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Evidence for alternative electron sinks to photosynthetic carbon assimilation in the high mountain plant species *Ranunculus glacialis*

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ABSTRACT

The high mountain plant species *Ranunculus glacialis* has a low antioxidative scavenging capacity and a low activity of thermal dissipation of excess light energy despite its growth under conditions of frequent light and cold stress. In order to examine whether this species is protected from over-reduction by matching photosystem II (PSII) electron transport (ETR) and carbon assimilation, both were analysed simultaneously at various temperatures and light intensities using infrared gas absorption coupled with chlorophyll fluorescence. ETR exceeded electron consumption by carbon assimilation at higher light intensities and at all temperatures tested, necessitating alternative electron sinks. As photorespiration might consume the majority of excess electrons, photorespiration was inhibited by either high internal leaf CO_2 molar ratio (C_i), low oxygen partial pressure (0.5% oxygen), or both. At 0.5% oxygen ETR was significantly lower than at 21% oxygen. At 21% oxygen, however, ETR still exceeded carbon assimilation at high C_i, suggesting that excess electrons are transferred to another oxygen consuming reaction when photorespiration is blocked. Nevertheless, photorespiration does contribute to electron consumption. While the activity of the water – water cycle to electron consumption is not known in leaves of *R. glacialis*, indirect evidence such as the high sensitivity to oxidative stress and the low initial NADP-malate dehydrogenase (NADP-MDH) activity suggests only a minor contribution as an alternative electron sink. Alternatively, the plastid terminal oxidase (PTOX) may transfer excess electrons to oxygen. This enzyme is highly abundant in *R. glacialis* leaves and exceeds the PTOX content of every other plant species so far examined, including those of transgenic tomato leaves overexpressing the PTOX protein. Finally, PTOX contents strongly declined during deacclimation of *R. glacialis* plants, suggesting their important role in photoprotection. *Ranunculus glacialis* is the first

reported plant species with such a high PTOX protein content.

Key-words: excess electron transport; over-reduction; photoprotection; photorespiration; plastid terminal oxidase (PTOX) itemperature and light stress.

Abbreviations: C_i, internal leaf CO_2 concentration; ETR, electron transport rate; NADP-MDH, NADP-malate dehydrogenase; NDH H, NAD(P)H-dehydrogenase; NPQ and q_N, non-photochemical quenching of chlorophyll fluorescence (i.e. thermal dissipation of absorbed light energy); Φ_{CO_2}, quantum yield of carbon assimilation; Φ_{PSII}, quantum yield of photosystem II; PAR, photosynthetic active radiation; PTOX, plastid terminal oxidase.

INTRODUCTION

Plants growing at high altitude in the Alps must cope with extreme and varying environmental conditions, such as high or low solar radiation and temperature ranging from freezing up to more than 30 °C (Moser *et al*. 1977; Streb, Feierabend & Bligny 1997; Körner 1999; Streb, Aubert & Bligny 2003c). Besides these environmental constraints the whole vegetation period may be limited to one or two months per year, necessitating efficient photosynthetic performance in order to complete the life cycle (Moser *et al*. 1977). Photosynthetic adjustment to such contrasting conditions is difficult since plants acclimated to low light intensity and moderately high temperatures increase the capacity of primary photosynthetic reactions, while plants acclimated to high light intensity or low temperature increase the capacity of carbon assimilation and utilization (Falk *et al*. 1996; Bailey, Horton & Walters 2004)

Ranunculus glacialis, an alpine plant species restricted to higher altitudes in the Alps, has a broad temperature optimum of photosynthesis. Net carbon assimilation in this plant is possible under contrasting environmental conditions (Moser *et al*. 1977; Körner 1999). Furthermore the actual photosynthetic activity of *R. glacialis* is mostly lim-

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ited by the available light intensity, whereas photosynthesis is not saturated in full sunlight (Moser *et al.* 1977; Körner & Diemer 1987).

Moser *et al.* (1977) showed that the majority of the net carbon gain of *R. glacialis* was achieved either at low temperatures (< 10 °C) and low light intensity or at high light intensity and moderate temperatures (> 10 °C). Nevertheless, photosynthetic performance in *R. glacialis* is also frequently challenged by periods of high light intensity and low temperature (Moser *et al.* 1977; Streb *et al.* 1997). Such conditions are generally known to cause an imbalance of electron transport and carbon assimilation due to enzymatic reactions being more sensitive to decreasing temperatures than light absorption and photosynthetic electron transport (Wise 1995; Falk *et al.* 1996). As a consequence, the photosynthetic electron transport chain may become strongly reduced inducing photoinhibition of PSII and of catalase and, in addition, generating reactive oxygen species which themselves can affect photosynthetic performance and induce more general cellular damage (Aro, Virgin & Andersson 1993; Wise 1995; Huner, Öquist & Sarhan 1998; Asada 1999; Shang & Feierabend 1999).

Ranunculus glacialis and other alpine plant species are well protected against high light- and low temperature-induced photoinhibition of PSII and of catalase (Streb *et al.* 1997). However, although not completely solved and understood, strategies of acclimation are not uniform among different plant species and *R. glacialis* in particular appears to be weakly protected by several well-known conventional mechanisms of acclimation (Streb *et al.* 1998). Light that is absorbed in excess of what can be used for metabolism may be dissipated as heat, as estimated by non-photochemical fluorescence quenching (q_N or NPQ), in order to keep the electron transport chain oxidized (Huner *et al.* 1998). Non-radiative energy dissipation depends on the transthylakoid proton gradient and is modulated by the appearance of the xanthophyll cycle pigment zeaxanthin interacting with the PsbS protein (Aspinall-O'Dea *et al.* 2002). In the alpine plant species *Geum montanum* cyclic electron transport around PSI can increase lumenal acidification and protective energy dissipation (Manuel *et al.* 1999). However, in comparison to *Soldanella alpina*, another alpine plant species, or to cold-acclimated rye, non-photochemical fluorescence quenching and zeaxanthin synthesis is less important in *R. glacialis* while the primary electron acceptor of PSII Q_A is still largely oxidized under varying conditions. Furthermore, when zeaxanthin formation was blocked by dithiothreitol, q_N decreased in both alpine plant species but Q_A reduction and the extent of PSII inhibition were much more affected in *S. alpina* than in *R. glacialis*. Therefore, calculated rates of PSII electron transport remained higher in *R. glacialis* than in *S. alpina* and cold-acclimated rye (Streb *et al.* 1998; Streb & Feierabend 1999). High rates of electron transport together with a cold-induced decrease of carbon metabolism increases the probability of electron transfer to oxygen in the Mehler reaction, thus forming superoxide and finally increasing oxidative stress (Wise 1995; Huner *et al.* 1998). This reactive oxygen is detoxified by an efficient chloroplastic antioxidative system, involving the antioxidants ascorbate and glutathione as well as the enzymes superoxide dismutase, ascorbate peroxidase and glutathione reductase (Noctor & Foyer 1998; Asada 1999). However, importantly, the capacity of these antioxidants and antioxidative enzymes is rather weak in *R. glacialis* and comparable to the capacity of non-cold-acclimated rye but much less efficient than in *S. alpina* or cold-acclimated rye (Streb *et al.* 1998; Streb, Shang & Feierabend 1999). This weak capacity is demonstrated by a high sensitivity of *R. glacialis* leaves to paraquat, which increases oxidative stress, and by diurnal oxidation of ascorbate and glutathione at the natural growing site (Streb *et al.* 1997, 1998). In conclusion, in order to keep the electron transport chain oxidized and to prevent reactive oxygen formation, electrons must be efficiently consumed in leaves of *R. glacialis*, either by the Benson–Calvin cycle or by other electron sinks. Interestingly, *R. glacialis* accumulated phosphoglycerate in cold and light, which may indicate a limitation of Ribulose-bis-phosphate regeneration while the amount of ATP was kept constant, suggesting, that sucrose synthesis was not limiting phosphate availability (Streb *et al.* 2003b).

