Irradiance values within the four matched growth cabinets were closely monitored and adjusted, where necessary, to ensure equal values in each cabinet; similarly, humidity was maintained at 60% in all cabinets.

Gas exchange measurements

Light-saturated photosynthesis (A_{sat}) and dark leaf R were measured on fully expanded leaves using a Li-Cor 6400 infra-red gas analyser (Li-Cor BioSciences, Lincoln, NE, USA). Leaves were exposed to a CO_2 concentration of 400 ppm (using the built-in Li-Cor 6400 CO_2 controller). A_{sat} was measured at 1500 μmol m^{-2} s^{-1}, after it had been determined that this light level was saturating, but not damaging, to all species. All measurements took place on leaves that had been exposed to at least 2 h of daytime illumination. The plants were covered with a black cloth for 30 min before the measurement of R; R was stable thereafter. When leaves were measured at a temperature different from their growth temperature, plants were transferred to the new temperature 1 h before measurements. Species grown at all four temperatures, but measured only at a set temperature (21°C), were: *S. dioica*, *S. uniflora*, *B. ramosus*, *B. erectus*, *Q. suber*, *Q. ilex* and *A. thaliana*. All other species were measured at 21°C (all treatments) and 7°C (7- and 21°C-grown plants only). Measurements were made after 10 d at treatment temperatures on PE leaves and again after ND leaves became fully expanded (*c.* 30–60 d, depending on the species and the temperature treatment; longer-lived species required more time (up to 60 d) to produce new, fully expanded leaves at low temperature than the shorter-lived herbaceous species, which never required longer than 30 d). All ND leaves were at the same stage of development, regardless of temperature treatment or species; care was taken to ensure that all leaves used were fully expanded. Plants grown at 7°C were also returned to 21°C for 1 wk and subsequently measured at 21°C. Measurements were performed on one or more leaves of three plants for each treatment, and the results were averaged.

Chemical analysis

The fresh mass and leaf area of samples were recorded (leaf area measurements were performed using a Li-Cor 3100 leaf area meter; Li-Cor Inc.) and then samples were freeze-dried in an Edwards EF4 Modulyo freeze-drier (Northern Scientific, York, UK). After the dry mass (DM) had been recorded, the replicate plant parts were pooled at each harvest (necessary because of the small mass of the individual leaf samples), ground to a fine powder using a hammer mill (31-700 Hammer Mill; Glen Creston, London, UK) and analysed by mass spectrometry (NA2100 Brewanalyser; CE Instruments, ThermoQuest Italia S.p.A, Milan, Italy) for total N concentration (Loveys *et al.*, 2003).

Soluble sugars were extracted from leaf tissue by hot ethanol extraction, and starch was extracted from the pellet (Loveys *et al.*, 2003). The glucose, fructose and sucrose contents were

then determined enzymatically using the method of Scholes *et al.* (1994).

Chlorophyll *a* fluorescence

Fluorescence was performed using the Waltz mini-PAM (Waltz, Effeltrich, Germany). Variable over maximal fluorescence (F_V/F_M) measurements were carried out on plants dark-adapted for at least 30 min. More extended dark treatments, whereby F_V/F_M was repeatedly measured, demonstrated that further dark adaptation was not required (data not shown). Reaction centre closure was achieved by applying a 0.8 s pulse of saturating light. F_V/F_M was calculated as the variable over the maximum fluorescence (Genty *et al.*, 1989).

Western blots

Protein concentrations were measured in leaves of several selected herbaceous species (*A. millefolium*, *A. ptarmica*, *A. thaliana*, *B. ramosus*, *B. erectus*, *P. costiniana*, *S. dioica* and *S. uniflora*). Leaf samples were snap frozen and ground at the temperature of liquid nitrogen (196°C) , and 50 mg of frozen tissue was solubilized by adding 1 ml of extraction buffer (65 mM Tris, pH 8.4, 11% glycerol, 4% sodium dodecyl sulfate (SDS), 1.7% β-mercaptoethanol), heated at 95°C for 10 min, centrifuged at 20 000 ***g*** and 18 μl of the supernatant was used for SDS-polyacrylamide gel electrophoresis. For comparisons of relative protein content, separation of extracted proteins was carried out using denaturing 10% or 4–12% NuPAGE Novex Bis-Tris polyacrylamide gels and 2-morpholinoethane sulfonic acid (MES)-SDS running buffer (Novex, San Diego, CA, USA). After electrophoresis, the proteins were transferred to a poly (vinylidene difluoride) (PVDF) membrane (Immun-Blot; Bio-Rad, Hercules, CA, USA). Commercially available primary antibodies (Agrisera, Vännäs, Sweden) were used to detect Rubisco (large subunit – derived from rabbit, diluted 1 : 50 000 for the primary and 1 : 50 000 for the secondary anti-chicken immunoglobulin), photosystem II (PSII) (D1 protein derived from chicken, diluted 1 : 25 000 for the primary and 1 : 50 000 for the secondary anti-chicken immunoglobulin), ATPase (β-subunit, derived from chicken, diluted 1 : 25 000 for the primary and 1 : 50 000 for the secondary anti-chicken immunoglobulin) and cytochrome oxidase (COX, subunit II, raised in rabbit, diluted 1 : 1000 for the primary and 1 : 10 000 for the secondary anti-rabbit immunoglobulin). The mitochondrial AOX was detected using a monoclonal antibody raised against the *Sauromatum guttatum* AOX (Elthon *et al.*, 1989; Gray *et al.*, 2004, used at a 1 : 500 dilution for the primary and a 1 : 500 dilution for the secondary anti-mouse immunoglobulin). Blots were immunostained with polyclonal chicken, rabbit or mouse antibodies, as required, and visualized using enhanced chemiluminescence (ECL; Amersham Biosciences Ltd, Bucks., UK) or the Pierce SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL, USA). The X-ray film was developed, scanned and analysed using Adobe Photoshop 5.5 (Adobe Systems Inc., San Jose, CA, USA) and ImageJ (US National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data were tested for normality and homogeneity of variance using a Shapiro–Wilk test and Levene's test in JMP version 4.0.4 (SAS Institute, Cary, NC, USA). Two way analysis of variance (ANOVA) was performed; where appropriate, overall means were differentiated using a one-way ANOVA followed by the Bonferroni posthoc test performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Correlation analysis for blots was performed using the Fit Model function of JMP, which performs a multiple regression based upon the general linear model. Data were divided by leaf developmental stage, and functional group was included as a factor in the analysis – although it did not significantly affect the trends in the data, it did account for a large amount of variation that otherwise masked trends.

Results

Responses of the different functional groups

The functional groups selected (forbs, grasses and evergreen trees/shrubs) were chosen to represent a wide range of leaf mass per area (LMA), *R* and *A* in order to test the effects of this range of LMA on the temperature acclimation of *R* and *A*. Our results confirm that the range of species selected were appropriately broad because functional group was highly correlated with *R*, whether measured on a DM or an area basis, and A_{sat} was highly correlated with functional group on a DM basis (Table 1). Functional group explained as much as 30% of the variation in these data.

