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ORIGINAL PAPER

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Responses of *Colobanthus quitensis* (Kunth) Bartl. to high light and low temperature

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Abstract Antarctic plants are exposed to high light and low temperature during their natural growing season. Therefore, the maintenance of an oxidative status compatible with normal metabolism is an important determinant for their survival. We hypothesized that *Colobanthus quitensis*, the only dicotyledonous plant from the Antarctic, has efficient energy-dissipating mechanisms, and/or high antioxidant capacity. A short time treatment (24 h) of high light and/or low temperature reduced maximum photochemical quantum yield (Fv/Fm), photochemical quenching (qP), and the quantum yield of PSII (ΦPSII). Concomitantly, an increase in the nonphotochemical quenching (qN) was observed. Superoxide dismutase (SOD) activity increased after cold acclimation. High light and low temperature did not significantly affect SOD, ascorbate peroxidase (APx), and glutathione reductase (GR) in nonacclimated plants. Total soluble antioxidants and carotenoids decreased after cold acclimation. Thus, it is likely that the main mechanism of *C. quitensis* to cope with energy imbalance is heat dissipation, as evidenced by the great increase in qN.

Introduction

Plants exposed to high light and low temperature absorb more energy than the amount that can be used by the Calvin cycle and other photochemical reactions. As a result, part of this excess energy is then transferred to O_2, leading to the accumulation of reactive oxygen species (ROS) (Kaiser 1987). ROS production occurs in different cellular compartments, such as the mitochondria, peroxisomes, and chloroplasts. The photosynthetic electron transport system constitutes the major source of ROS in plants (Foyer et al. 1994). Two main forms of ROS are apparently involved in the initiation of photooxidative damage in vascular plants: superoxide radical O_2^- and singlet oxygen $^1O_2$ (Salin 1987). Accumulation of the superoxide radical in the chloroplast is usually kept under control by the consecutive action of the enzymes superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APx, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2); meanwhile, singlet oxygen production is controlled by carotenoids (Cogdell and Frank 1987; Krinsky 1989; Palozza and Krinsky 1992). However, when ROS production exceeds its scavenging by antioxidants, ROS accumulate and cause cellular damage by oxidizing lipids, proteins, and nucleic acids.

ROS participation in low temperature damage is supported by the correlation that exists between hydrogen peroxide accumulation and damage by cold in plants such as cucumber (Omran 1980), winter wheat (Anderson et al. 1994), and maize (Prasad et al. 1994). ROS participation in damage by high light causes increased degradation of proteins, such as glutamine synthetase, phosphoglycolate phosphatase, and the large subunit of Rubisco, together with a higher carbonyl group content in stromal proteins (Stieger and Feller 1997).

An antioxidant system able to control ROS accumulation under conditions that promote oxidative stress may be an important determinant for the survival of plants in extreme environments, such as polar regions and mountains. In this context, *Colobanthus quitensis* (Kunth) Bartl. is specially interesting, because it is the only dicotyledonous plant that has successfully colonized the maritime Antarctic (Xiong et al. 1999, 2000; Bravo et al. 2001; Alberdi et al. 2002). Among the traits of *C. quitensis* partially responsible for its survival in this region are the ability to avoid the formation of ice in its tissues by supercooling, conservation of 30% of its optimum photosynthetic rate at 0°C, and reversible photoinhibition

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at low temperature (Edwards and Lewis-Smith 1988; Xiong et al. 1999; Bravo et al. 2001). The maximum photosynthetic rate is reached at 15°C, while light saturation is achieved in the range of 50–100 μmol photons m^2 s^{-1}, depending on temperature (Edwards and Lewis-Smith 1988). These traits altogether improve the fitness of *C. quitensis* for cold environments.

During its growing season in the Antarctic summer, *C. quitensis* must cope with episodes of high light (1,500 μmol $m^{-2}s^{-1}$) and constant low air temperature (−2 to 5°C), conditions that are known to cause an energy imbalance and high PSII excitation pressure which promote oxidative stress in plants (Huner et al. 2002). We hypothesize that *C. quitensis* has an efficient energy-dissipating system or elevated levels of antioxidants under high light and/or low temperature conditions. To test this hypothesis, we subjected nonacclimated plants to a combination of high light and low temperature for 24 h and analyzed fluorescence quenching. The effect of high light and/or low temperature in antioxidant content and enzyme activities involved in superoxide detoxification in cold-acclimated and nonacclimated *C. quitensis* was also studied.