In order to investigate the relationship between photosynthetic electron transport and the consumption of electrons by carbon assimilation under various conditions of temperature and light, both were analysed simultaneously with an infrared gas analysing system and a modulated chlorophyll fluorometer. As the rates of calculated electron transport exceeded electron consumption by the Benson–Calvin cycle, the importance of alternative electron sinks was investigated. Photorespiration was estimated by carbon assimilation at varying CO_2 and O_2 partial pressures and the activity of the malate valve by enzyme measurements. Furthermore, the alternative plastid terminal oxidase (PTOX, also known as IMMUTANS) was quantified by immunoblotting. PTOX is able to transfer electrons from plastoquinone to oxygen without generating reactive oxygen species (Cournac *et al.* 2000; Josse *et al.* 2003). While PTOX is involved in carotenoid synthesis and chlororespiration, it can potentially protect the chloroplast electron transport chain from over-reduction (Carol & Kuntz 2001). Since carotenoid contents are low in *R. glacialis* in comparison with other alpine plant species (Streb *et al.* 1997), it seems unlikely that PTOX play a major role for carotenoid synthesis. However, PTOX is only a minor protein in all plant species so far examined and the capacity of PTOX-dependent consumption of excess electrons appears to be low (Peltier & Cournac 2002; Ort & Baker 2002). This restriction might be overcome in *R. glacialis* leaves.

MATERIALS AND METHODS

Plant material of *Ranunculus glacialis* (L) was collected in the French Alps in the years 2001–04 at the Galibier pass at approximately 2700 m elevation as described previously (Streb *et al.* 1997).

Gas exchange and fluorescence measurements

Net CO_2 uptake by leaves was measured together with chlorophyll fluorescence emission using a LICOR 6400 (liCor Inc., Lincoln, NE, USA) equipped with a leaf chamber fluorometer 6400–40. The system was maintained in a closed thermostated chamber, in which temperatures could be varied from approximately 6 to 30 °C. The CO_2 molar ratio in the thermostated chamber was always close to that of the air in the leaf chamber.

Dark levels of chlorophyll fluorescence emission (F_o and F_m) were recorded at the beginning of the experiments after 30 min dark acclimation. Fluorescence emission in the light was taken every 5 min in actinic light (F_t) during a saturating light flash of 2 s duration (F_m') and followed by a short period in far red light (F_o'). F_v'/F_m' ($F_m' - F_o'/F_m'$), a parameter for the efficiency of excitation capture by open PSII reaction centres and photochemical fluorescence quenching q_P [$(F_m' - F_t)/(F_m' - F_o')$] were measured as previously described in Schreiber, Schliwa & Bilger (1986) and Genty, Briantais & Baker (1989). The relative reduction state of Q_A, the primary electron acceptor of PSII, was approximated as $1 - q_P$. As discussed in Manuel *et al.* (1999) absolute values for Q_A reduction would require the knowledge of connectivity of PSII reaction centres. Φ_{PSII} was measured according to Genty *et al.* (1989). Φ_{PSII} was calibrated against Φ_{CO2} in a 0.5% oxygen atmosphere. Φ_{CO2} is the quantum yield of gross photosynthesis as approximated by net carbon assimilation + dark respiration. For Φ_{CO2} calculation, leaf absorbance of 80% PAR was measured with a LI 800 portable spectroradiometer with an 1800–12 S external integration sphere and absorbed light was taken into consideration. The following relation was measured: $\Phi_{PSII} = 10.47 \times \Phi_{CO2} + 0.015$, $r^2 = 0.98$. ETR values in air were recalculated as described by Ghashghaie & Cornic (1994) (ETR = $\Phi_{CO2} \times$ PPFD $\times$ 4). The difference between the calculation of ETR used in this investigation and the ETR calculation according to Krall & Edwards (1992) is very small. However, ETR rates calculated with the currently used method are usually 5–10% smaller than calculations with the classical method of Krall & Edwards (1992), resulting in a conservative estimation of ETR (Ghashghaie & Cornic 1994).

Enzyme measurements

In order to measure maximum extractable enzyme activity several sampling, extraction and measuring protocols were tested and only highest enzyme activities are presented.

Leaf material was collected at the Galibier pass, transported on ice to the laboratory and analysed immediately or used for incubations (approximately 20 min after collection). For NADP-MDH activity (EC. 1.1.1.82) leaves were frozen in liquid nitrogen directly at the sampling site or after incubation. For some experiments cut leaves were incubated in water in a Petri dish for 6 h at 6 °C in darkness or in light at 1000 μmol m^{-2} s^{-1} PAR, as described previously (Streb *et al.* 2003b).

Approximately 0.3 g fresh leaf material was ground with mortar and pestle. For glycolate oxidase activity (EC 1.1.3.1) leaves were extracted in 5 mL 50 mM phosphate-buffer pH 7.5 on ice and centrifuged for 8 min at 8000 *g* and 4 °C. The supernatant was immediately measured spectrophotometrically at 25 °C or additionally at 4 °C in an assay mixture containing 37 mM triethanolamine pH 7.8, 0.01% Triton X 100, 0.4 mM flavinmononucleotide, 3.3 mM phenylhydrazinhydrochloride and 5 mM glycolate as described previously (Streb *et al.* 1997). As glycolate oxidase might be associated with membranes and sensitive to oxidation (Schäfer & Feierabend 2000) additional extracts were prepared in the presence of 0.5% Triton X 100 and 10 mM dithiothreitol (DTT). This extraction resulted in slightly higher enzyme activities.

NADP-MDH activity was extracted and measured according to Trevanion, Furbank & Ashton (1997). Frozen leaves were ground in liquid nitrogen to a fine powder and transferred to 3 mL of an extraction buffer containing 25 mM HEPES/KOH pH 7.5, 10 mM MgSO$_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5% polyvinyl pyrrolidone (PVP) and 0.05% Triton X 100. The extract was thawed and centrifuged for 10 min at 20 000 *g* and 4 °C. Initial NADP-MDH activity was measured immediately after centrifugation and an aliquot was incubated for 30 min at 25 °C in a solution containing 60 mM Tricine-KOH pH 9.0, 0.6 mM EDTA, 180 mM KCl, 150 mM DTT and 0.0025% Triton X 100. Maximum activity was confirmed by the addition of thioredoxin to the incubation medium. Initial and maximum NADP-MDH activity was measured in a 1-mL cuvette containing 25 mM Tricine-KOH pH 8.3, 1 mM EDTA, 15 mM KCl, 5 mM DTT, 0.2 mM NADPH and 20 μL extract.

All experiments were repeated independently at least three times and the standard error was calculated.

Immunoblotting

Leaves of *R. glacialis* were collected at the Galibier pass (2700 m) and immediately frozen in liquid nitrogen. In addition whole plants were planted in pots together with soil from the sampling site, transported to low elevation (200 m) and grown for 3 weeks in a temperature-controlled room at approximately 22 °C with natural sunlight of reduced intensity (approximately 100–200 μmol m^{-2} s^{-1} PAR), essentially as described by Streb *et al.* (2003a). Leaves of these plants were either used for western blot analysis in the morning (low light) or illuminated for 6 h at 1000 μmol m^{-2} s^{-1} PAR (high light) at 25 °C. Leaves of other alpine and lowland plant species were investigated for comparison: *Geum montanum* (L.) from three different elevations (2100–2600 m), *Geum reptans* (L.) and *Arabis alpina* (L.) growing at the same sampling site as *R. glacialis*, *Aquilegia vulgaris* (L.) cultivated at 200 m elevation, *Trollius europaeus* (L.) collected at 1700 m elevation, *Ranunculus auricomus* (L.) collected at 200 m elevation and leaves of transgenic *Lycopersicon esculentum* (Mill.) cv. Micro Tom

(PTOX +) overexpressing the PTOX gene from *Arabidopsis thaliana* and cultivated as described elsewhere (Josse 2003).

