Respiratory carbon metabolism in the high mountain plant species *Ranunculus glacialis*

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Abstract
Very little is known about the primary carbon metabolism of the high mountain plant *Ranunculus glacialis*. It is a species with C3 photosynthesis, but with exceptionally high malate content in its leaves, the biological significance of which remains unclear. 13C/12C-isotope ratio mass spectrometry (IRMS) and 13C-nuclear magnetic resonance (NMR) labelling were used to study the carbon metabolism of *R. glacialis*, paying special attention to respiration. Although leaf dark respiration was high, the temperature response had a Q10 of 2, and the respiratory quotient (CO2 produced divided by O2 consumed) was nearly 1, indicating that the respiratory pool is comprised of carbohydrates. Malate, which may be a large carbon substrate, was not respired. However, when CO2 fixed by photosynthesis was labelled, little labelling of the CO2 subsequently respired in the dark was detected, indicating that: (i) most of the carbon recently assimilated during photosynthesis is not respired in the dark; and (ii) the carbon used for respiration originates from (unlabelled) reserves. This is the first demonstration of such a low metabolic coupling of assimilated and respired carbon in leaves. The biological significance of the uncoupling between assimilation and respiration is discussed.

Key words: Carbon isotopic labelling, malate, photosynthesis, *Ranunculus glacialis*, respiration.

Introduction
Alpine plant species must tolerate low temperatures and high incident solar radiation during a short growing season (Streb et al., 1997; Körner, 1999a). These conditions would induce photoinhibition in many lowland species which are not adapted to these extreme conditions (Streb et al., 1997; Allen and Ort, 2001), and can limit plant assimilation even in alpine species. Alpine species frequently have higher respiratory rates than many lowland species (Atkin et al., 1996), which suggests comparatively greater allocation to maintenance than growth, and might limit their productivity. Respiratory losses are thus an important component of the carbon budget of alpine plants (Atkin and Tjoelker, 2003). Furthermore, a greater proportion of assimilated carbon is allocated to roots and perennial organs in alpine plants (Körner, 1999b; Larcher, 2003).

Despite its importance, little is known about the respiratory metabolism of high mountain species, in particular, there are still many uncertainties regarding the metabolic pathways leading from CO2 assimilation to CO2 release, and the importance of reserve recycling to feed respiration.

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Abbreviations: δ13C, carbon isotope composition; δ13C, carbon isotope discrimination; ETR, electron transport rate; IRMS, isotope ratio mass spectrometry; NADP-MDH, NADP-malate dehydrogenase; NMR, nuclear magnetic resonance; PEPcase, phosphoenolpyruvate-carboxylase; PPFD, photosynthetic active photon flux density; Q10, proportional changes in respiration per 10°C rise in temperature; RQ, respiratory quotient.

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(Körner, 1999a). In this study, attention was focused on the respiratory metabolism of *Ranunculus glacialis* (Ranunculaceae), a common alpine plant and one of the species found at the highest altitudes in Europe (>2500 m).

Those alpine species examined to date are well protected against high light-induced inhibition of primary photochemistry at low temperatures (i.e., photoinhibition), although strategies of adaptation differ between species (Wildi and Lütz, 1996; Streb et al., 1998). In cold conditions, light absorbed in excess may increase the electron pressure at photosystem II (PSII) because light utilization by sink reactions is limited by low temperature and, consequently, the probability of oxidative stress and damage increases (Wise, 1995; Huner et al., 1998; Allen and Ort, 2001). *Ranunculus glacialis* copes efficiently with excess electron availability by maintaining photorespiration at low temperature and having a high capacity to transfer electrons to an alternative oxidase [plastid terminal oxidase (PTOX) in the thylakoid membrane] (Streb et al., 2005), thus preventing photoinhibition. In addition, *R. glacialis* leaves show high concentrations of two metabolites, malate and ranunculin, neither of which is commonly found at such high concentrations in other C₃ species (Streb et al., 2003). These two metabolites may be involved in cold acclimation of photosynthesis and/or oxidative protection, by increasing the capacity of the malate shuttle causing a decrease in chloroplast electron pressure (Scheibe, 2004). Malate may also serve as an additional carbon sink and CO₂ store (Crecelius et al., 2003). Although carbon fixation metabolism has not been fully elucidated in *R. glacialis*, it is highly unlikely that alpine plants are carbon limited (Körner, 1999b).

Respiratory metabolism and the distinct metabolic fates of fixed carbon are uncertain in *R. glacialis*, as in many alpine plants. The large pool of malate (and ranunculin, see above), which may be synthesized during light-saturated photosynthesis as storage compounds to overcome limitations of sugar metabolism, might serve as respiratory reserves. Only lowland plants have been the subject of ¹³C pulse-labelling experiments showing that reserves can contribute significantly to respiration feeding. About 50% of the CO₂ respired by *Phaseolus vulgaris*, and 57% by *Fagus sylvatica* leaves, after light exposure derives from previously assimilated carbon (Nogués et al., 2004, 2006), while the other half is remobilized from previously fixed carbon sources.