Materials and methods

Plant material and growth conditions

C. quitensis (Kunth) Bartl. (Cariophyllaceae) was collected in Robert Island, maritime Antarctic (62°22′S; 59°43′W). Plants were propagated vegetatively in plastic pots using a soil:peat mixture (3:1) and were fertilized with 0.12 g l^{-1} Phostrogen (Solaris, Buckinghamshire, UK) once every 2 weeks. Plants were placed in a growth chamber with a photon flux density (PFD) of 100 μmol $m^{-2}s^{-1}$ (cool-white fluorescent tubes F40CW, General Electric, Charlotte, N.C.) at 15°C (100/15) at 21/3 h light/dark photoperiod. Cold acclimation consisted of transferring plants described above to growth chambers set at 100 μmol $m^{-2}s^{-1}$ PFD and 4°C (100/4) for 21 days. High light and low temperature treatments were performed at 1,600 μmol $m^{-2}s^{-1}$ at the top of the plant in a growth chamber set at 4°C (1600/4). The light source was a 1,000-W halogen lamp, located over the plants, with a transparent water circulation filter inserted between the lamp and plants. Low-temperature treatments were made by transferring plants from growth chambers at 100 μmol $m^{-2}s^{-1}$ and 15°C to chambers with the same light intensity set at 4°C (100/4). Samples were taken at 1, 3, 6, 12, and 24 h of treatment. Values reported correspond to the mean of three replicates.

Photoinhibitory treatment and fluorescence measurements

The combined effect of low temperature and high light on the photochemical efficiency of *C. quitensis* was studied. Nonacclimated plants were transferred to a growth chamber at 4 ± 1.5°C or maintained at 15°C, and PFD (100 and 1,600 μmol $m^{-2}s^{-1}$) for 24 h. After each treatment, plants were removed from the climatic chamber for fluorescence measurements at room temperature. Fluorescence signals were generated by a pulse-amplitude modulated fluorometer (FMS II, Hansatech Instruments, UK) according to Schreiber et al. (1986). Fully developed leaves (attached to the plant) from nonacclimated plants were dark-adapted for 30 min (to obtain open centers) by covering the plant attached to the fluorescence probe with a piece of black cloth. Signal recordings and calculations were performed using the data analyses and control software provided with the instrument (Hansatech Instruments, Norfolk, UK). Minimal fluorescence (Fo), that is, the fluorescence of dark-adapted leaves, was determined applying a weak modulated light (0.4 μmol $m^{-2}s^{-1}$). Maximal fluorescence (Fm) was induced by a short saturating pulse 0.8 s of 9,000 μmol $m^{-2}s^{-1}$ of actinic light. Steady state fluorescence (Fs) was obtained after illumination of leaves from the exterior, and by emission of a new saturating light pulse from the FMSII, the light-adapted fluorescence maximum (Fm'), quantum yield of PSII (ΦPSII), and photochemical (qP) and nonphotochemical (qN) quenching were calculated according to Maxwell and Johnson (2000).

Enzyme extraction and determinations

Fresh leaves (0.1 g) were frozen in liquid nitrogen and homogenized in an Eppendorf tube with 100 mM phosphate buffer. The buffer pH and volume used depended on the enzyme and are indicated in the respective paragraph. The homogenate was centrifuged at 14,500 *g* and the supernatant was separated for measurements.

Superoxide dismutase (EC 1.15.1.1)

The extraction buffer (300 μl, pH 7.8) contained 2 mM EDTA and 1% PVP (polyvinylpyrrolidone) at 4°C. SOD activity in the homogenate was determined according to McCord and Fridovich (1969) with modifications by Schöner and Krause (1990), by following the rate of reduction of ferricytochrome c at 550 nm. One unit of SOD is defined as the amount of enzyme that inhibits in 50% the reduction of ferricytochrome c at pH 7.8 and 25°C.