Intrinsic membrane proteins were extracted from approximately 0.5 g of plant material. After grinding in liquid nitrogen to a fine powder, the plant material was suspended in 5 mL 50 mM Tris/HCl pH 8.0, 50 mM *β*-mercaptoethanol and 1 mM PMSF, agitated at 4 °C for 30 min and centrifuged at 45 000 *g* for 20 min at 4 °C. The pellet was re-suspended in extraction buffer with the addition of 150 mM NaCl. The extraction and centrifugation was repeated. The pellet was again re-suspended in extraction buffer with the addition of 1% sodium dodecyl sulphate (SDS). After another repetition of the extraction and centrifugation step, the supernatant containing intrinsic membrane proteins was further used. Aliquots of 100 and 300 *μ*L were taken for the determination of protein content according to Lowry *et al.* (1951) by using the DC protein assay of BioRad (Bio-Rad Laboratories Inc., France). The remaining supernatant was diluted four times with 100% acetone. Proteins were precipitated at −20 °C overnight. The pellet was re-suspended in a storage buffer for SDS-polyacylamide gel electrophoresis (PAGE) electrophoresis, containing 2.5% glycerol, 0.75% SDS and 15 mM Tris/HCl pH 6.8 at a concentration of 5 mg mL^{-1} total protein and stored at −20 °C until use.

For immunoblotting, 30–60 *μ*g proteins were fractionated by SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membrane according to Sambrook, Fritsch & Maniatis (1989). Polyclonal antibodies against PTOX of *Arabidsopsis thaliana* were produced as described previously (Cournac *et al.* 2000) and used at a final dilution of 1/15 000. Antibodies against NDH H were used at 1/1000 final dilution and antibodies against IE 37, used as control for loading, were used at 1/150 000 final dilution. Immuno-

detection was performed using the ECL Western blotting kit (Amersham Bioscience Europe GmbH, Succursale, France) as recommended by the suppliers. Blots were repeated twice from extraction of three different leaves.

RESULTS

Carbon assimilation and electron transport under atmospheric conditions

Figure 1 shows light response curves of carbon assimilation under atmospheric conditions (390 ppm CO_2, 21% O_2) for different leaf temperatures. Carbon assimilation at light intensities above 400 μmol m^{-2} s^{-1} PAR was similar at 15 and 23 °C leaf temperature and not saturated by the highest light intensity applied. However, maximum carbon assimilation decreased significantly at 10 °C and saturated at 700 μmol m^{-2} s^{-1} PAR. The light compensation point was the highest at 23 °C and decreased at lower temperatures, thus allowing net-carbon assimilation at very low light intensities in cold (Fig. 1 insert).

ETR under atmospheric conditions was estimated from the Φ_{PSII}/Φ_{CO2} relation measured under non-photorespiratory conditions (0.5% oxygen) as described by Ghashghaie & Cornic (1994). The ETR was relatively high under all experimental conditions (Fig. 2a). However, saturated ETR was more than three times as high at 23 °C than at 10 °C and intermediate at 15 °C. The ETR saturated at 400 μmol m^{-2} s^{-1} PAR at 10 °C, at 700 μmol m^{-2} s^{-1} PAR at 15 °C and at 1200 μmol m^{-2} s^{-1} PAR at 23 °C leaf temperature, respectively (Fig. 2a). In order to relate electron consumption by carbon assimilation to the number of electrons transported at the level of PSII, assimilation rates were subtracted from ETR after correcting net-assimilation by
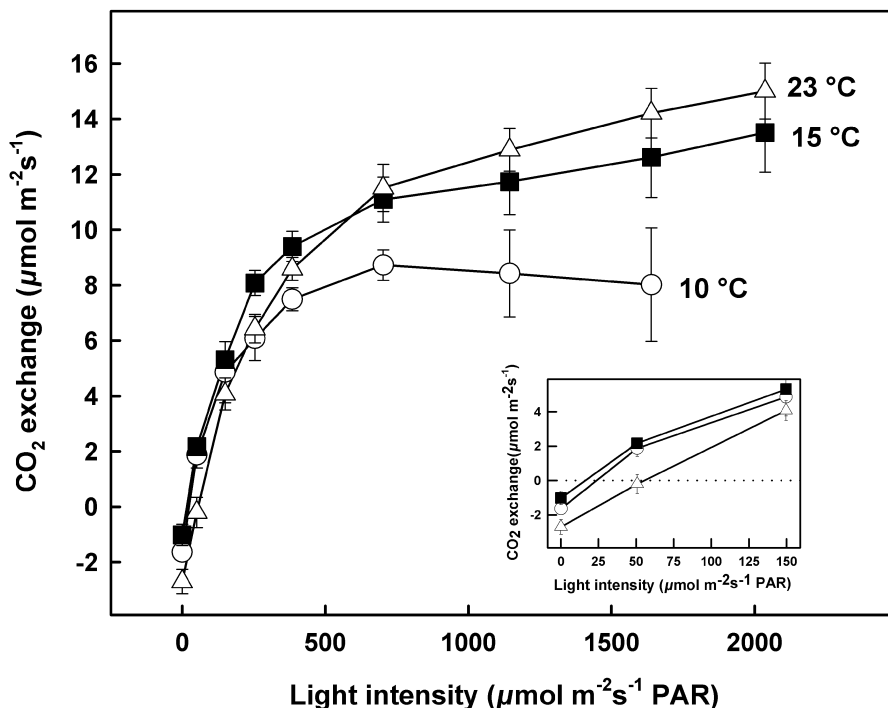

Figure 1. Light response curve of carbon assimilation in intact leaves of *R. glacialis*. Carbon assimilation was measured under atmospheric conditions (390 ppm CO_2, 21% oxygen) at different PPFD and at 23 °C (△), 15 °C (■) and 10 °C (○) leaf temperature. Insert: extension of the scale at low light intensities, demonstrating the light compensation point.

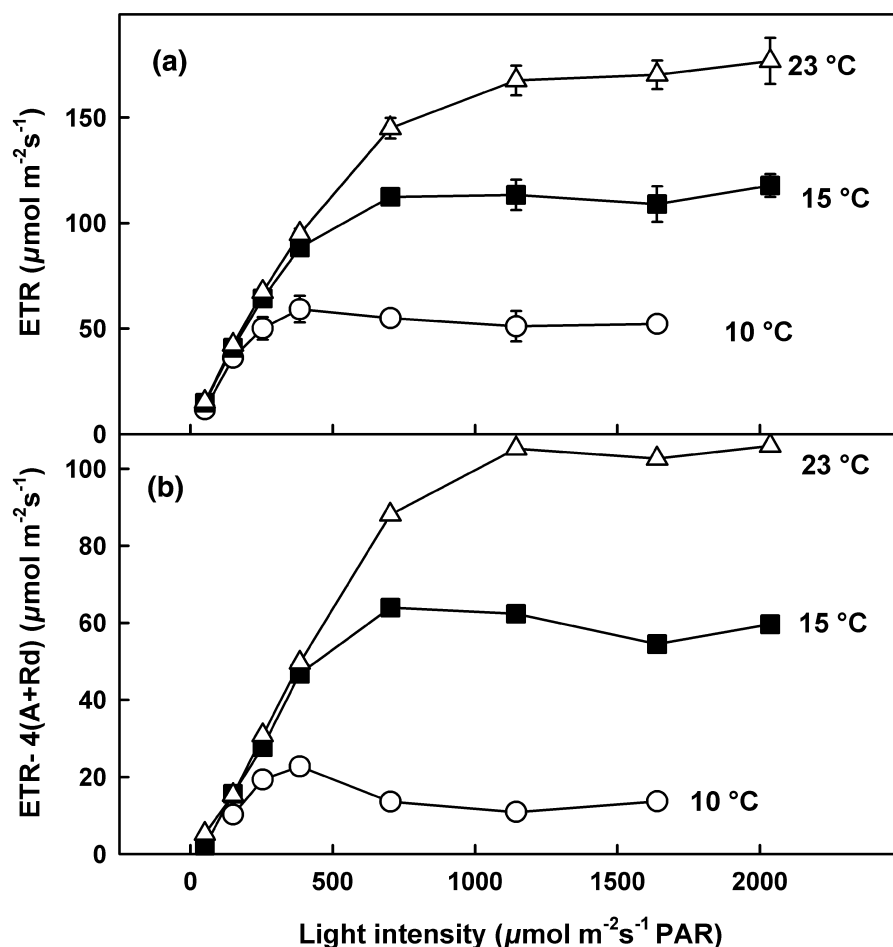**Figure 2.** (a) Light response curve of photosynthetic electron transport at PSII (ETR) in μmol electrons m^{-2} s^{-1} in intact leaves of *R. glacialis*. ETR was measured under atmospheric conditions (390 ppm CO_2, 21% oxygen) at different PPFD and at 23 °C ($\triangle$), 15 °C ($\blacksquare$) and 10 °C ($\bigcirc$) leaf temperature. (b) ETR exceeding carbon assimilation at different temperatures and light intensities. Excess ETR was calculated, assuming that carbon assimilation consumes four electrons for every CO_2 fixed and by considering leaf dark respiration (R_d) (Excess ETR = ETR − ($4A − R_d$)). Data for carbon assimilation and R_d were taken from Fig. 1.