To assess whether contrasting functional groups differed in their ability to thermally acclimate *R* and *A*, we measured rates of leaf gas exchange of plants grown under temperatures ranging from 7 to 28°C, in PE and ND leaves at the shifted temperature. The data were expressed relative to 21°C controls to provide an index of temperature acclimation. On this relative basis, plant functional group had no significant effect on the temperature responses of A_{sat} and *R* when measured at 21°C (Table 1, Fig. 1), indicating that, however different the absolute rates of A_{sat} and *R*, the temperature acclimation response of A_{sat} and *R* was very similar across the groups of species tested. The $R^2_{adj.}$ of functional group for all the parameters measured (F_V/F_M, $R : A_{sat}$, and A_{sat} at growth temperature and 21°C, *R* at growth temperature and 21°C, leaf mass per area, nitrogen content, soluble sugars, total nonstructural carbohydrates (TNC) and stomatal conductance, all relative to plants grown

Table 1 Statistical data (R^2_{adj}, *F*-value and *P*-value) on the correlation between functional group and leaf parameters

Parameter	$R^2_{adj.}$	*F*-value	*P*-value
A_{sat}, area basis	0.023	2.59	0.078
R, area basis	0.14	11.8	< 0.0001
A_{sat}, DM basis	0.29	31.3	< 0.0001
R, DM basis	0.26	26.5	< 0.0001
A_{sat}, area basis, relative to 21°C	0.008	1.54	0.56
R, area basis, relative to 21°C	0.022	2.52	0.084
A_{sat}, DM basis, relative to 21°C	0.000	0.85	0.43
R, DM basis, relative to 21°C	0.042	3.97	0.021
F_V/F_M at 21°C	0.034	2.30	0.104
LMA relative to 21°C	0.002	1.16	0.32
N, area basis, relative to 21°C	−0.006	0.56	0.57
soluble sugars, area basis, relative to 21°C	0.046	4.36	0.015
TNC, area basis, relative to 21°C	0.041	3.95	0.022
gs, area basis, relative to 21°C	−0.011	0.24	0.78
A_{sat}, area basis, 7 vs 21, relative to 21°C	0.010	1.29	0.28
R, area basis, 7 vs 21 relative to 21°C	0.020	1.58	0.22
R/A_{sat}, 7 vs 21	0.061	2.60	0.085

Data on *R* and A_{sat} are first expressed directly, to show the strong effect of functional group on the absolute rates of these parameters. All other data are expressed relative to 21°C data to eliminate species effects on absolute values and evaluate the effect of functional group on relative changes. All values presented here are a result of regression correlations performed in JMP.
A_{sat}, photosynthetic carbon assimilation at 1500 μmol mol^{-1} photons (saturating light); DM, dry mass; F_V/F_M, variable over maximal fluorescence; gs, stomatal conductance; LMA, leaf mass per area; N, nitrogen; *R*, leaf dark respiration; TNC, total nonstructural carbohydrates.

at 21°C) was always less than 0.05, meaning that it explained < 5% of the variance in the data (Table 1). In none of the parameters measured was the effect of functional group significant at $P < 0.01$. When two-way ANOVAs were performed, in none of these parameters was the interaction between temperature and functional group significant at $P < 0.01$ (Table S3). We therefore concluded that species belonging to these different functional groups show similar acclimation trends in response to short- and long-term changes in their thermal environment, and in our subsequent analyses we pooled the responses of the 19 test species to see if it was possible to define general principles in the thermal response of leaf *A* and *R*.

Leaf mass per area, leaf nitrogen and carbohydrates

Functional group as a factor had little effect on the temperature responsiveness of leaf physical parameters (Table 1, Supplementary material Fig. S1). As expected, LMA was unaffected by temperature after 10 d under different temperature regimes but was increased in leaves that developed at low temperature (Table 2). Owing to the intended large variation in LMA of warm-grown control leaves among the species chosen (trees and shrubs generally have a high LMA, whereas forbs and grasses tend to have a low LMA), this increase was only significant when the data were expressed relative to 21°C-grown control plants (Table 2) and functional group had no significant effect on the normalized data (Table 1). The temperature response of the absolute concentration per unit leaf area of N was very similar to that of absolute LMA (the correlation of N vs LMA was $R^2_{adj.} = 0.60$), with no change in PE leaves and an increase in ND leaves at low temperature (Table 2).

Soluble sugar concentrations were significantly ($P < 0.05$) and negatively affected by increasing temperature (Table 2). This was particularly pronounced in the ND leaves, with the soluble sugar concentration in plants grown at 7°C being 1.5–3 times higher than in plants grown at 21°C. The TNC concentration showed a pattern similar to those of soluble sugars, but the positive effect of exposure to 7°C on TNC concentration was even more pronounced (Table 2, Supplementary material Fig. S1). Functional group did have a significant effect on the relative change of these parameters in response to temperature – forbs, in general, accumulated more sugars at low temperature. However, this effect explained < 5% of the variance in the data (Table 1).

Thermal acclimation of A_{sat} and *R*: measurements made at a set temperature (21°C)

Because the temperature responses of *A* and *R* and F_V/F_M were not influenced by functional group (Table 1), we used the combined collection of species to assess the extent to which general principles could be defined in the temperature acclimation of *A* and *R*. Table 3 shows the interspecies average of absolute rates of gas exchange for all species pooled, with