Ascorbate peroxidase (EC 1.11.1.11)

To prepare the homogenate for APx, 400 μl of extraction phosphate buffer (pH 7.5) containing 1 mM EDTA and 5 mM ascorbic acid at 4°C were used. APx activity was assayed according to Rao et al. (1996) by monitoring OD at 290 nm for 2 min in a 1-ml solution containing 0.5 mM ascorbate and 200 μM H_2O_2.

Glutathione reductase (EC 1.6.4.2)

The extraction phosphate buffer (300 μl, 100 mM potassium phosphate, pH 7.6) contained 0.05% Triton X-100, 2% PVP, 1 mM ascorbic acid and 2 mM EDTA. Enzyme activity was determined by the procedure of Foyer and Halliwell (1976), as modified and described by Donahue et al. (1997). This method is based on the spectrophotometric determination of the NADPH oxidation rate, at 340 nm, catalyzed by GR activity present in the sample. One unit of GR is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH per minute at 25°C at pH 7.6.

Superoxide dismutase activity staining gels

SOD isozymes were determined according to Rao et al. (1996). Equal amounts of SOD activity (1 Unit) were subjected to native PAGE at 12% at 80 mV for 6 h at 4°C. Because SOD isozymes are differentially sensitive to KCN and H_2O_2, isozyme identification as Cu/ZnSOD or MnSOD was achieved by incubation with 3 mM KCN or 5 mM H_2O_2 for 30 min before staining for SOD activity.

Total antioxidants

Aerial tissue (0.1 g) was homogenized in an Eppendorf tube with liquid nitrogen and extracted with phosphate buffer 100 mM pH 7.4. The homogenate was centrifuged at 14,500 *g* and the

supernatant was separated and used in the determination. The method is based on the ability of soluble antioxidants present in the sample to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzotiazoline sulfonate] (ABTS) to $ABTS^{+}$ by metmyoglobin which is followed at 600 nm (Calbiochem kit no. 615700).

Pigments

Carotenoids and chlorophyll were determined according to Lichtenthaler and Wellburn (1983). Aerial tissue (0.1 g) was powdered in liquid nitrogen and then extracted with ethanol at 96% under low light. Absorbance was measured at 470, 649, and 665 nm.

Statistical analysis

Values reported correspond to the means of three independent assays. A two-way (temperature×light) ANOVA was used for qP, qN, Fv/Fm, and quantum yield of PSII. For enzyme activities, differences among treatments and their interactions were determined by three-way-ANOVA (light×time×growth temperature) analyses ($P < 0.05$). These analyses were followed by a Tukey test.

Results

Photochemical efficiency

Short-term effect of high light, low temperature or the combination of both on the photochemical efficiency of nonacclimated *C. quitensis* (15°C and 100 μmol $m^{-2}s^{-1}$) was studied using modulated pulse fluorescence (Table 1). High light (1,600 μmol $m^{-2}s^{-1}$) combined with low temperature (4°C) produced a 41% decrease in the maximum photochemical quantum yield (Fv/Fm) and a 67% decrease in the steady state quantum yield of PSII (ΦPSII) (Table 1). The higher decrease in the steady state quantum yield compared to maximum quantum yield may reflect a fast recovery of plants in darkness after exposure to photoinhibitory conditions. The excitation pressure of PSII (1-qP) in plants under high light and low temperature was fourfold higher than in control plants, indicating a significant higher proportion of closed reaction centers in plants exposed to this combination of factors. Nonphotochemical quenching in these nonacclimated plants remained low at low light intensity. Unexpectedly, the highest qN value was observed in plants exposed to high light at 15°C, being 61% higher than at high light and low temperature (4°C). Thus, heat dissipation is maximized at high light intensity, but somehow impaired by low temperature. Interactions among factors (temperature and light) were highly significant ($P < 0.05$) for all fluorescence parameters, with the exception of qP.