dark respiration (R_d) and assuming that four electrons are consumed for the fixation of 1 molecule of CO_2. According to Valentini *et al.* (1995) this calculation corresponds to $J_O + 4L$ where J_O is the electron flux to oxygen and L the electron consumption by carbon assimilation, assuming that photorespiration and carbon assimilation are the only reactions competing for electrons. As shown in Fig. 2b, ETR exceeded the number of electrons consumed by carbon assimilation by up to 100 electrons m^{-2} s^{-1} at 23 °C, 60 electrons m^{-2} s^{-1} at 15 °C and 20 electrons m^{-2} s^{-1} at 10 °C, respectively. The highest difference between ETR and electron consumption was observed at light intensities close to saturation of electron transport.

Increasing light intensities enhanced successively relative Q_A reduction ($1 - q_P$) at all temperatures (Fig. 3a). In contrast, the same saturation values for F_v'/F_m' were observed for all three leaf temperatures (Fig. 3b).

Carbon assimilation and electron transport under non-photorespiratory conditions

In order to evaluate the importance of photorespiration for the consumption of excess electrons at moderate (22 °C) and cold (6 °C) temperature, carbon assimilation and ETR were measured as a function of C_i at both ambient (21%

oxygen) and depleted (0.5% oxygen) oxygen molar ratio. At 22 °C leaf temperature carbon assimilation was not completely saturated at a C_i of 1300 ppm, irrespective of oxygen partial pressure (Fig. 4a) while at 6 °C leaf temperature carbon assimilation saturated at approximately 500 ppm C_i (Fig. 5a). However, at both leaf temperatures, carbon assimilation was similar for both oxygen molar ratios when the C_i was higher than 800 ppm CO_2, suggesting that photorespiration was largely suppressed under these conditions. At a C_i lower than 800 ppm CO_2 (22 °C) and 500 ppm CO_2 (6 °C) a low oxygen molar ratio stimulated carbon assimilation indicating photorespiratory activity.

ETR was significantly higher at 21% oxygen compared to 0.5% oxygen at both leaf temperatures (Figs 4b & 5b). At low C_i the difference between ETR at normal and depleted oxygen molar ratio (ETR 21% O_2 − ETR 0.5% O_2) was the highest and decreased with increasing C_i (Figs 4c & 5c). Assuming that photorespiration in 21% oxygen is maximal at low C_i (100 ppm and suppressed at high C_i (800–1200 ppm) the difference in ETR (ETR 21% O_2 − ETR 0.5% O_2) between high and low C_i indicates a consumption of approximately 55 μmol electrons m^{-2} s^{-1} at 22 °C and of approximately 20 μmol electrons m^{-2} s^{-1} at 6 °C by photorespiration (Figs 4c & 5c). However, even at the highest C_i ETR in 21% oxygen exceeded ETR in 0.5%
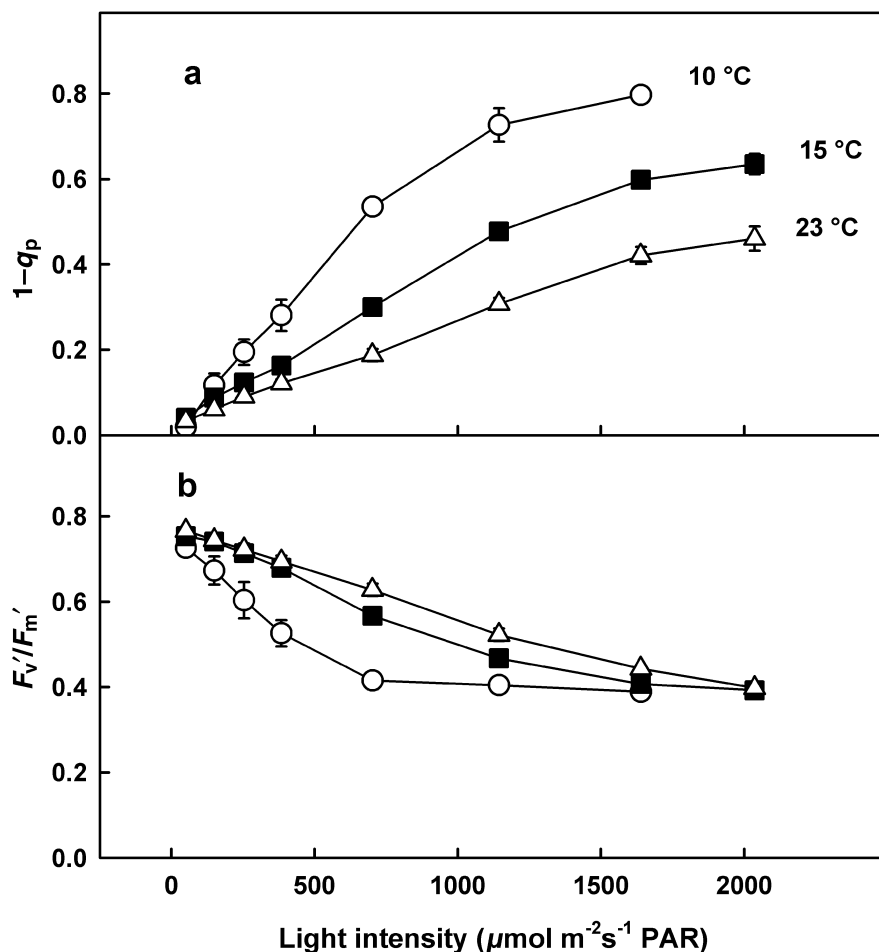
Figure 3. Light response curve of different fluorescence parameters in intact leaves of *R. glacialis*. $1 - q_P$ and F_v'/F_m' were measured under atmospheric conditions (390 ppm CO_2, 21% oxygen) at different PPFD and at 23 °C ($\triangle$), 15 °C ($\blacksquare$) and 10 °C ($\bigcirc$) leaf temperature. The parameter $1 - q_P$ reflects the relative reduction state of the primary electron acceptor Q_A of PSII. Changes in the parameter $F_v'/F_m' = F_m' - F_o'/F_m'$ indicates changes in the efficiency of non-photochemical fluorescence quenching.

oxygen by 16 μmol electrons m^{-2} s^{-1} at both temperatures (Figs 4c & 5c) showing alternative electron flow to oxygen independent of photorespiration. The ETR exceeding electron consumption by carbon assimilation at 21% oxygen is shown in Figs 4c and 5c (insert). At 22 °C leaf temperature and low C_i (100 ppm) excess ETR was as high as 105 μmol electrons m^{-2} s^{-1} while at 6 °C leaf temperature and low C_i excess electron transport was up to 56 μmol electrons m^{-2} s^{-1}. At both temperatures excess ETR at high C_i (800–1200 ppm) was between 15 and 20 μmol electrons m^{-2} s^{-1}, in agreement with the calculation of excess ETR by the difference between normal and depleted oxygen molar ratio.