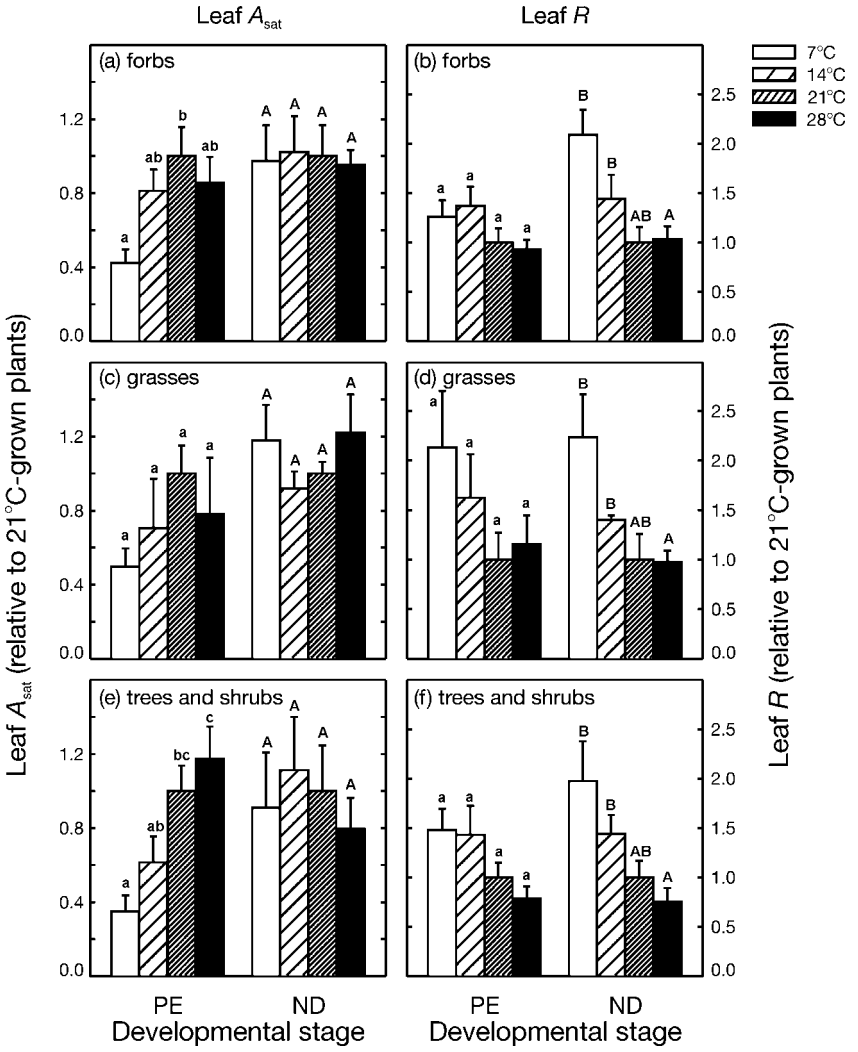

Fig. 1 Relative rates of net, area-based light-saturated photosynthesis (A_{sat}) and leaf dark respiration (R) in pre-existing (PE, 21°C-grown leaves that were exposed to 7, 14, 21 or 28°C for 10 d before measurement) and newly developed (ND, leaves that developed under the four growth temperature treatments before measurement) leaves. Relative rates were calculated for each species individually and then averaged. In all cases, rates of CO_2 exchange were measured at 21°C, irrespective of the growth temperature treatment. In all cases, values are expressed as a proportion of rates exhibited by controls maintained at 21°C. Values are the mean of four to eight species (± standard error). Different letters within a leaf developmental stage indicate statistical differences at $P < 0.05$, as a result of posthoc Bonferroni analysis within a one-way analysis of variance (ANOVA).

relative rates of leaf gas exchange being shown in Fig. 2 (where values at each growth temperature are expressed as a proportion of the rates exhibited by 21°C control plants). For the pooled species, a 10-d 7°C treatment of PE leaves resulted in a significant decline in the average rates of A_{sat} exhibited by PE leaves, when measured at the common temperature of 21°C (Table 3, Fig. 2, Supplementary material Table S2). For example, the mean rates of A_{sat} exhibited by the PE leaves of 7°C-treated plants were approx. 40% of those of the 21°C-grown controls. In addition, the pooled F_V/F_M was significantly depressed (Fig. 3). For PE leaves exposed to a temperature of 14°C for 10 d, the average rates of A_{sat} were 50–80% of those of the 21°C-grown controls, but F_V/F_M was unaffected at this temperature. Exposure of PE leaves to 28°C for 10 d had no significant effect on 21°C-measured values of A_{sat} or F_V/F_M.

After new leaves formed at each growth temperature (i.e. at 7, 14, 21 and 28°C), the rates of A_{sat} measured at 21°C on an area basis were recovered in 7°C-treated plants such that there was no significant difference between temperature treatments when measured at 21°C (Fig. 2). This recovery was only complete when A_{sat} was measured on an area basis; on a DM basis, 7°C ND leaves had A_{sat} levels 40–60% lower than the 21°C controls (Table 3). Increased LMA and N per area (Table 2), may thus be partially responsible for the recovery seen in A_{sat} on an area basis. F_V/F_M, in contrast, which was depressed in PE leaves after 10 d at 7°C, recovered in the ND leaves, correlating well with the recovery of A_{sat} in the ND leaves (Fig. 3).

In contrast to A_{sat}, 21°C-measured rates of leaf R on an area basis in PE leaves increased, rather than decreased, following 10 d of exposure to 7 and 14°C (Fig. 2), with the increase in R being greater in 7°C-treated plants (here, the rates were 60% higher than in 21°C-grown controls) than plants treated with 14°C for 10 d (the rates were 50% higher than in the 21°C-grown controls). Exposure to 28°C had no significant effect on rates of leaf R of PE leaves (compared with 21°C-grown controls). The leaf R at 21°C of ND leaves formed at 7°C was twice as high as controls (Fig. 2). Increased leaf

Table 2 Leaf dry mass per area (LMA), and levels of nitrogen (N), soluble sugars, total nonstructural carbohydrates (TNC) and stomatal conductance (gs), in pre-existing (PE; 21°C-grown leaves that were exposed to 7, 14, 21 or 28°C for 10 d before measurement) and newly developed (ND; leaves that developed under the four growth temperature treatments before measurement) developmental stages

		Growth temperature			
Leaf trait	Developmental stage	7°C	14°C	21°C	28°C
LMA	PE	61.27 ± 7.88^{b}	57.61 ± 8.37^{a}	51.67 ± 7.69^{a}	51.25 ± 7.33^{a}
($g\ m^{-2}$)	ND	82.27 ± 13.48^{A}	77.73 ± 13.46^{A}	59.95 ± 9.12^{A}	65.28 ± 9.61^{A}
LMA	PE	1.27 ± 0.08^{b}	1.18 ± 0.05^{ab}	1.00 ± 0^{a}	1.03 ± 0.06^{a}
(relative to 21°C)	ND	1.73 ± 0.15^{C}	1.32 ± 0.12^{B}	1.00 ± 0^{A}	1.14 ± 0.10^{A}
N concentration	PE	1.83 ± 0.19^{a}	1.60 ± 0.12^{a}	1.67 ± 0.15^{a}	1.71 ± 0.16^{a}
($g\ m^{-2}$)	ND	3.03 ± 0.40^{B}	2.52 ± 0.37^{AB}	1.88 ± 0.20^{A}	2.08 ± 0.28^{A}
N concentration	PE	1.16 ± 0.09^{a}	1.06 ± 0.07^{a}	1.00 ± 0^{a}	1.06 ± 0.08^{a}
(relative to 21°C)	ND	1.82 ± 0.20^{B}	1.40 ± 0.13^{B}	1.00 ± 0^{A}	1.17 ± 0.11^{AB}
Soluble sugars	PE	4.12 ± 0.53^{a}	3.28 ± 0.40^{a}	3.07 ± 0.47^{a}	2.96 ± 0.43^{a}
($g\ m^{-2}$)	ND	5.64 ± 0.84^{B}	4.56 ± 0.70^{AB}	3.21 ± 0.47^{A}	3.28 ± 0.48^{A}
Soluble sugars	PE	1.63 ± 0.21^{b}	1.23 ± 0.08^{ab}	1.00 ± 0^{a}	1.02 ± 0.08^{a}
(relative to 21°C)	ND	2.57 ± 0.42^{C}	1.73 ± 0.17^{B}	1.00 ± 0^{A}	1.07 ± 0.09^{A}
TNC	PE	9.30 ± 1.09^{b}	9.05 ± 1.33^{b}	6.65 ± 1.20^{ab}	4.84 ± 0.64^{a}
($g\ m^{-2}$)	ND	9.51 ± 1.24^{B}	7.56 ± 0.92^{AB}	4.92 ± 0.74^{A}	4.65 ± 0.64^{A}
TNC	PE	2.10 ± 0.42^{b}	1.67 ± 0.20^{ab}	1.00 ± 0^{a}	1.00 ± 0.13^{a}
(relative to 21°C)	ND	2.86 ± 0.49^{B}	2.05 ± 0.32^{B}	1.00 ± 0^{A}	0.99 ± 0.09^{A}
gs	PE	0.07 ± 0.02^{a}	0.11 ± 0.02^{ab}	0.16 ± 0.03^{ab}	0.17 ± 0.04^{b}
($mmol\ m^{-2}\ s^{-1}$)	ND	0.11 ± 0.02^{A}	0.18 ± 0.03^{A}	0.19 ± 0.04^{A}	0.16 ± 0.03^{A}
gs	PE	0.52 ± 0.10^{a}	0.79 ± 0.13^{ab}	1.00 ± 0^{ab}	1.29 ± 0.23^{b}
(relative to 21°C)	ND	0.64 ± 0.14^{A}	1.00 ± 0.11^{AB}	1.00 ± 0^{AB}	1.17 ± 0.34^{B}