In an experiment to study the dilution of ¹³C in alpine plants receiving ¹³C-depleted air for 2 years (Körner, 1999a), *Carex curvula* was reported to use hardly any mobile carbon pools of more than 1 year old for leaf growth. Also, a similar ¹³C signal was present in young tissues at the base of old leaves and in the tips of a new generation of leaves. On the basis of this observation, it was concluded that both were supplied from the same carbon source. However, the ¹³C signal of the CO₂ respired by leaves was not measured.

In this study, the substrates involved in the high respiration rate of *R. glacialis* are investigated, i.e. (i) the contribution of recent photoassimilates to respiration; and (ii) the possible involvement of malate as a respiratory substrate. ¹³C/¹²C labelling techniques are used to elucidate the turnover of primary photosynthetic products and their further use as respiratory substrates. Leaves were initially labelled with CO₂ depleted in ¹³C, and the isotopic ratio was tracked in the respired CO₂ and in metabolites. The difference in δ¹³C between atmospheric CO₂ (−11.5‰) and commercially available (¹³C-depleted) CO₂ (−51.2‰) was exploited to follow the fate of existing carbon reserves. Consequently, the abundance of ¹³C in the CO₂ used for the labelling was in the same order of magnitude as that found in nature. This allowed the contribution of carbon already stored (reserves) to be followed up. This would not have been possible if heavily labelled (i.e. several per cent of ¹³C) carbon had been used. Subsequently, *R. glacialis* leaves were also incubated in a solution containing KH¹³CO₃ or KH¹²CO₃ for pulse-chase labelling. Metabolite turnover was estimated by ¹³C-nuclear magnetic resonance (NMR) spectroscopy after illumination. The findings demonstrate that in *R. glacialis*, malate is probably not involved in feeding respiration, and that most of the CO₂ respired in darkness does not come from recently assimilated carbon.

**Materials and methods**

**Plant material**

From 2002 to 2005, *R. glacialis* (L.) plants were collected from the Galibier Pass in the French Alps, at ~2700 m elevation, as described previously (Streb et al., 2005). For gas exchange measurements, the petiole was cut and maintained under water throughout the experiments, otherwise leaves were floated in water. All the measurements were done at the Station Alpine Joseph Fourier, except the isotope ratio mass spectrometry (IRMS; Orsay) and NMR (Grenoble) measurements. The δ¹³C of CO₂ of the air at the Galibier Pass was approximately −11.5±0.1‰.

**Gas exchange and chlorophyll fluorescence measurements (experiment I)**

Net CO₂ uptake by leaves was measured simultaneously with chlorophyll fluorescence emission using a LI-6400 (Li-Cor Inc, Lincoln, NE, USA) equipped with a leaf chamber fluorometer 6400-40. Electron transport rate (ETR) values under atmospheric conditions were recalculated as described by Ghashghaie and Cornic (1994) [ETR=ΦCO₂×photosynthetic active photon flux density (PPFD)×4]. The temperature inside the closed leaf chamber of the LI-6400 was maintained by a thermostat between ~6 °C and 30 °C. The CO₂ molar fraction (i.e. ~400 ml l⁻¹) was always close to that of the air in the leaf chamber (Streb et al., 2005).

**Labelling procedures for IRMS (experiment II)**

**Closed system for dark respiration:** Before labelling, leaves were placed in a respiration chamber to measure unlabelled dark-respired
CO₂. The respiration chamber, included in the closed system, was first flushed with CO₂-free air to ensure that only the CO₂ respired in the chamber was accumulated. Gas samples for analysis were collected from this chamber in 50 ml glass balloons (Scott Glass, Mainz, Germany). Balloons were taken to the Institut de Biotechnologie des Plantes (IBP) at the Université Paris Sud, and the gas inside the balloon was introduced into an elemental analyser (EA) NA-1500 (Carlo-Erba, Milan, Italy), using a pump, through a 15 ml loop, as described by Tcherkez et al. (2003). Molar fractions of respired CO₂ were measured with an infrared gas analyser IRGA (Finor, Mairyh, Germany) also placed in the closed system.