Enzyme activities contributing to electron consumption pathways

Photorespiration appears to be one major sink of excess electrons in *R. glacialis* leaves, even at low temperature. However, in comparison with crop plants such as *Secale cereale,* the enzyme activity of glycolate oxidase is low in *R. glacialis* leaves (Streb *et al*. 1997). Therefore, glycolate oxidase activity was re-investigated using several extraction and measuring procedures (see Material and Methods).

The possible consumption of electrons by the photorespiratory pathway was estimated, assuming that the photorespiratory turnover cannot exceed glycolate oxidase activity and that the oxidation of 1 mol glycolate consumes 6 mol electrons in the whole photorespiration pathway. This estimation is based on the assumption that CO_2 liberated during photorespiration is re-assimilated by the Benson–Calvin cycle and most likely is an overestimation. In the absence of re-assimilation the ratio of glycolate oxidation to electron consumption would be only 4 (Ghashghaie & Cornic 1994). As shown in Table 1 photorespiration can consume up to 48 μmol m^{-2} s^{-1} electrons at 25 °C and 18 μmol m^{-2} s^{-1} electrons at 4 °C based on glycolate oxidase activity. These values correspond well with the calculation of photorespiration based on the difference in ETR between 21% oxygen and 0.5% oxygen at high (800–1200 ppm) and low C_i (100 ppm) at both leaf temperatures, as shown above. Glycolate oxidase activity is not induced by light during the day, excluding a rapid adjustment of photorespiration capacity (Table 1).

In order to evaluate the capacity of the malate valve to export excess electrons out of the chloroplast, which potentially could support electron and oxygen consumption by mitochondrial respiration, the initial and total activity of
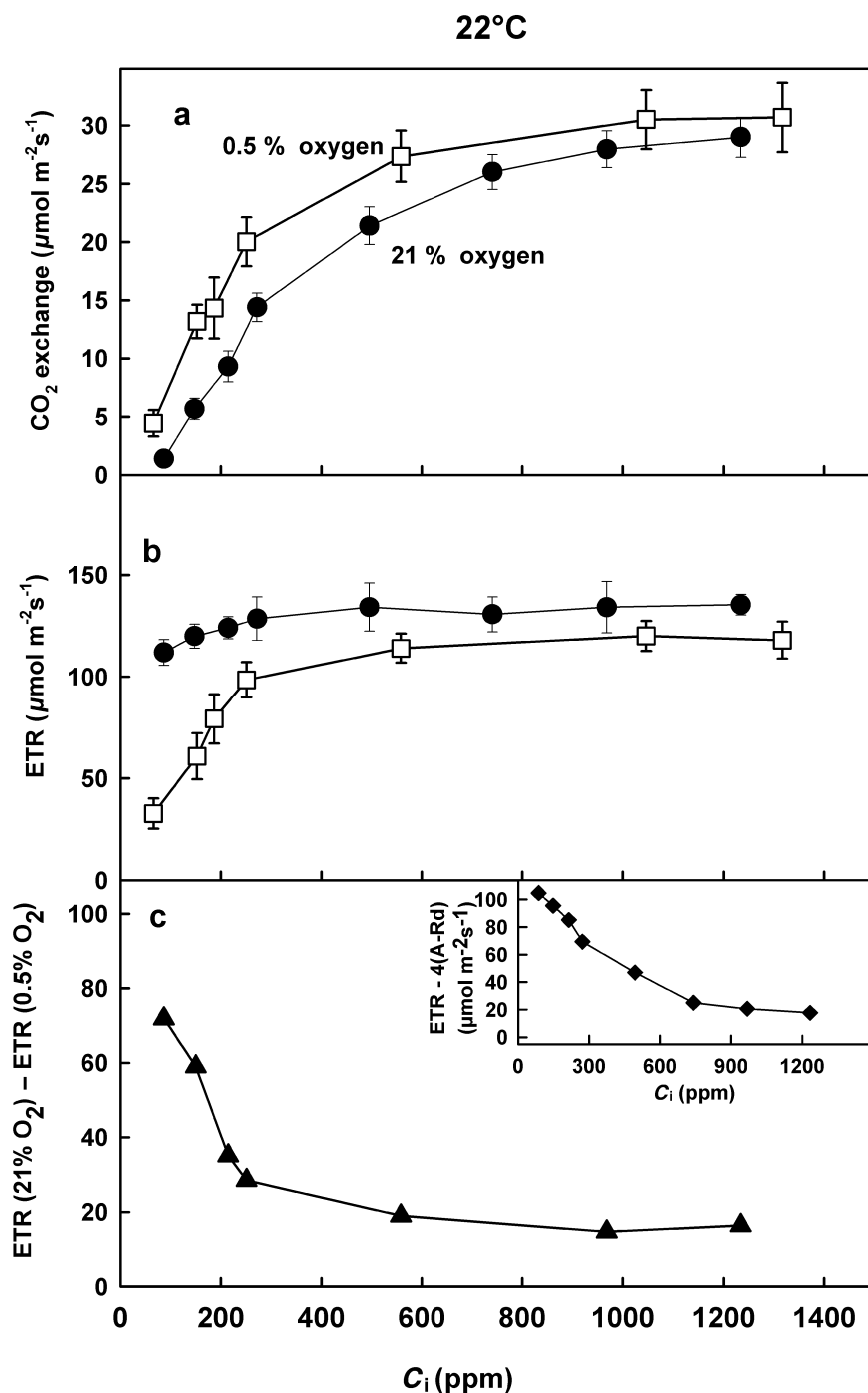
22°C

Figure 4. Effects of internal leaf CO_2 concentration (C_i) on (a) carbon assimilation (b) electron transport (c) the difference between ETR at 21% oxygen and ETR at 0.5% oxygen (ETR 21% oxygen − ETR 0.5% oxygen) and (c) (insert) on excess ETR not used for carbon assimilation (ETR − 4($A − R_d$)). ETR is calculated as μmol electrons m^{-2} s^{-1}. Leaves of *R. glacialis* were illuminated with 600 μmol m^{-2} s^{-1} PAR at 22 °C leaf temperature and at 21% oxygen (●) or under non-photorespiratory conditions at 0.5% oxygen (□).

NADP-malate dehydrogenase (NADP-MDH) was measured. As shown in Table 2, maximum NADP-MDH activity is similar in the morning and at midday as well as after exposure to darkness or artificial light. Hence, also NADP-MDH capacity was not adjusted to varying light conditions. Nevertheless the capacity of NADP-MDH would be high enough to consume between 30 and 50 μmol electrons m^{-2} s^{-1} (Table 1). However, initial NADP-MDH activity was much lower, allowing only the consumption of approximately 10 μmol electrons m^{-2} s^{-1} in light (Table 2) which is clearly less than excess ETR to oxygen. Nevertheless, the

increase of initial NADP-MDH activity during the day and the higher initial NADP-MDH after illumination at low temperature in comparison with treatment in darkness indicates a slight increase of the reduction potential of the chloroplast and a partial participation of NADP-MDH in decreasing excess electrons (Table 2).