Nitrogen values are corrected for TNC. Values are the mean of 19 species (± standard error), with all functional groups combined for values relative to 21°C, values were normalized for each species individually and then averaged overall. Different letters within a leaf developmental stage indicate statistical differences at $P < 0.05$, as a result of a Bonferroni posthoc test, following a one-way analysis of variance.

mass content per unit area may play some part in this, but all functional groups still showed elevated *R* of 7°C-grown plants at 21°C when *R* was expressed on a DM basis (Table 3).

Collectively, the data in Table 3 and Fig. 2 highlight the contrasting responses of A_{sat} and *R* (measured at a set temperature) to short- and long-term changes in growth temperature, with development of new leaves being crucial to the recovery of A_{sat} on an area basis and the largest increase in *R* in plants grown at 7°C. The general trends observed for A_{sat} and *R*, described above, were similar for the three functional groups examined (Fig. 1, Supplementary material Table S2).

Thermal acclimation: measurements made at each respective growth temperature and the balance between *R* and A_{sat}

Functional group as a factor had little effect on the temperature responsiveness of A_{sat} and *R* measured at growth temperature. To assess the extent of homeostasis of leaf gas exchange when measured at each new growth temperature, we measured the rates of A_{sat} and leaf *R* in 21°C control plants at 21°C and in 7°C-treated plants at 7°C (Fig. 4). After 10 d at 7°C, PE leaves measured at 7°C exhibited rates of A_{sat} that were significantly lower than those of PE leaves kept at 21°C, with 7°C-treated and -measured rates being, in general, 25% of the rates exhibited by 21°C control plants (Fig. 4). The formation of ND leaves resulted in the 7°C measured rates of A_{sat} recovering to nearly three-quarters of 21°C control rates (Fig. 4). This resulted in an average increase of nearly three-fold, with some species increasing more than five-fold (*A. melanoxylon* and *C. ladanifer*, Supplementary material Table S2).

The rates of leaf *R* were also reduced in 10-d 7°C-treated and -measured plants in comparison with those at 21°C (Fig. 4); in PE leaves, the rates of leaf *R* at 7°C were 50% of those of the plants in the control treatment, with further recovery occurring once ND leaves formed at each growth temperature. Overall, these data paralleled the changes observed at a set measuring temperature of 21°C – the recovery of A_{sat} at 7°C is less complete than the recovery of *R*.

Because of the asynchrony in the response of A_{sat} and *R* to temperature, the balance between A_{sat} and *R* was significantly affected by changes in growth temperature (Figs 5 and 6), although not by functional group (Table 1). In PE leaves grown and measured at 7°C, A_{sat} was reduced to a much greater extent than *R*, although it did recover substantially in ND leaves at 7°C (Fig. 5). To assess what impact shifts to cold and back to the warm had on the balance between *R* and A_{sat}, we calculated the ratios of leaf *R* to A_{sat} for 21°C-developed/measured PE leaves (at day 10), 21°C-developed PE leaves

Table 3 Light-saturated photosynthesis (A_{sat}), and leaf dark respiration (R) in pre-existing leaves (PE) after 10 d under the new temperature regime and in leaves newly developed at the new temperature (ND)

	Pre-existing leaves				Newly developed leaves			
	7°C	14°C	21°C	28°C	7°C	14°C	21°C	28°C
A_{sat} (μmol of CO_2 m^{-2} s^{-1})	6.35 ± 0.81^{a}	11.51 ± 1.32^{b}	15.34 ± 1.37^{b}	15.3 ± 1.3^{b}	14.44 ± 1.79^{A}	14.8 ± 1.85^{A}	14.32 ± 1.7^{A}	13.08 ± 1.23^{A}
A_{sat} (μmol kg^{-1} of DM s^{-1})	124.63 ± 22.75^{a}	238.64 ± 43.10^{ab}	357.22 ± 54.03^{b}	311.33 ± 36.07^{b}	205.26 ± 41.72^{A}	229.87 ± 34.77^{A}	300.83 ± 51.34^{A}	255.09 ± 40.77^{A}
A_{sat} (rel. to 21°C, DM basis)	0.35 ± 0.04^{a}	0.63 ± 0.05^{b}	1.00 ± 0^{c}	1.02 ± 0.09^{e}	0.56 ± 0.07^{A}	0.72 ± 0.07^{A}	1.00 ± 0^{B}	0.89 ± 0.13^{B}
R (μmol of CO_2 m^{-2} s^{-1})	1.59 ± 0.15^{b}	1.54 ± 0.18^{b}	1.07 ± 0.11^{ab}	0.95 ± 0.08^{a}	2.39 ± 0.32^{C}	1.78 ± 0.23^{B}	1.30 ± 0.18^{AB}	1.09 ± 0.13^{A}
R (μmol kg^{-1} of DM s^{-1})	24.65 ± 3.41^{a}	24.89 ± 3.96^{a}	21.61 ± 3.37^{a}	18.64 ± 2.62^{a}	28.12 ± 2.31^{B}	23.15 ± 1.92^{AB}	21.40 ± 3.54^{AB}	16.99 ± 2.17^{A}
R (rel. to 21°C, DM basis)	1.28 ± 0.11^{b}	1.17 ± 0.08^{ab}	1.00 ± 0^{ab}	0.93 ± 0.07^{a}	1.40 ± 0.19^{B}	1.07 ± 0.12^{AB}	1.00 ± 0^{A}	0.88 ± 0.16^{A}

In all cases, the rates of CO_2 exchange were measured at 21°C, irrespective of the growth temperature treatment. Values are the mean of 15–19 species (± standard error); three replicates were averaged to provide individual species data, and these averages were then used to calculate overall means. Different letters within a functional group indicate statistical differences at $P < 0.05$, as a result of a one-way analysis of variance followed by the Bonferroni posthoc test.