Carbon isotope compositions were calculated as deviations of the carbon isotope ratio \(^{13}C/^{12}C\) of the sample from a standard (Pee Dee Belemnite):

\[
\delta^{13}C = 1000 \left( \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right)
\]

Open system for isotopic labelling: After the initial measurement of dark-respired CO₂, leaves were removed from 'the respiration chamber' (described above) and placed in a specially designed gas exchange chamber for isotope labelling, as described by Nogue’s et al. (2004). The chamber was connected in parallel to the sample air hose of the LI-6400. This aluminium chamber (20×12×6 ×10\(^{-6}\) m\(^3\)), fitted with a clear plastic lid, holds two leaves (total leaf surface ~0.005 m\(^2\)). Two fans were enclosed in the chamber and gave a boundary layer conductance to water of ~6.7 mol m\(^{-2}\) s\(^{-1}\). Leaf temperature in the chamber was maintained at ~20 °C by cool watering the jacket around the leaf chamber, and was measured with a copper–constantan thermocouple plugged into the thermocouple sensor connector of the LI-6400 chamber/IRGA. Ingoing air was passed through the chamber at a rate of 1.0 l min \(^{-1}\) monitored by the LI-6400. Molar fractions of CO₂ and humidity were measured with the infrared gas analyser (IRGA) of the LI-6400. The PPFD inside the chamber was maintained at 500 μmol m\(^{-2}\) s\(^{-1}\). For labelling, CO₂ was obtained from a bottle (Air Liquide, Grigny, France) with a δ\(^{13}C\) value of ~51.2±0.1‰. After photosynthetic measurements, the outgoing air of the chamber was flushed and the air at CO₂ ~300 ml 1\(^{-1}\) was accumulated in 50 ml glass balloons and analysed as described above for the measurement of photosynthetic carbon isotope discrimination (Δ\(^{13}C\)). The Δ\(^{13}C\) measured was ~20.4±0.7‰ (this corresponds to a p\(/\text{pa}\) value of ~0.7, data not shown).

After labelling, leaves were removed from the labelling chamber and replaced in the respiration chamber for measurements of dark-respired CO₂ (labelled), as described above.

The respiratory quotient \((RQ)\) was calculated from the ratio of carbon produced to oxygen consumed, as described by Nogue’s et al. (2004). At the end of the measurements, leaves were immediately frozen in liquid nitrogen, lyophilized, and powdered for metabolite analysis.

The proportion \((p)\) of ‘new’ carbon (inherited from the labelling) in CO₂ resired in darkness after illumination was calculated following Nogue’s et al. (2004):

\[
p = \frac{\delta^{13}C_{\text{after}} - \delta^{13}C_{\text{before}}}{\delta^{13}C_{\text{fixed}} - \delta^{13}C_{\text{before}}}
\]

where \(\delta^{13}C_{\text{before}}\) and \(\delta^{13}C_{\text{after}}\) are the carbon isotope compositions of dark-respired CO₂ before and after the labelling, respectively, \(\delta^{13}C_{\text{fixed}}\) is the isotope composition of fixed CO₂, which is given by

\[
\delta_0 - \frac{\Delta^{13}C}{1 + \Delta^{13}C}
\]

where \(\delta_0\) is the isotope composition of the outlet air. Because the delta value of the outlet air does not differ greatly from that of the inlet air (small draw-down of CO₂ through the chamber) and \(\Delta^{13}C\) is nearly 20‰, \(\delta^{13}C_{\text{fixed}}\) is close to ~71‰ (Nogue’s et al., 2004). For metabolites, the relationship giving \(p\) is the same as for CO₂.

Carbohydrate extraction and quantification: The extraction procedures for starch and sucrose, glucose, and fructose were similar to those described by Tcherkez et al. (2003). Leaf powder was suspended with 1 ml of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). After centrifugation, starch was extracted from the pellet by HCl solubilization. Soluble proteins of the supernatant were heat denatured and precipitated, and soluble sugars and organic acids of the protein-free extract were separated by high-performance liquid chromatography (HPLC). After lyophilization, purified metabolites were suspended in distilled water, transferred to tin capsules (Courtage Analyse Service, Mont Saint-Aignan, France), and dried for isotope analysis. Isotope analysis of metabolites was performed using the same elemental analyser (EA) and IRMS as described above.

Labelling procedures for NMR (experiment III)

NMR measurements of labelling were done with 5 g of fresh leaves collected in the morning from the Galibier Pass. Leaves were cut into small pieces and incubated in a Petri dish with 50 ml of 5 mM KH\(^{13}CO₃\). The solution was vacuum infiltrated and either immediately washed with distilled water (0 h control), dried, and frozen, or illuminated at 1000 μmol m\(^{-2}\) s\(^{-1}\) PPFD at 25 °C for 4 h and 8 h. After 4 h of incubation, one sample was used for the chase experiments. In this sample, KH\(^{13}CO₃\) was replaced with KH\(^{12}CO₃\) and samples were treated under the same conditions (light and temperature) as the labelled control. Frozen samples were extracted in perchloric acid, and metabolite contents were measured by NMR and identified as described in Streb et al. (2003). For estimation of metabolite content, relative peak height was used and related to a known standard of 250 μmol maleic acid, which was added to each extraction. The detection limit of a given compound was ~5–10 μmol per sample.

Results were calculated as the percentage of total carbon in the NMR spectrum and as the percentage change of metabolite content during the experiment in relation to the untreated control. The experiments were repeated three times.