Table 1 Effect of irradiance and temperature on the fluorescence parameters in *Colobanthus quitensis*. Plants were grown at 15°C and 100 μmol $m^{-2}s^{-1}$ (t_0). Plants were transferred to the different treatments of light intensity combined with low temperatures. Pulse-modulated fluorescence profiles were determined using FMS II (Hansatech). Different *letters* indicate statistically significant differences within *rows* (Tukey test; $P < 0.05$)

Fluorescence parameters	t_0	24 h of treatment		
	15/100	4/100	15/1600	4/1600
Fv/Fm	0.85 ± 0.01a	0.83 ± 0.01a	0.63 ± 0.01b	0.50 ± 0.01c
ϕPSII	0.75 ± 0.01a	0.64 ± 0.01b	0.50 ± 0.02c	0.25 ± 0.02d
qP	0.89 ± 0.02a	0.77 ± 0.02b	0.63 ± 0.02c	0.53 ± 0.03d
1−qP	0.11 ± 0.01a	0.23 ± 0.01b	0.37 ± 0.02c	0.47 ± 0.03d
qN	0.11 ± 0.01a	0.07 ± 0.01a	0.79 ± 0.02b	0.48 ± 0.03c

SOD activity and isozymes

SOD activity in nonacclimated *C. quitensis* (NA) presented small but significant oscillations during the 1st 6 h of exposure to low temperature and high light (Fig. 1A). After this time, plants exposed to high light and low temperature presented a significantly lower value than plants at low light at both temperatures ($P < 0.05$). Acclimation to low temperature for 21 days resulted in a significant 40% increase in SOD activity when compared with nonacclimated plants ($P < 0.05$). Cold-acclimated plants treated with high light had a significantly lower SOD activity after 1 h of exposure and along the 24 h of assay than cold-acclimated plants maintained at low light (Fig. 1B) ($P < 0.05$). The three-way ANOVA showed significant interactions among the three factors (growth temperature, light, and time).

Three major bands with SOD activity were detected by the electrophoretic analysis of native protein extracts from both cold-acclimated and nonacclimated *C. quitensis*. Differential sensitivity of these bands' activity to incubation of gels with KCN and H_2O_2 revealed that the band with the lower mobility corresponded to an MnSOD and the other two bands with higher electrophoretic mobility corresponded to FeSOD (Fig. 2).

APx activity

APx activity in nonacclimated plants did not vary significantly during low-temperature treatment for 24 h at low light; yet, low temperature and high-light treatment significantly ($P < 0.05$) decreased APx activity after 1 h of treatment and then increased by 40% at 24 h (Fig. 1C). Cold-acclimated plants exhibited 25% less APx activity than nonacclimated plants (Fig. 1C,D). High-light and low-temperature treatment in cold-acclimated plants slightly decreased APx activity between 1 and 12 h of treatment (Fig. 1C) ($P < 0.05$).

GR activity

C. quitensis GR activity presented similar response patterns in cold-acclimated and nonacclimated plants exposed to low temperature and high light. GR activity significantly ($P < 0.05$) decreased at 3 h of exposure, and then recovered and increased at 12 h, and finally re-