Fig. 2 Relative area-based rates of (a) light-saturated photosynthesis (A_{sat}) and (b) leaf dark respiration (R) of pre-existing (PE, 21°C-grown leaves that were exposed to 7, 14, 21 or 28°C for 10 d before measurement) and newly developed (ND, leaves that developed under the four growth temperature treatments before measurement) leaves. Relative rates were calculated for each species individually and then averaged. In all cases, rates of CO_2 exchange were measured at 21°C, irrespective of the growth temperature treatment, and values are expressed as a proportion of rates exhibited by controls maintained at 21°C. Values are the mean of 19 species (± standard error). Different letters within a leaf developmental stage indicate statistical differences at $P < 0.05$, as a result of posthoc Bonferroni analysis within a one-way analysis of variance (ANOVA).

exposed to 7°C for 10 d (measured at 7°C), 7°C ND leaves (measured at 7°C) and finally 7°C ND leaves transferred back to 21°C for 7 d (and measured at 21°C) (Fig. 6). Exposure of 21°C-developed leaves to 7°C for 10 d resulted in $R : A_{sat}$ ratios increasing approx. three-fold. Although significant decreases in $R : A_{sat}$ occurred in 7°C ND leaves, $R : A_{sat}$ remained about twice that of the 21°C control leaves. However after 1 wk at 21°C, the $R : A_{sat}$ of 7°C ND leaves was identical to that of the 21°C controls (Fig. 6).

Photosynthetic and respiratory proteins

To investigate the biochemical changes that underpin the asynchrony in the acclimation potential of A and R, we

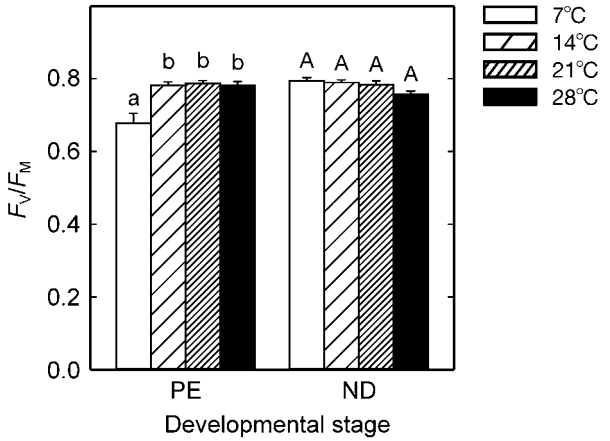

Fig. 3 Variable over maximal fluorescence (F_V/F_M) in pre-existing (PE, 21°C-grown leaves that were exposed to 7, 14, 21 or 28°C for 10 d before measurement) and newly developed (ND, leaves that developed under the four growth temperature treatments before measurement) leaves. Values are the mean of 19 species (± standard error). Different letters within a graph indicate statistical differences at $P < 0.05$, as a result of posthoc Bonferroni analysis within a one-way analysis of variance (ANOVA).

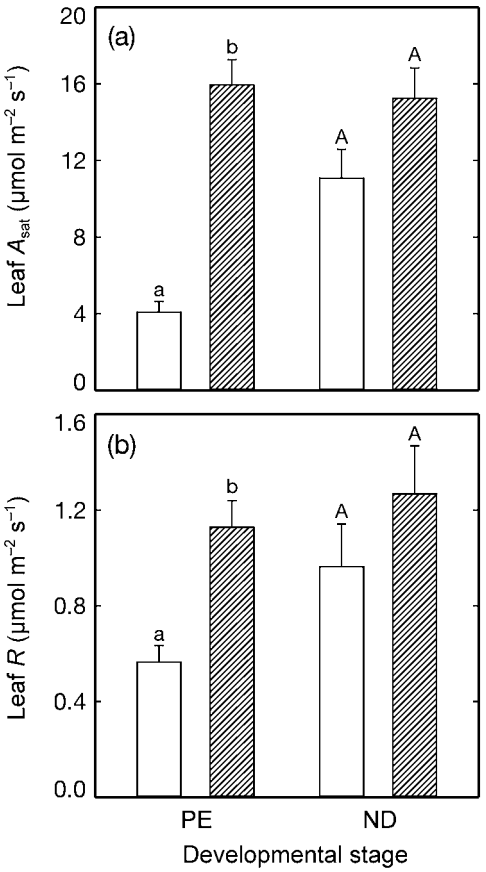

Fig. 4 Area-based rates (a) light-saturated photosynthesis (A_{sat}) and (b) leaf dark respiration (R) in pre-existing (PE, 21°C-grown leaves that were exposed to 7, 14, 21 or 28°C for 10 d before measurement) and newly developed (ND, leaves that developed under the four growth temperature treatments before measurement) leaves. Measurements were made at the growth temperature (i.e. 7- and 21°C-treated plants at 7°C (open bars) and 21°C (hatched bars), respectively). Values are the mean of 15 species (± standard error). Different letters within a graph indicate statistical differences at $P < 0.05$, as a result of posthoc Bonferroni analysis within a one-way analysis of variance (ANOVA).

measured the concentrations of three chloroplastic (the β-subunit of ATPase, the D1-subunit of PSII and the large subunit of Rubisco) and two mitochondrial (subunit II and alternative oxidase of cytochrome oxidase) proteins in extracts of leaves from several forb and grass species (Fig. 7). Values were expressed relative to abundance in leaves of 21°C-grown control plants for both PE and ND leaves. Exposure of 21°C-grown plants to 7°C for 10 d resulted in a decline in the relative abundance of the D1 subunit of PSII when compared with 21°C-grown control plants; the downward trend with temperature was significant ($P = 0.005$, ANOVA F-ratio = 8.87; Fig. 7b), although the scatter in the data set was too large to resolve differences between specific temperatures. By contrast, the relative abundance of the large subunit of Rubisco in PE leaves increased nearly three-fold when plants were exposed to 7°C for 10 d. The amount of ATPase β-subunit was not significantly affected by temperature after 10 d. After the development of new leaves at low temperature, the abundance of the D1 subunit of PSII in plants grown at 7°C recovered to control levels (Fig. 7b) and the abundance of the ATPase β-subunit was higher at low temperature than at high temperature ($P = 0.05$, ANOVA F-ratio = 4.23, Fig. 7a). The abundance of the Rubisco large subunit remained significantly higher in ND leaves of the colder treatments ($P = 0.035$, ANOVA F-ratio = 4.9, Fig. 7c), although was not as high as in PE leaves.

Changes in growth temperature had little effect on the relative abundance of COX in either the PE or the ND leaves (Fig. 7d). This contrasted with the large increases in relative abundance of AOX that occurred when PE leaves were shifted to the cold and when ND leaves formed in the cold (Fig. 7e). The relative AOX abundance increased by 50% in 7°C-grown plants after 10 d of treatment and by > 300% in the ND leaves at 7°C. This increase at low temperature was significant overall ($P = 0.016$, ANOVA F-ratio = 5.04). Not all individual species followed the general trend: *Arabidopsis* showed the reverse of the response of all other species, with an increase in COX and no change in AOX (Supplementary material Table S2).