For other NMR measurements (in stems, pseudobulbs, and roots), 10 g of plant material was immediately frozen after collection and extracted as previously described (Streb et al., 2003) with the addition of 250 μmol maleic acid as internal standard.

Enzyme measurements and malate determination (experiment IV)

Enzyme activities of NAD-malate dehydrogenase (NAD-MDH), phosphoenolpyruvate-carboxylase (PEPcase), and NADP-malic were measured, and also the malate content was determined as described by Crecelius et al. (2003), using ~0.3 g of fresh weight leaf material. This material was collected at the Galibier Pass, transported on ice to the laboratory, and immediately analysed. For malate determination on samplings, leaves were immediately frozen in liquid nitrogen at the sampling site and extracted in liquid nitrogen.

Results

Carbon assimilation and electron transport (experiment I)

The changes in net carbon assimilation, respiration, and ETR under atmospheric gaseous conditions at three leaf temperatures are shown in Table 1. Net carbon assimilation was similar at these three temperatures, ~11–12 μmol m\(^{-2}\) s\(^{-1}\). Respiration rates were quite high (~2 μmol m\(^{-2}\) s\(^{-1}\) at
Table 1. Experiment I. Net assimilation rate ($A_n$, µmol m$^{-2}$ s$^{-1}$), internal CO$_2$ concentration ($c_i$, ppm), ETR (µmol m$^{-2}$ s$^{-1}$), respiration rate in the dark ($R_d$, µmol m$^{-2}$ s$^{-1}$) and the $-R_d/A_n$ ratio (%) at three temperatures in R. glacialis leaves

<table>
<thead>
<tr>
<th>Parameter</th>
<th>10 °C</th>
<th>18 °C</th>
<th>30 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_n$</td>
<td>11.5±1.4</td>
<td>12.5±0.9</td>
<td>11.7±0.7</td>
</tr>
<tr>
<td>$c_i$</td>
<td>235.6±5.8</td>
<td>243.4±9.2</td>
<td>300.2±8.7</td>
</tr>
<tr>
<td>ETR</td>
<td>144.4±11.7</td>
<td>153.2±15.7</td>
<td>122.0±10.4</td>
</tr>
<tr>
<td>$R_d$</td>
<td>−1.6±0.2</td>
<td>−2.2±0.3</td>
<td>−4.2±0.4</td>
</tr>
<tr>
<td>$-R_d/A_n$</td>
<td>15.8±3.1</td>
<td>17.0±1.3</td>
<td>36.1±4.0</td>
</tr>
</tbody>
</table>

18 °C (‘ambient temperature’), and reached a value of ~4 µmol m$^{-2}$ s$^{-1}$ at 30 °C, i.e. exhibited a classical $Q_{10}$ value of ~2. Note that the respiration/assimilation ratio increased as the temperature increased: at 10 °C, the respiration rate represents 16% of the assimilation rate, and this proportion increases up to 36% at 30 °C.

Labelling of respired CO$_2$ (experiment II)

The carbon isotope composition ($\delta^{13}C$) of CO$_2$ respired by leaves in the dark was around $-23.4_{\text{oo}}$ prior to labelling (measured in the closed system; Fig. 1). The respiration rate was ~2.4 µmol m$^{-2}$ s$^{-1}$ before labelling. Thereafter, leaves were placed in an open system for isotopic labelling with $^{13}$C-depleted CO$_2$ ($\delta^{13}C$ of $-51.2_{\text{oo}}$) during a range of light periods. During labelling, the assimilation rate was ~9.0 µmol m$^{-2}$ s$^{-1}$. CO$_2$ respired in darkness after labelling was only slightly depleted: the carbon isotope composition of CO$_2$ was around $-25.1_{\text{oo}}$ after 1 h labelling (i.e. 45 mmol C m$^{-2}$ of new carbon was assimilated) and reached about $-31.6_{\text{oo}}$ after 24 h continuous labelling (during which >735 mmol C m$^{-2}$ of new carbon was assimilated by leaves). This means that the percentage of new carbon in CO$_2$ increased from ~3% after 1 h of labelling to ~20% only after 24 h. Furthermore, the $\delta^{13}C$ of respired CO$_2$ did not change during the following days in continuous darkness after the longest (i.e. 24 h or 735 mmol C m$^{-2}$) labelling period (Fig. 1, inset). However, respiration probably used carbohydrates, as indicated by the respiratory quotient of ~1 (or slightly higher, which may point to the contribution of organic acids), even after 4 d in the dark.