Western blots of PTOX

Using PTOX antibodies (Cournac *et al.* 2000), a prominent signal was detected upon Western blot analysis in leaf pro-
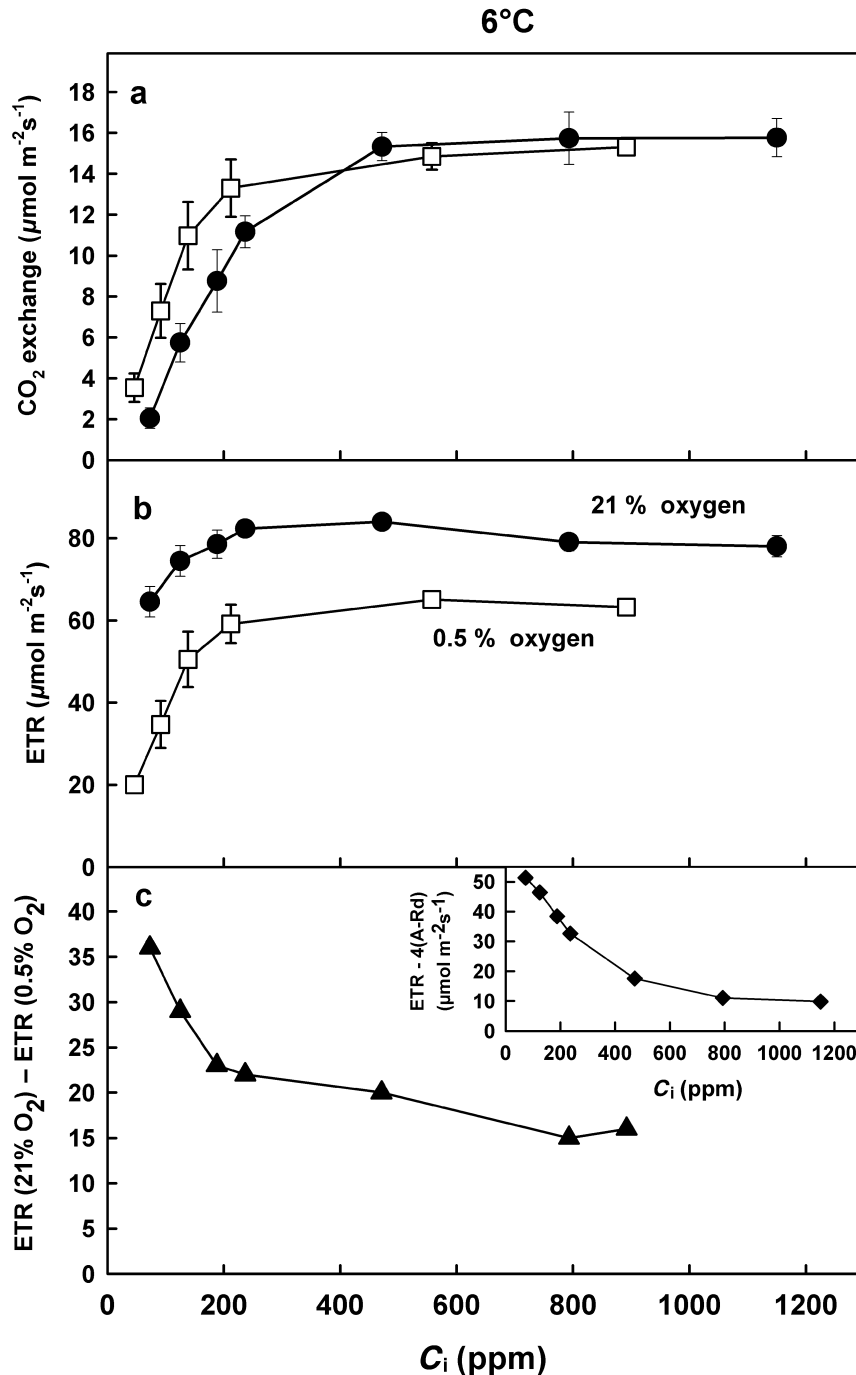
Figure 5. Effects of internal leaf CO_2 concentration (C_i) on (a) carbon assimilation (b) electron transport, and (c) the difference between ETR at 21% oxygen and ETR at 0.5% oxygen (ETR 21% oxygen – ETR 0.5% oxygen) and (c) (insert) on excess ETR not used for carbon assimilation (ETR – $4(A - R_d)$). ETR is calculated as μmol electrons $m^{-2}\,s^{-1}$. Leaves of *R. glacialis* were illuminated with 600 μmol $m^{-2}\,s^{-1}$ PAR at 6 °C leaf temperature and at 21% oxygen (●) or under non-photorespiratory conditions at 0.5% oxygen (□).

tein extract from *R. glacialis* and other alpine plant species collected at the same sampling site (*Geum montanum, Geum reptans* and *Arabis alpina;* altitude 2600 m) (Fig. 6). This result was surprising since PTOX is normally hardly detectable in total protein extracts from previously examined plants (Carol & Kuntz 2001; Josse 2003). Interestingly, this apparent abundance of PTOX correlates with altitude in *G. montanum*. At 2100 m, PTOX is only detectable in a purified chloroplast fraction, not in a total protein extract. Thus, this higher PTOX content might be important for the acclimation to high mountains, in particular for

the transport of excess electrons from plastoquinone to oxygen.

An even more prominent PTOX signal was found in *R. glacialis* (Fig. 6) with respect to the other tested plants. In order to investigate if this apparent high PTOX content is specific for *R. glacialis* or a property of the plant family Ranunculaceae, the PTOX content of several species of this family was compared. As can be seen from Fig. 7 the PTOX content is much higher in *R. glacialis* in comparison with the other species of this plant family on the basis of equal protein content.

Table 1. Glycolate oxidase activity of *R. glacialis* leaves collected in the morning or at midday at the Galibier pass in μkat m^{-2}

	Activity at 25 °C (μkat m^{-2})	E-consumption at 25 °C (μmol m^{-2} s^{-1})	E-consumption at 4 °C (μmol m^{-2} s^{-1})
Morning	7.9 ± 0.1	47.4 ± 0.6	18
Midday	7.3 ± 1.4	43.8 ± 8.4	16.6

Glycolate oxidase activity was measured at 25 and at 4 °C. Glycolate oxidase activity at 4 °C declined by $62 \pm 8\%$ in comparison with the measurement at 25 °C. For the calculation of electron consumption (μmol m^{-2} s^{-1}) by photorespiration, it is assumed that photorespiration cannot proceed faster than the enzymes involved in the cycle and that 6 μmol electrons are consumed by photorespiration after the oxidation of 1 μmol glycolate. This calculation includes CO_2 reassimilation by the Benson–Calvin cycle, otherwise electron consumption is lower (Ghashghaie & Cornic 1994)

Table 2. Initial and maximal NADP-MDH activity in μkat m^{-2} in *R. glacialis* leaves collected in the morning or at midday at the Galibier pass

	Morning	Midday	6 h darkness	6 h 1000 μmol m^{-2} s^{-1} PAR
Total activity (μkat m^{-2})	23.1 ± 7.3	23.6 ± 5.4	16.7 ± 3.1	16.3 ± 1.8
e-consumption (μmol m^{-2} s^{-1})	46	47	33	32
Initial activity (μkat m^{-2})	2.7 ± 0.3	4.1 ± 1.2	3.2 ± 0.8	6.5 ± 0.9
e-consumption (μmol m^{-2} s^{-1})	5.4	8.2	6.4	13

Initial NADP-MDH activity is also shown for leaves incubated for 6 h at 6 °C in darkness or at 1000 μmol m^{-2} s^{-1} PAR. Electron consumption of NADP-MDH is calculated with the assumption that two electrons are taken for the reduction of 1 molecule of oxaloacetate to malate.

To exclude the possibility that our antibody against PTOX (raised against the protein from *Arabidopsis*) reacts simply better with PTOX from *R. glacialis* than with PTOX from other plant species, we used transgenic tomato plants over-expressing approximately 50-fold the PTOX gene from *Arabidopsis* (Josse 2003). Since it is unlikely that our antibodies react better with PTOX from *R. glacialis* than from *Arabidopsis*, and since a stronger signal is observed in *R. glacialis* than in these transgenic plants (Fig. 8), we conclude that the PTOX level is exceptionally high in *R. glacialis*.