Taken together, the data in Fig. 7 show that associated with the decline and subsequent recovery of 21°C-measured rates of A_{sat} (for 7°C-treated plants) in forbs and grasses (in PE and ND leaves, respectively; Fig. 2) was a decrease and recovery of thylakoid membrane proteins, with relative amounts of the Rubisco large subunit increasing in abundance in both leaf types (relative to 21°C controls). At the same time, there was

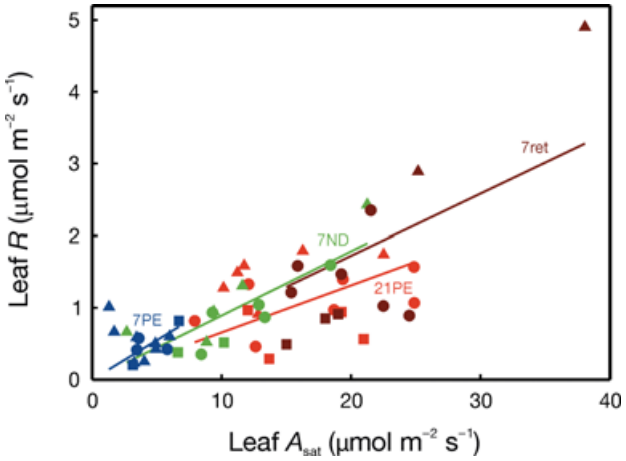

Fig. 5 Rates of leaf dark respiration (R) to light-saturated photosynthesis (A_{sat}) at 21°C and 7°C growth temperatures for differing time-periods. Treatments: red, 21PE, grown and measured at 21°C on day 10; blue, 7PE, 21°C developed leaves that were shifted to 7°C for 10 d and then measured at 7°C; green, 7ND, leaves newly developed at 7°C, measured at 7°C. Dark red, 7ret, leaves newly developed at 7°C, transferred to 21°C for 1 wk and measured at 21°C. Triangles, trees and shrubs; squares, grasses; circles, forbs. Regression lines are constrained to pass through the origin.

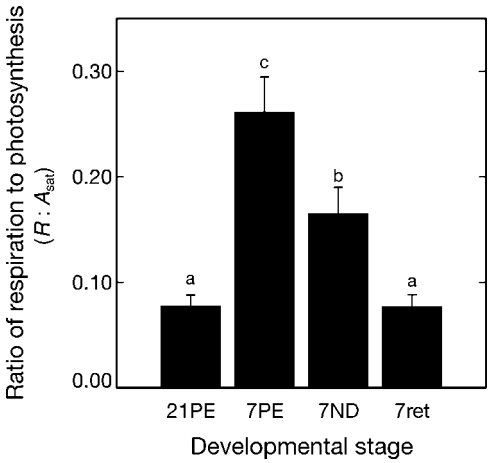

Fig. 6 Ratio of leaf dark respiration to light-saturated photosynthesis ($R : A_{sat}$) for leaves exposed to 21°C and 7°C growth temperatures for different time-periods. Treatments: 21PE, grown and measured at 21°C on day 10; 7PE, 21°C developed leaves that were shifted to 7°C for 10 d and then measured at 7°C; 7ND, leaves newly developed at 7°C, measured at 7°C; 7ret, leaves newly developed at 7°C, transferred to 21°C for 7 d and measured at 21°C. Values are the mean of 15 species (± standard error). Different letters within a developmental stage indicate statistically different groups at $P < 0.05$.

a significant decline in the F_V/F_M of PE leaves followed by recovery in ND leaves (Fig. 3). Similarly, the significant increases in 21°C-measured rates of leaf R exhibited by cold-acclimated leaves (Fig. 2) were associated with an increase in the abundance of AOX, but not of COX.

Discussion

Our study encompassed a range of temperate species from three plant functional groups that varied widely in LMA, A and R rates. Despite the wide range of species, there were no major differences between groups. Previous studies have reported interspecific variation in the degree of short-term respiratory and photosynthetic thermal acclimation in leaves (Xiong *et al.*, 2000; Loveys *et al.*, 2003; Atkin *et al.*, 2006). However, a recent study reported no systematic difference of acclimation potential of root R among species and/or different N availabilities (Atkinson *et al.*, 2007). Our current study suggests that the same is true for leaf R and A in plant species representative of the three functional groups.

If the thermal acclimation of R and A_{sat} are indeed constant across functional groups, this may allow large-scale models (e.g. Cox *et al.*, 2000) to assume a constant degree of thermal acclimation among plant taxa, simplifying the inclusion of photosynthetic and respiratory acclimation. However, incorporating acclimation of R and A into models is complicated by the fact that the optimal temperature (T_{opt}) of A of the plants will shift, depending on the growth temperature. Temperature response curves for A have been published for only some of the species used in this study. *P. major* and *P. euryphylla* have a T_{opt} of A_{sat} that is generally stable in the range of 20–27°C for both species, regardless of growth regime (Atkin *et al.*, 2006). Moreover, cold-developed leaves exhibit higher rates of A_{sat} than their warm-grown counterparts, irrespective of the measurement temperature used to compare rates in cold- and warm-grown plants (Atkin *et al.*, 2006). The temperature range of T_{opt}, and the increased A_{sat} in cold-grown plants, is also seen in *A. thaliana* (Strand *et al.*, 1997). *Q. suber* has been shown to acclimate to low temperature in a similar way to most other C3 trees (Ghouil *et al.*, 2003), and *Q. ilex* shows evidence of photodamage when grown at 5°C that is manifested as a reduced photosynthetic rate when measured at 20°C (Oliveira & Peñuelas, 2004). *A. melanoxylon* suffers photoinhibition at these temperatures, similarly to *Quercus* spp. (Watson *et al.*, 2004). In the present study, photoinhibition occurred in PE leaves in most species after transfer to 7°C (Supplementary material Table S2), and this coincided with a drop in A_{sat} measured at 21°C. The recovery of A_{sat} at 21°C after the development of new leaves at 7°C coincided with the recovery of F_V/F_M (Supplementary material Table S2). No specific data were found in the literature for the other species in this study, although other *Eucalyptus* spp. have been shown to have a T_{opt} near 20°C, depending on the thermal history and age of the leaf (Battaglia *et al.*, 1996). This temperature is near the T_{opt} of many temperate species: most C3 plants have a T_{opt} in the range of 15–30°C (Larcher, 1995). Thus, all of our species for which data are available in the literature have a broad T_{opt} of A_{sat}, as predicted for C3 plants by models (Kubien & Sage, 2007). We chose to measure the plants at the common temperature of 21°C because, although there