Labelling of metabolites (experiments II and III)

After labelling with $^{13}$C-depleted CO$_2$ (with a $\delta^{13}C$ of $-51.2_{\text{oo}}$) (see the above section) and collecting dark-respired CO$_2$ for isotope analysis, the major metabolites were extracted and analysed by IRMS. Sucrose and fructose were the only compounds labelled after 1 h labelling (Table 2), and the proportion of ‘new’ carbon in these compounds was 4.4% and 3.8%, respectively, after 1 h of labelling. After 3 h, fructose was labelled (it was $4_{\text{oo}}$ depleted in $^{13}$C compared with unlabelled fructose, i.e. it comprised ~8.0% of ‘new’ carbon); sucrose was probably also labelled; however, the concentration was too low to purify enough material for isotope analysis. Note that leaf starch content (~2.5%) is low in this species compared with other cultivated plants (e.g. ~10% in French bean; Nogués et al., 2004). In addition, starch content did not increase during labelling nor did its isotopic composition change (Table 2), indicating that only a very small fraction of carbon is allocated to transitory starch in leaves, the main
part being directed to other compounds, such as soluble carbohydrates.

To determine the turnover of photosynthetic products, the NMR spectra of all soluble carbohydrates were recorded after the pulse–chase experiments with \([13C]\) and \([12C]\)bicarbonate. Malate and ranunculin represented most of the total soluble carbon, together accounting for \(>50\%\) before labelling (Table 3). The contribution of \([13C]\)malate to total soluble carbon decreased during labelling and increased during the chase procedure, while the malate content remained fairly constant throughout the experiment (Table 3A, B), showing that this organic acid was neither synthesized nor degraded.

The highest turnover rates of metabolites were observed for glycerate and unknown compounds, as seen by the relatively high content of these metabolites during the pulse–chase experiments (Table 3B). High turnover rates were also detected for fructose and sucrose, and, to a lesser extent, for glucose and inositol (Table 3B). However, glycerate and inositol accounted for only a very small percentage of total soluble carbon and, therefore, they were quantitatively unimportant from the point of view of photosynthetically generated products (Table 3A). By contrast, after \(8\ h\) of labelling, sucrose, glucose, and fructose accounted for up to \(46\%\) of total soluble carbon and were therefore regarded as the major photosynthetic products, of which sucrose was the most important product with respect to assimilated carbon (Table 3A).

### Metabolite allocation within the plant (experiment III)

To study the metabolites exported from leaves to sink tissues during active photosynthesis and to determine the amount of carbohydrates in them, stems, pseudobulbs formed at leaf bases, and roots were collected early in the morning before sunrise and at midday in full sunlight, and their metabolic profile was determined by NMR spectroscopy. Glucose was the major sugar present in stems, while sucrose accumulated in the roots (Fig. 2). The major soluble metabolite in pseudobulbs was gluco (note that insoluble fructans and starch, which cannot be measured by NMR, might also be present in this organ). In contrast to the leaves, malate concentration was very low in other plant organs (data not shown) and ranunculin content was much lower than in leaves.

### Malate production and use (experiment IV)

The malate content of leaves was measured as a function of time and leaf age. Leaves showed an exceptionally high malate content (Table 4), to the best of our knowledge much higher than reported for other alpine or non-alpine \(C_5\) species (Crecelius et al., 2003; Streb et al., 2003). To check whether malate content varied in a diurnal rhythm, concentrations were measured enzymatically throughout the day (Table 4A). Malate content increased slightly from the morning to midday, and decreased in the evening, but changes were small, indicating no significant synthesis of this compound during the light period and no respiration of...
it during the night. Furthermore, malate concentrations did not vary when leaves were kept for 3–4 d in darkness at ambient temperature (data not shown), showing that this compound was not degraded by respiration. Enzyme activities of PEPcase and of malic enzyme were under the detection limit of the assay, thereby excluding the possibility of significant malate synthesis and degradation by this pathway (data not shown). However, malate accumulated during the growing season, showing higher values in August than in July (Table 4B). In addition, the concentration of this compound did not differ greatly between young, mature, senescent, or brown (i.e. about to fade) leaves when collected at the same time of the year (Table 4C). Taken together, these observations indicate that malate may accumulate without further use by leaf metabolism.

Several enzyme activities were measured. NAD-MDH activity was high in the morning and, regardless of temperature, decreased to almost the same level of activity after several hours of light exposure (Table 5). Activity also decreased to a similar level in the dark. Malate may be involved in the transfer of redox equivalents between cell compartments of illuminated leaves. The decrease in activity in darkness is also consistent with the lack of malate use by dark respiration.

### Discussion

Although being a cornerstone of plant carbon balance and growth (the respiration rate may account for nearly 40% of the assimilation rate; Table 1), the respiratory carbon metabolism of the high mountain plant species *R. glacialis* is not well known. The relationships between carbon fixation and respiration were investigated using mass spectrometry and NMR after manipulating $^{12}$C/$^{13}$C stable isotopes in *R. glacialis* leaves.

#### The origin of leaf dark-respired CO$_2$

The CO$_2$ respired in the dark by *R. glacialis* leaves immediately after a light period originated mainly from the oxidation of carbohydrates (the respiratory quotient is nearly 1; Fig. 1B) and had a $\delta^{13}$C value of about $-23\%_{oo}$ (Fig. 1C), similar to what is reported in beans (Duranceau et al., 1999; Ghashghaie et al., 2003; Tcherkez et al., 2002; Nogue's et al., 2003).