Unfortunately no current test is available to measure the function of PTOX for decreasing excitation pressure of PSII and for protection against photoinhibition. However, it was shown previously that *R. glacialis* leaves partially deacclimate against high light-induced photoinhibition when grown at 22 °C and low light for 3 weeks (Streb *et al.* 2003a). The content of PTOX was therefore compared between deacclimated leaves of *R. glacialis* and leaves collected in the mountains. As shown in Fig. 8 the content of PTOX decreases markedly in plants grown at 22 °C. Furthermore, the level of PTOX protein was not rapidly established during 6 h high light exposure at 25 °C. In Fig. 8 PTOX contents were also compared with those of NAD(P)-dehydrogenase (NDH H) which catalyses cyclic electron transport around PSI and those of IE 37, an inner
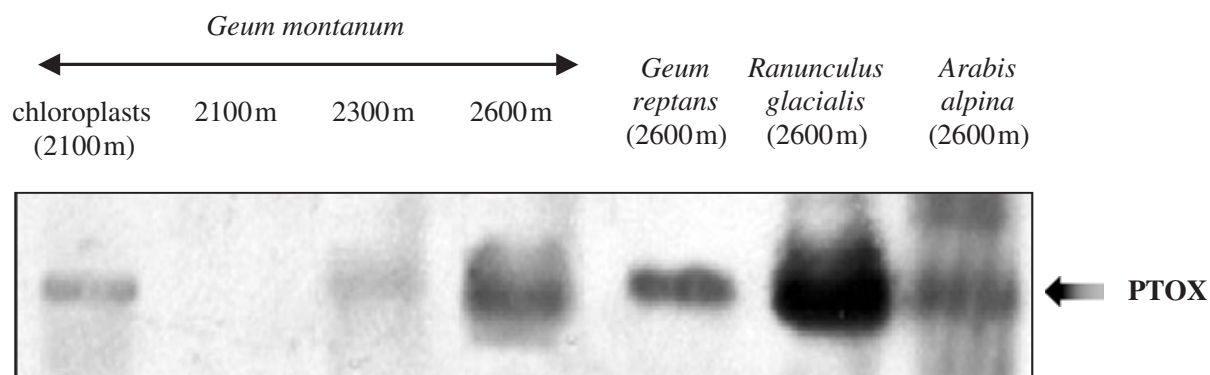

Figure 6. Immunodetection of the 37 kDa subunit of plastid terminal oxidase (PTOX) after SDS-PAGE separation of 60 μg lipid soluble protein from leaves or chloroplasts of different alpine species. *Ranunculus glacialis*, *Geum reptans* and *Arabis alpina* were collected at 2600 m altitude from the same sampling site. *Geum montanum* was collected at 2100, 2300 and 2600 m altitude. The position of PTOX is indicated. The antibody was diluted to a final concentration of 1/15 000.

Ranunculus glacialis	*Aquilegia vulgaris*	*Trollius europaeus*	*Ranunculus auricomus*

← PTOX

Figure 7. Immunodetection after SDS-PAGE electrophoresis of 30 µg membrane proteins of the 37 kDa subunit of PTOX (final dilution of the antibody 1/15 000) in several species of the family Ranunculaceae. *Ranunculus glacialis* was collected at 2600 m at the Galibier pass, *Aquilegia vulgaris* and *Ranunculus auricomus* were cultivated at 200 m elevation and *Trollius europaeus* was collected at 1700 m elevation.

envelope membrane protein probably acting as methyl-transferase, as control protein for loading. While the content of IE 37 was similar in all samples tested the content of NDH H is elevated in *R. glacialis* leaves from the Galibier site and decreases during deacclimation (Fig. 8).

DISCUSSION

Ranunuculus glacialis has a broad temperature optimum for photosynthetic assimilation, a high photosynthetic capacity and photosynthesis is not saturated by high light intensities (Moser *et al.* 1977; Körner & Diemer 1987). This raises the question of whether chilling tolerance in light is achieved by the ability to balance carbon assimilation and photosynthetic electron transport. A broad temperature optimum of carbon assimilation in air (21% oxygen, 390 ppm CO_2) at moderate light intensity was also observed

in this study (not shown). Furthermore, the ability of net carbon uptake at low temperature and low light intensity and the fact that photosynthesis is not saturated by high light intensity at moderate temperature were confirmed (Fig. 1). However, a comparison of ETR and the consumption of electrons by carbon assimilation showed a significant difference at all temperatures tested (Fig. 2). At leaf temperatures above 15 °C more than 50% of transported electrons are not used for carbon assimilation from ambient air. At a leaf temperature of 10 °C excess ETR is slightly lower.

The ETR is a function of Φ_{PSII} which depends both on the reduction state of Q_A as estimated by q_P and on the efficiency of non-photochemical quenching as reflected by changes of F_v'/F_m' ($\Phi_{PSII} = q_P \times F_v'/F_m'$) (Genty *et al.* 1989). As shown in Fig. 3, F_v'/F_m' saturates at the same high light intensity, independently of temperature. In contrast, $Q_A(1 -$
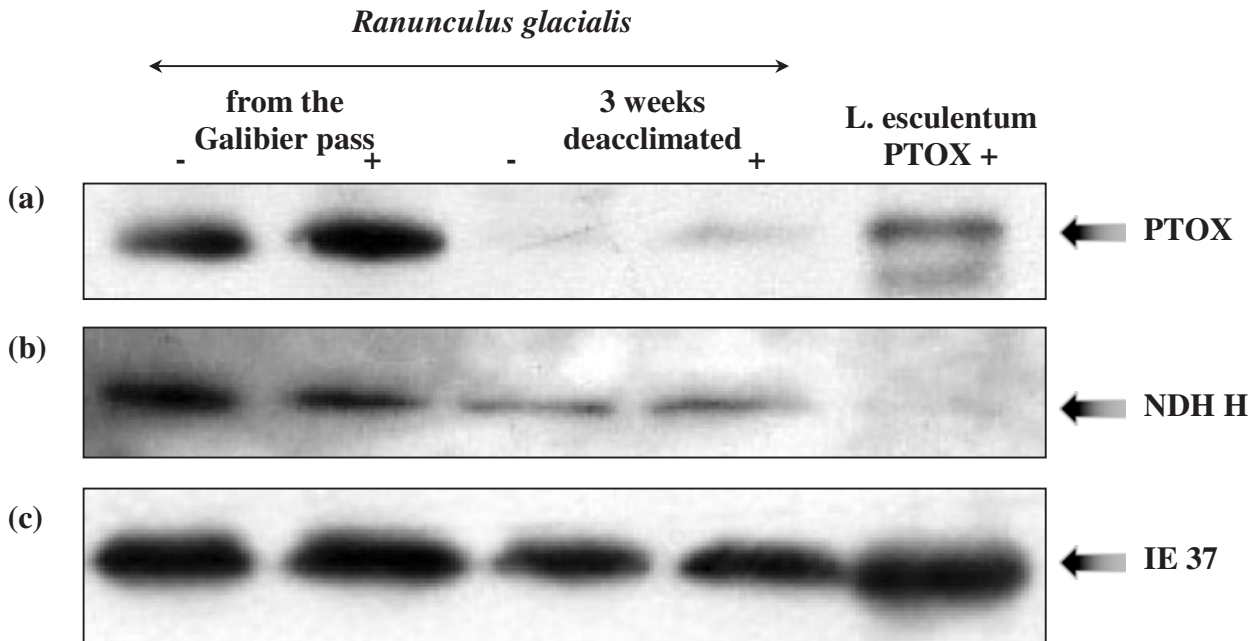
Ranunculus glacialis

Figure 8. Immunodetection after SDS-PAGE electrophoresis of 30 µg membrane proteins of (a) the 37 kDa subunit of PTOX (final dilution of the antibody 1/15 000); (b) the H subunit of NAD(P)-dehydrogenase (NDH H) (final dilution of the antibody 1/1000); and (c) of the IE 37 protein (final dilution of the antibody 1/150000) in leaves of *R. glacialis* and *Lycopersicon esculentum*. *R. glacialis* leaves were either collected at the Galibier pass or transferred to low elevation and grown for 3 weeks at elevated temperature and low light intensity in order to deacclimate the plants. Leaves were either directly analysed (–) or illuminated at 1000 µmol m^{-2} s^{-1} PAR for 6 h at 25 °C (+). Leaves of transgenic *L. esculentum* overexpressing the PTOX gene from *Arabidopsis* were analysed for comparison.

q_P) is relatively more reduced at lower temperatures, particularly in higher light intensities, suggesting that under these conditions ETR is controlled mainly by the consumption of electrons and less by nonradiative dissipation of excitation energy (i.e. non-photochemical fluorescence quenching). This confirms previous observations that non-photochemical fluorescence quenching is of minor importance for photoprotection in *R. glacialis* (Streb *et al.* 1998). Nevertheless, although 80% of Q_A is reduced at the lowest temperature and the highest light intensity applied (Fig. 3) *R. glacialis* maintains Q_A in a much more oxidized state than other alpine plant species or cold-acclimated rye under comparable conditions (Streb *et al.* 1998; Streb & Feierabend 1999), implying efficient electron consumption.