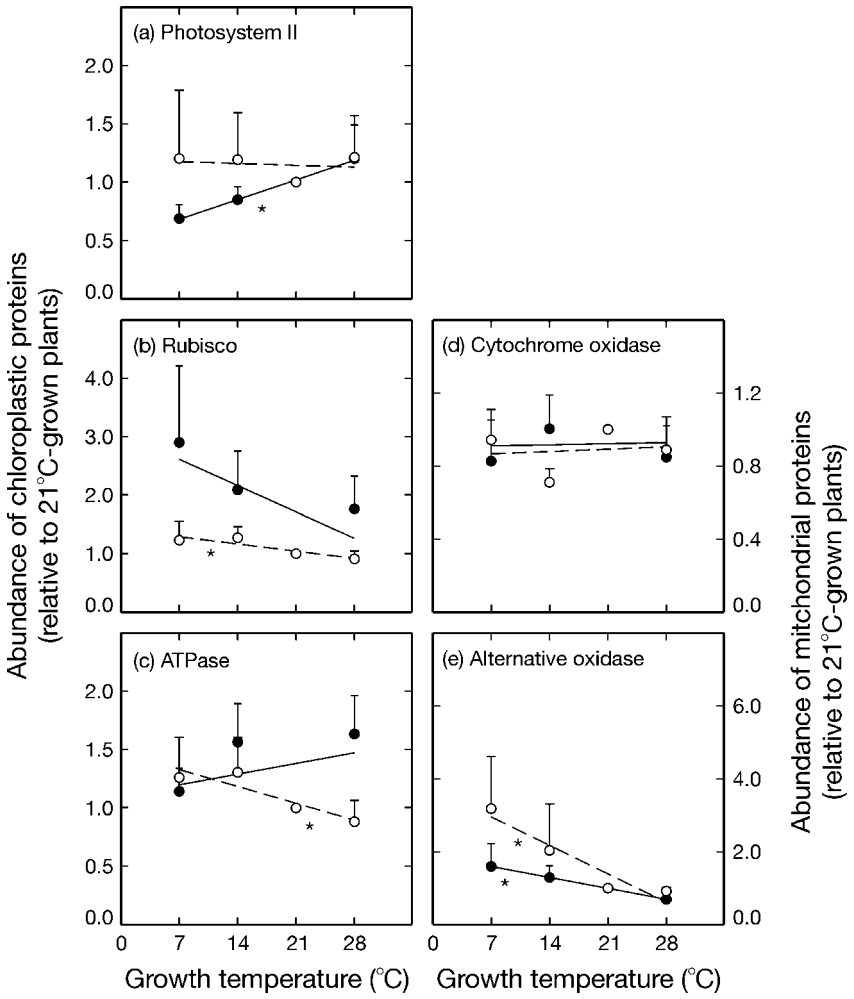

Fig. 7 Relative abundance of photosynthetic (a, b and c) and respiratory (d and e) proteins (calculated on an area basis). In all cases, abundance is expressed relative to plants grown at 21°C. PE (closed circles) leaves were those that developed at 21°C and were subsequently exposed to 7, 14, 21 or 28°C for 10 d, whereas ND (open circles) leaves are those that subsequently developed at each respective temperature. Values represent the mean of five to eight grass and forb species (± standard error). Stars represent regression lines where the trend was significantly different from 0, regressions were performed in JMP, using the general linear model. Antibodies used were as follows: (a) photosystem II, subunit a (raised in chicken; Agrisera); (b) Rubisco large subunit (raised in chicken; Agrisera); (c) ATPase β-subunit (raised in chicken; Agrisera); (d) cytochrome oxidase, subunit 2 (raised in rabbit, Agrisera); (e) alternative oxidase (AOX) (raised in mouse).

will be a shift in A_{sat} with acclimation to different growth temperatures (e.g. Strand *et al.*, 1997; Atkin *et al.*, 2006), from published data cited above, 21°C is probably close to the T_{opt} of all species used in the study. It thus possible to assess the degree of acclimation by measuring rates of gas exchange at 21°C (irrespective of the growth temperature) and normalizing the 21°C-measured rates (exhibited by 7-, 14- and 28°C-acclimated plants) to rates exhibited by 21°C-grown control plants.

Overall, the response of leaf R to long-term temperature change in the three functional groups was similar to that reported in previous studies (e.g. Loveys *et al.*, 2003), in that R generally exhibited greatest acclimation to the new growth temperature in ND leaves (Figs 1,2,4). When shifted to 7°C, the recovery of A_{sat} and R was also more complete in ND leaves compared with PE leaves exposed to the cold for 10 d (Fig. 4). Several studies have found that following the development of new leaves at low temperature, the $R:A$ ratio is restored (Ziska & Bunce, 1998; Dewar *et al.*, 1999; Loveys *et al.*, 2002, 2003; Atkin *et al.*, 2006). However, our results also showed that $R:A$ did not remain constant when plants were exposed to temperatures down to 7°C for extended periods (Fig. 6). This low temperature range probably explains the difference between this and other studies that used higher temperature ranges (e.g. Loveys *et al.*, 2003, grew plants at 18–28°C). Past studies using *Arabidopsis* have also shown that leaf-level $R:A$ fails to return to warm-grown values, even after developing new leaves at 5°C (Savitch *et al.*, 2001; Strand *et al.*, 2003). This also holds true above the normal growth range: previous studies have found that $R:A$ is unable to fully acclimate to very high average daily temperatures (Loveys *et al.*, 2003; Atkin *et al.*, 2006, 2007). The directionality of the temperature shift is also important: although even ND leaves were not able to confer full homeostasis of the $R:A$ ratio at 7°C, leaves that had developed at 7°C were capable of achieving the same $R:A$ ratio as plants grown at 21°C after only 1 wk of being moved from 7 to 21°C. Homeostasis of $R:A$ is therefore possible over a broad range of temperatures, but not beyond a certain optimum temperature range for a given species. Many current global carbon cycle models assume homeostasis of $R:A$ when predicting future CO_2 fluxes between plants and the atmosphere (e.g. Gifford,

2003); we show that this is valid, but only when plants are in the linear portion of the temperature curve (i.e. near the T_{opt}).

Biochemical underpinnings of photosynthetic acclimation

In our study, N concentration per unit mass increased in ND leaves at 7°C, paralleling the recovery of *A* (the correlation between the relative increase of N in ND leaves and the relative increase of A_{sat} in ND leaves at 7°C had an $R^2_{adj.} = 0.68$). Carbohydrates have also been shown to play a role in the regulation of *A*, either through biochemical suppression (Myers *et al.*, 1999) or through their effect on gene expression (Smeekens, 2000). We found that the accumulation of TNCs was associated with growth at low temperature in PE and ND leaves, but that soluble sugar concentration did not correlate with photosynthesis or photosynthetic acclimation ($R^2_{adj.} = 0.02$). In this experiment, the drop of *A* in PE leaves at low temperature was also not correlated with the drop in stomatal conductance, and the intercellular [CO_2] remained high enough to support *A* (Supplementary material Table S2). Thus, sugar accumulation and stomatal conductance did not appear to limit *A* directly in any of the species used for this study, either following transfer to low temperatures or during recovery.