### Table 4.

<p>| Experiment IV. Malate contents (µmol g$^{-1}$ FW) of <em>R. glacialis</em> leaves collected in the morning, at midday, and in the evening at the Galibier Pass (A), collected at the same time during July and August of 2003–2005 (B), and in young, mature, senescent, and completely brown leaves collected in 2005 (C) Data are the means of three replicates ±SE. |</p>
<table>
<thead>
<tr>
<th>Malate content</th>
</tr>
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<tbody>
<tr>
<td>(A) Morning</td>
</tr>
<tr>
<td>Midday</td>
</tr>
<tr>
<td>Evening</td>
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<tr>
<td>(B) July 2003</td>
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<tr>
<td>August 2003</td>
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<tr>
<td>July 2004</td>
</tr>
<tr>
<td>August 2004</td>
</tr>
<tr>
<td>(C) Young</td>
</tr>
<tr>
<td>Mature</td>
</tr>
<tr>
<td>Senescent</td>
</tr>
<tr>
<td>Brown</td>
</tr>
</tbody>
</table>

### Table 5.

<table>
<thead>
<tr>
<th>Experiment IV. NAD-MDH activity (µmol m$^{-2}$ s$^{-1}$) in <em>R. glacialis</em> leaves collected in the morning or at midday at the Galibier Pass NAD-MDH is also shown for leaves collected in the morning and incubated for 6 h at 1000 µmol m$^{-2}$ s$^{-1}$ or in the dark, at either 25 °C or 6 °C. Data are the means of three replicates ±SE.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAD-MDH activity</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Morning</td>
</tr>
<tr>
<td>Midday</td>
</tr>
<tr>
<td>6 h 1000 25 °C</td>
</tr>
<tr>
<td>6 h dark 25 °C</td>
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<tr>
<td>6 h 1000 6 °C</td>
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<tr>
<td>6 h dark 6 °C</td>
</tr>
</tbody>
</table>
Nogués et al., 2004). Although quite high, the respiration rate increased with temperature following a classical $Q_{10}$ value of 2 (Table 1).

However, the respiratory metabolism in R. glacialis differs from species previously investigated (Nogués et al., 2004, 2006), in that the relative contribution of the newly fixed (labelled) carbon (by photosynthetic activity) versus that from stored carbohydrates (unlabelled carbon) to dark respiration is much lower, with much higher $\delta^{13}C$ values of dark-respired CO$_2$ after labelling. As the measured photosynthetic discrimination ($\Delta^{13}C$) of the leaves was $\sim$20\% and the carbon isotope composition ($\delta^{13}C$) of dark-respired CO$_2$ by leaves may have been $13^{13}C$-enriched by the respiratory fractionation ($\varepsilon_{\text{dark}}$) of $\sim$4–6\% (compared with the respiratory substrates; Ghayshi et al., 2003), the $\delta^{13}C$ of the respiratory substrates (mainly carbohydrates) should have approached $-67\%$ (this is $-51 - \Delta^{13}C + \varepsilon_{\text{dark}}$) if the substrates used to sustain respiration had been fully labelled (with a CO$_2$ of $\delta^{13}C$ of $-51\%$), but this was not the case (Fig. 1C). The contribution of labelled C atoms to CO$_2$ production is 18–20\% after 24 h labelling (see Materials and methods). In French bean, this proportion can be as high as 50\% for the same amount of incorporated carbon during illumination (Nogués et al., 2004). After illumination periods of realistic duration (i.e. $\sim$12 h), labelling of the CO$_2$ subsequently respired is much less labelled, indicating that dark-respired CO$_2$ comprises only $\sim10\%$ ‘new’ carbon (Fig. 1). In other words, ‘old’ carbon atoms (reserves) are the predominant contributor to respiratory activity, so the respiratory pool is likely to have a very slow turnover.

Reserves in this plant species are made up of carbohydrates and may include some organic acids (values of respiratory quotient slightly higher than 1). However, the latter proportion is probably small, as malate is not used by respiration: the malate pool did not decrease significantly during the night (Table 4) and the activity of malate-processing enzymes did not correlate with a plausible use by respiration during the night (Table 5). Therefore, presumably, leaf carbohydrate reserves feed the respiratory pool in this species. It is possible that sucrose is the major substrate for respiratory CO$_2$ production, as the labelling level after 1 h of illumination is similar (nearly 3\%) in CO$_2$ and sucrose. However, it remains plausible that the respiratory pool is fed by other carbon sources: in bean, it has been shown by a similar technique that starved plants had a similar labelling level in respired CO$_2$ while sucrose was much more labelled, as compared with control (non-starved) plants (Nogués et al., 2004). Some of the unlabelled molecules might also be sucrose remobilized from the vacuole (Dubinia et al., 2001).