Besides carbon assimilation, photorespiration is the main sink of photosynthetically generated electrons, particularly if re-assimilation of liberated CO_2 is taken into account. Under these conditions the synthesis of one mole of glycolate-phosphate would consume 6 mol of electrons (Ghashghaie & Cornic 1994). This assumption was used to calculate potential electron consumption by the photorespiratory pathway based on the enzyme activity of glycolate oxidase, suggesting that photorespiratory activity cannot be faster than maximum activities of enzymes involved in the pathway. Maximum glycolate oxidase activity, however, indicated that up to 50 μmol electrons m^{-2} s^{-1} can be consumed at 25 °C and approximately 20 μmol electrons m^{-2} s^{-1} at 4 °C, respectively (Table 1).

Excess electrons are indeed transported to oxygen at both temperatures as shown by lower ETR in an oxygen-depleted atmosphere in comparison with an atmosphere with 21% oxygen (Figs 4 & 5). Moreover, at elevated C_i there is little (above 800 ppm CO_2 at 22 °C) or no (above 500 ppm CO_2 at 6 °C) enhancement of CO_2 assimilation at 0.5% oxygen as compared to 21% oxygen. This suggests that Rubisco oxygenase activity is greatly suppressed under these conditions. However, even when Rubisco oxygenase activity is suppressed approximately 16–20 μmol electrons m^{-2} s^{-1} are still transported to oxygen at both temperatures (based on calculations at the highest ci either by ETR exceeding carbon assimilation in 21% oxygen or by the difference in ETR between 21% oxygen and 0.5% oxygen, Figs 4c & 5c). Maximum ETR to photorespiration was estimated between low and high C_i by the difference in ETR between 21% oxygen and ETR in 0.5% oxygen. This calculation shows that photorespiration consumes approximately 50 μmol electrons m^{-2} s^{-1} at 22 °C and approximately 20 μmol electrons m^{-2} s^{-1} at 6 °C leaf temperature. Both values correspond well with maximum glycolate oxidase activity at these temperatures, as shown above. In conclusion, at low and ambient temperatures, another oxygen-consuming pathway beside photorespiration must exist, consuming up to 50% of excess ETR not used for carbon assimilation at both temperatures.

It is generally assumed that the water–water cycle consumes excess electrons with a capacity of up to 30% of total photosynthetic electron flow (Asada 1999; Ort & Baker 2002). Assuming that the water–water cycle consumes all electrons in excess one must suggests that it operates with maximal activity in *R. glacialis* leaves at 22 °C. However, although the contribution of the water–water cycle to electron consumption is not known, indirect evidence suggests that a high activity is unlikely as: (1) the concentration of antioxidants and the activity of antioxidative enzymes are very low; (2) the antioxidants become oxidized during the day, suggesting that their reduction is limited by enzyme activities; and (3) *R. glacialis* leaves are very sensitive to even low concentrations of paraquat, contrary to other plant species acclimated to low temperature (Streb *et al.* 1997, 1998; Streb & Feierabend 1999). Paraquat enhances the reduction of oxygen at PSI to superoxide and leads to marked photobleaching in *R. glacialis* (Streb *et al.* 1998). Furthermore, oxygen reduction at PSI requires a high reduction potential of the chloroplast. Before this reduction potential would be high enough to reduce oxygen in the Mehler reaction the malate valve would be activated protecting the chloroplast from over-reduction (Backhausen *et al.* 2000; König *et al.* 2002). Under these conditions, however, one can expect that the chloroplastic NADP-MDH would be highly activated (Miginiac-Maslow *et al.* 2000; Scheibe 2004). As shown in Table 2, initial NADP-MDH activity increases during the day or during illumination at low temperature, indicating accumulation of excess electrons in the chloroplast but is far from its maximum activity. Nevertheless, the malate valve could potentially transport excess electrons out of the chloroplast to be used during oxidative phosphorylation in the mitochondria (Scheibe 2004) if the enzyme is fully activated. The actual activity of NADP-MDH, however, allows only partial consumption of excess electrons in the chloroplast.

As alternative to the above discussed electron sinks, PTOX might potentially consume excess electrons in the thylakoid membrane. PTOX has been shown to be able to transfer electrons from plastoquinone directly to oxygen and thus to avoid reduction of the plastoquinone pool during light induction (Joët *et al.* 2002; Josse *et al.* 2003). However, the potential electron consumption by PTOX is currently thought to be very low, consuming maximally 0.3% of total photosynthetic saturated electron transport (Ort & Baker 2002). Nevertheless, a role of PTOX as final electron acceptor has previously been assumed for photosystem I-deficient algae (Cournac *et al.* 2000) and *Nicotiana tabacum* antisense plants lacking catalase and ascorbate peroxidase (Rizhsky *et al.* 2002). Since no practical test for PTOX activity in *R. glacialis* was available, it was not possible here to unequivocally demonstrate the proposed function of PTOX as alternative electron sink in *R. glacialis* leaves. However, several unique properties of *R. glacialis* leaves suggest that PTOX could fulfil this function in this plant species. PTOX is present in *R. glacialis* leaves in much higher concentrations than in other alpine plant species, other species from the Ranunculaceae family or transgenic tomato leaves overexpressing PTOX from *Arabidopsis thaliana*, against which the antibody was constructed (Figs 6–8). Furthermore, mRNA transcripts were found in major leaves of *R. glacialis* but not in other alpine plant

species (Josse 2003). In addition, *R. glacialis* contains high amounts of NDH H, an enzyme mediating cyclic electron transport around PSI (Peltier & Cournac 2002) when compared with transgenic tomato leaves.

Ranunculus glacialis plants can be partially deacclimated against chilling-induced photoinhibition by growing the plants for several weeks at elevated temperature and low light intensity (Streb *et al.* 2003a). Under these conditions the PTOX protein is largely lost in *R. glacialis* as well as the NDH H protein and its synthesis cannot be induced by short periods of high light (Fig. 8). However, even if PTOX and NDH H are contributing significantly to light and stress protection in *R. glacialis* it must be assumed that its activity is strictly regulated in order to allow carbon assimilation and to prevent excess electron consumption by PTOX. Finally, PTOX and NDH H activity would change the photosynthetically produced NADPH/ATP ratios markedly in favour of ATP, necessitating sinks for ATP overproduction. It should be noted, that ATP/ADP ratios remained constant at high light and low temperature, while PGA accumulation indicates a limitation of carbon assimilation (Streb *et al.* 2003b). The investigation of PTOX regulation awaits, however, on the development of a suitable assay for its activity.

CONCLUSION

Although carbon assimilation is very flexible in *R. glacialis,* electron transport exceeds energy consumption by the Benson–Calvin cycle markedly. Excess electrons are partially consumed by photorespiration, even at low temperature. This confirms previous observations, that photoinhibition of PSII is enhanced when photorespiration is blocked by phosphinothricin (Streb *et al.* 1998). However, the capacity of photorespiration is insufficient for the consumption of all electrons in excess. It is hypothesized, that the high abundance of PTOX and its decline during deacclimation indicates that chlororespiration is the second major electron sink in *R. glacialis* with oxygen as final acceptor. Oxygen reduction by PTOX has the advantage that no harmful reactive oxygen species, that would damage *R. glacialis* leaves, are produced. While PTOX might consume electrons separated by PSII, NDH H might cycle electrons around PSI. *Ranunculus glacialis* is the first plant species with such extraordinary high protein content of PTOX, and therefore offers a new perspective on plant acclimation to high light intensity and low temperature.

ACKNOWLEDGMENTS

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