Although the overall N concentration of the leaves increased in the cold, the extra N was not distributed equally among proteins; in the chloroplast, the response of the D1 subunit of PSII to 7°C was different from that of the ATPase β-subunit and the Rubisco large subunit. The initial drop of *A* in PE leaves (whether measured at 7 or 21°C) in this study was accompanied by a reduction in the amount of the D1 subunit of PSII. This initial drop in abundance of the D1 subunit of PSII is not surprising because growth and the consumption of reducing power are more limited than light acquisition and energy production in the cold (Boese & Huner, 1990; Ensminger *et al.*, 2006). This imbalance can cause photoinhibition; the drop in F_V/F_M observed in the PE leaves of the plants transferred to 7°C in this study is consistent with photoinhibition, and the recovery of F_V/F_M after the development of new leaves is consistent with cold acclimation relieving photoinhibition (Hurry & Huner, 1992; Gray *et al.*, 2003). The amount of the D1 subunit of PSII returned to original levels of abundance after new leaves had developed at 7°C, in concert with the acclimation of *A*. The processes of photosynthesis that are not involved directly in light capture, including the Rubisco large subunit, are more inhibited by the cold, and the amount of Rubisco large subunit protein tends to increase in ND leaves at low temperature (Hurry *et al.*, 1994; Goulas *et al.*, 2006). In this experiment, the Rubisco large subunit protein was more abundant in the PE leaves grown for 10 d at 7°C, and significantly more abundant in the leaves that developed at low temperatures. Previous studies have also shown an increase in ATPase subunits free in the stroma in PE leaves exposed to cold treatment, indicative of a loss of functional ATPase complexes following a shift to colder temperatures (Goulas *et al.*, 2006) that is consistent with a loss of photosynthetic competence. In the current study we found no significant change in the total amount of the β-subunit of ATPase after 10 d at different temperatures (Fig. 7). However, we did find the amount of the ATPase β-subunit to be significantly elevated in ND leaves in the colder treatments. This increase in amount of ATPase β-subunit in ND leaves may help to balance light capture and the more biochemical reactions of photosynthesis at low temperature. Cold temperature slows enzymes, such as ATPase and Rubisco, more than the light-capture processes of PSII, and the acclimation process increases resource allocation to the former, restoring photosynthetic flux. Thus, the increase in DM content and N per leaf area in the cold is not the result of a general increase in all proteins, but a specific acclimation response of key proteins.

Biochemical underpinnings of respiratory acclimation

Substrate availability plays a role in determining the rate of leaf *R*; however, previous experiments have not found sugar content to be important in respiratory acclimation at low temperature (snow gum, Atkin *et al.*, 2000; Arabidopsis, Talts *et al.*, 2004). We also found no direct role for sugars in respiratory control, as the respiratory rates in ND leaves were not correlated with sugar levels ($R^2_{adj.} = -0.01$, Tables 2 and 3). Leaf N was correlated with *R* in ND leaves ($R^2_{adj.} = 0.48$). However, recent work assessing acclimation of *R* in roots found that acclimation is not contingent on increases of N concentration (Atkinson *et al.*, 2007). If the same is true for leaves, then increases in *R* in cold acclimated tissues may reflect reductions in the adenylate restriction of respiratory metabolism, via increased rates of ATP turnover, increased ADP concentrations and/or uncoupling of electron transport from proton translocation across the inner mitochondrial membrane. The latter could be facilitated, in part, via increases in AOX activity. Although we did not measure AOX activity *in vivo*, we did observe increases in AOX protein abundance in cold-developed tissues of several herbaceous species (Fig. 7e). Previous studies have also reported increases in AOX protein and/or activity after long-term exposure to cold temperatures (Vanlerberghe & McIntosh, 1992; Gonzalez-Meler *et al.*, 1999). The exception to this trend in our study was Arabidopsis, which increased COX instead of AOX in the cold (Supplementary material Table S2); this is consistent with the increased cytochrome pathway activity seen in isolated Arabidopsis mitochondria (Armstrong *et al.*, 2006). As Arabidopsis is both fast-growing and cold-tolerant, it may be that increases in respiratory flux are associated with a recovery in ATP synthesis and/or use in this species, whereas in other slower-growing and/or less cold-tolerant species, recovery of respiration in the cold is more associated with an

increase in AOX activity. Increases in AOX activity, where they occur, could serve several roles, including increasing oxidation of excess redox equivalents and/or ensuring that the tricarboxylic acid cycle remains active under conditions of low ATP demand (Atkin *et al.*, 2005). Whatever the roles of the AOX, our results show that while recovery of respiratory flux is often associated with increases in AOX, exceptions do occur, suggesting divergent responses in the ability to maintain growth in the cold. It is possible that neither the increase in AOX nor COX was directly responsible for the acclimation of respiration at cold temperatures; increased AOX may reduce excess reactive oxygen species and COX may help to ensure efficient ATP synthesis in *Arabidopsis*.

Concluding statements

The results demonstrate striking similarities in the degree and the biochemical underpinning of acclimation of R and A in three functional groups, despite the large differences in overall rates of metabolism, chemical composition and leaf structure among these functional groups. Importantly, our results show that homeostasis of $R:A$ is not achieved across all growth temperatures, suggesting that large-scale models cannot assume constancy of $R:A$ ratios under all climatic scenarios. However, our results suggest that large-scale models can assume similar degrees of thermal acclimation potential among functional groups. Based on our results, and those of the previously published studies (e.g. Loveys *et al.*, 2003; Atkin *et al.*, 2006), it seems likely that $R:A$ is homeostatic at moderate temperatures but that the ratio increases when leaves experience very low or high growth temperatures. Although care must always be taken when scaling up growth chamber studies (the data gathered in this study could not have been obtained in the field), our results do provide important insights into how the A and R of species representative of different functional groups respond to long-term changes in growth temperature. Our results suggest that while coupled climate-carbon models may be able to make broad generalizations about the thermal acclimation potential of terrestrial ecosystems, they cannot assume that the $R:A$ ratio remains constant over a very wide range of temperatures.

Acknowledgements

This work was supported by a Natural Environment Research Council (NERC) research grant to OKA and VH (NER/A/S/2001/01186), and grants from the Swedish Council for Forestry and Agricultural Research and the Nordic Academy of Advanced Studies Temperature Stress Network to VH. We would like to thank David Sherlock for expert technical assistance, Dr Gordon Gray, University of Saskatchewan, for the kind gift of the AOX antibody and the staff of AgriSera for assistance with the global antibodies.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 Distribution and general habitat of species used in this experiment, but not in Loveys *et al.* (2002).

Table S2 Full table of parameters broken down by species.

Table S3 Statistical parameters, results of two-way analysis of variance (ANOVA), performed on the average results obtained for each species with all functional groups combined.

Fig. S1 Relative leaf mass per area (LMA) and total non-structural carbohydrate content (TNC) in pre-existing (PE, 21°C-grown leaves that were exposed to 7, 14, 21 or 28°C for 10 d before measurement) and newly developed (ND, leaves that developed under the four growth temperature treatments before measurement) leaves. In all cases, values are expressed as a proportion of values to 21°C controls. Values are the mean of four to eight species (± standard error). Different letters within a leaf developmental stage indicate statistical differences at $P < 0.05$, as a result of posthoc Bonferroni analysis within a one-way analysis of variance (ANOVA).

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