In addition to the proportion of ‘new’ carbon atoms in respiratory CO$_2$, it is noted that R. glacialis differs from bean as far as the synthesis of sucrose is concerned. Indeed, after 1 h labelling, sucrose was nearly 3\% labelled in R. glacialis while it was nearly 30\% labelled in bean. Clearly, this effect cannot be fully explained by a ‘dilution effect’ of the labelled fixed carbon by the amount of unlabelled sucrose in the leaf: the amount of sucrose before labelling is $\sim1\%$ of dry weight (Table 2), i.e. 28–30 mmol C m$^{-2}$ in R. glacialis, while is near 10–15 mmol C m$^{-2}$ in bean. Thus, it is likely that metabolic fluxes associated with sucrose synthesis, i.e. the balance between the contribution of new assimilates versus the remobilization of unlabelled molecules, are somewhat different in these two species.

Furthermore, the respiratory pool feeding dark respiration may be quite large because (i) there is no indication that it can be filled up after 24 h of photosynthesis (Fig. 1C) and (ii) although the respiratory quotient did not change after 4 d in darkness, the isotope composition of CO$_2$ evolved in darkness did not change for 4 d when leaves were maintained in continuous darkness after the 24 h labelling illumination (Fig. 1C, inset). Interestingly, the second point is in contrast to previously published results in Phaseolus vulgaris where the isotopic composition of CO$_2$ evolved in darkness decreases rapidly with the respiration rate, while leaves consume less oxygenated substrates. Ranunculus glacialis leaves darkened for several days (i.e. starved leaves) do not use lipids, as indicated by the respiratory quotient of $\sim1.0$ after 4 d (data not shown).

**Carbon fixation pathway**

Since carbon incorporated during labelling is hardly found in respired CO$_2$ (see the above section), what is the fate of these carbon atoms in the leaf? When metabolites were examined by NMR after H$^{13}$CO$_3^-$ labelling, the higher turnover rates were detected in glycerate, sucrose, fructose, and unknown compounds (Table 3). The large turnover rate of glycerate, which is an intermediate of the photorespiratory cycle, is probably the result of high rates of photorepiration under these experimental conditions, as shown by Streb et al. (2005). However, it should be noted that after 8 h of labelling, glycerate accounted for only 1\% of the total soluble carbon in leaves.

As expected, the major products of photosynthetic primary carbon metabolism were sucrose, fructose, and, to a lesser extent, glucose and ranunculin: sucrose accounted for up to 15\% and fructose for up to 8\% of total soluble carbon in the leaves, indicating a concentration higher than 1 mM (Table 3). After 8 h of labelling, these contents rose to 20\% and 11\% of total soluble carbon for sucrose and fructose, respectively. This increase was in accordance with mass spectrometry data, which indicated that after 1 h of illumination, these two sugars are $^{13}C$ depleted as a consequence of labelling.

Newly assimilated carbon was directed mainly to sucrose and fructose synthesis and was hardly used to replenish respiratory pools (see above). However, the carbohydrate concentration increased only very slowly in leaves (Table 2). This observation supports the assumption that carbohydrate
export is very efficient in this plant. After synthesis, sucrose and fructose were efficiently exported by leaves, as indicated by the accumulation of these metabolites in roots and pseudobulbs (Fig. 2). Streb et al. (2003) reported that the unidentified compounds belonged to several metabolite types, among which no prominent signals were detected during labelling or chase experiments, indicating equally high turnover rates for all of them.

Leaves contained very high amounts of malate. However, malate concentration did not follow a circadian rhythm, neither did enzyme activities. In addition, malate was not labelled when 13C-depleted CO2 was incorporated (near 20°C). Clearly, the results rule out a C4-type metabolism. The Δ13C in the present study was higher than the Δ13C value of 16.6‰ calculated using δ13C of the total organic matter by Körner et al. (1988, 1991) for the same species at the same altitude. The lower value reported by Körner and co-workers may be the result of (i) the use of an assumed value δ13C of ~7.7‰ for CO2 in air; and (ii) post-photosynthetic fractionation adulterating the isotope signature of the photosynthetically fixed carbon.

Role of malate
Malate is not used by respiration and has a slow turnover when leaves are labelled with either 13C-depleted CO2 or H13CO3-. A biological explanation for the accumulation of malate in R. glacialis leaves is not apparent. In rye (Secale cereale), leaves acclimated to low temperature have an increased malate content (Crecelius et al., 2003; Streb et al., 2005). However, contrary to cold-acclimated rye, the activity of the ‘malate shuttle’ and the activities of PEPcase and malic enzyme in R. glacialis were low or below the detection limit of the present assay, indicating that malate does not protect the leaves of this species against damage caused by cold conditions.

However, malate accumulated during the vegetation period, and NAD-MDH activity was relatively high, suggesting that it might be used mainly for redox transfer reactions between cell compartments and may possibly accumulate as an osmoticum before leaf senescence. In this respect, the NAD-MDH activity might support the photosynthesis cycle with reducing equivalents, as photorespiration is very active in R. glacialis leaves in the light (Körner et al., 2005). More data are now required to determine the biological function of malate in this plant and its involvement in redox-reductive reactions. The present results demonstrate that malate accumulates but is not used further by leaf metabolism and that it is probably stored in cell vacuolar compartments. In general, it is noted that many alpine plants produce more assimilates than can be invested in structural growth (Körner, 1999b).

Two respiratory physiotypes
The carbon recently assimilated during photosynthesis accounts for ~50% of the carbon in the CO2 lost by dark respiration after illumination of P. vulgaris leaves and F. sylvatica twigs (Nogués et al., 2004, 2006). However, most of the carbon released by dark respiration from R. glacialis leaves after illumination (~80%) does not come from newly assimilated carbon. Similarly, most of the carbon in sucrose is not inherited from newly fixed carbon atoms. However, the increase in the respiratory rate as a function of assimilated carbon makes it clear that there is a tight link between photosynthesis and respiration. It is argued that this increase could result from an increase in the availability of substrate fed directly from photosynthesis and also from a non-labelled pool(s).

These contrasting values indicate that there might be two distinct naturally occurring ‘respiratory physiotypes’: for a given amount of assimilated carbon, parsimonious plants restrict the turnover of the respiratory pool, favouring either recycling of reserves (R. glacialis) or investment in respiration (French bean). It has recently been found that the thermo-Mediterranean palm Chamaerops humilis (Arecaceae), like R. glacialis, also respired a small proportion of ‘new’ carbon after illumination (A Pardo et al., unpublished data; Table 6). Presumably, the respiratory strategy may be related to the biological constraints of the environment. Interestingly, Atkin and Tjoelker (2003) have identified two types of respiratory acclimation to temperature in plants, one of which is underpinned by adjustments

Table 6. The δ13C of the CO2 respired before and after labelling (%oo), δ13C of the CO2 used during the labelling (%oo), the Δ13C measured during the labelling period (%oo), and the percent of new carbon in the CO2 respired after the labelling are shown for four C3 plant species

<table>
<thead>
<tr>
<th>Plant species</th>
<th>δ13C before (%oo)</th>
<th>δ13C after (%oo)</th>
<th>δ13C labelling (%oo)</th>
<th>Δ13C (%oo)</th>
<th>New C in CO2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranunculus glacialis</td>
<td>-23.4±0.7</td>
<td>-28.0±0.8</td>
<td>-51.2±0.1</td>
<td>20.4±0.7</td>
<td>9.6±1.1</td>
</tr>
<tr>
<td>Chamaerops humilis</td>
<td>-21.8±0.9</td>
<td>-23.2±0.6</td>
<td>-51.2±0.1</td>
<td>15.2±0.3</td>
<td>8.7±1.3</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>-22.0±0.6</td>
<td>-44.8±0.8</td>
<td>-51.2±0.1</td>
<td>20.1±0.2</td>
<td>50.3±0.9</td>
</tr>
<tr>
<td>Fagus sylvatica</td>
<td>-18.8±0.7</td>
<td>-44.5±0.9</td>
<td>-51.2±0.1</td>
<td>17.0±0.6</td>
<td>56.8±1.0</td>
</tr>
</tbody>
</table>
in the $Q_{10}$ (called type I) and the other by changes in the enzymatic capacity of the respiratory system (called type II); type I acclimation enables rapid changes in respiratory flux at high temperatures to occur following changes in the thermal environment, meanwhile type II acclimation is likely to be maximal upon the development of new leaves and roots following a change in temperature.

For plants growing slowly in harsh environments, such as *R. glacialis* (alpine conditions) and *C. humilis* (hot and dry conditions), most of the newly assimilated carbon is not respired; these species may be called 'low respiratory turnover plants'. In fast-growing and cultivated plants and trees, such as *P. vulgaris* and *F. sylvatica*, nearly 50% of the respiratory CO$_2$ comes from recently assimilated carbon; these may be called 'high respiratory turnover plants'. However, further studies on the range of the leaf respiratory response in other species are required to check the validity of this hypothesis and to relate it to the Atkin and Tjoelker model.

Conclusions

It has been shown that turnover of the respiratory pool in the alpine plant *R. glacialis* is very slow. In other words, this species devotes much energy to recycling and metabolite accumulation (i.e. malate and sucrose) in the absence of photosynthetic limitation. The immediate allocation of carbon to carbohydrate metabolites such as sucrose is normal, but the immediate allocation to malate and ranunculin is much more unusual; nevertheless, it produces a very large amount of carbon when integrated over the life of the leaf. The function of these two metabolites is unclear, but they may provide, along with carbohydrates, a large pool of carbon reserves that can be remobilized, thereby ensuring a carbon source regardless of the (somewhat unpredictable) alpine conditions.

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