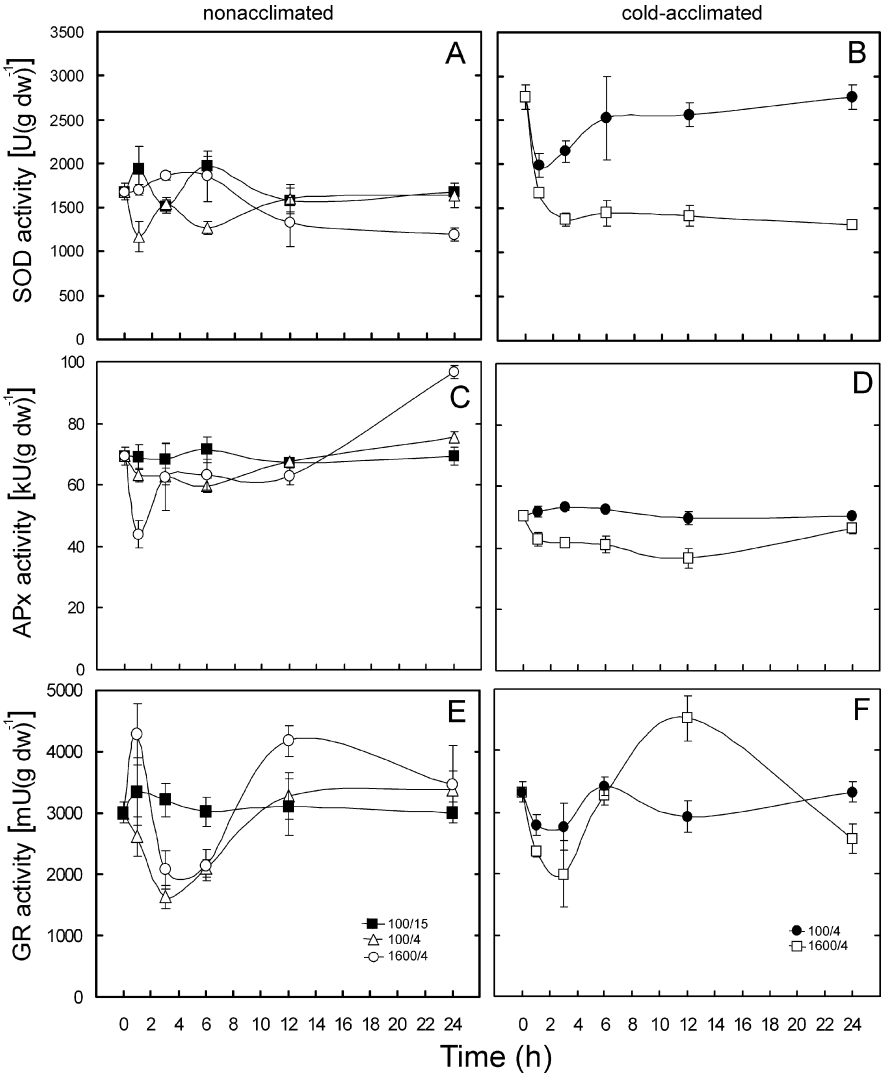

Fig. 1A–F SOD, APx and GR activities in *Colobanthus quitensis*. Graphs **A**, **C** and **E** correspond to assays with nonacclimated plants (initial condition 100/15): ■ nonacclimated plants, control treatment (100/15), △ low temperature and low light treatment (100/4), and ○ low temperature, high light treatment (1600/4). Graphs **B**, **D** and **F** correspond to assays with cold-acclimated plants (initial condition 100/4): ● cold-acclimated plants control (100/4), □ low temperature and high light treatment (1600/4). *Symbols* correspond to the average of three measurements and *bars* to the standard error

turned to values similar to their respective controls at 24 h (Fig. 1D,E). GR activity in cold-acclimated plants showed no statistically significant differences when compared with nonacclimated plants ($P > 0.05$).

Total antioxidants and carotenoids

Nonacclimated plants treated with high light and/or low temperature presented small variations in their TA content, but stabilized near control values at 24 h of treatment (Fig. 3A). Cold acclimation for 21 days resulted in a statistically significant 43% lower TA content with respect to nonacclimated plants (Fig. 3A,B). Cold-acclimated plants exposed to high light and low temperature presented a small but significant ($P < 0.05$) decrease in TA from the first hour of treatment and along the assay (Fig. 3B).

Total carotenoids in nonacclimated plants did not vary after exposure for 24 h to low temperature but stabilized to a significantly ($P < 0.05$) lower value after 6 h under high light and low temperature treatment (Fig. 3C). Cold-acclimated plants had 55% less carotenoids than nonacclimated plants and, when exposed to high light and low temperature, presented a small but

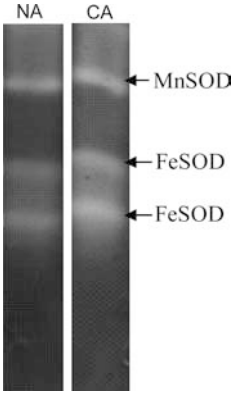

Fig. 2 Superoxide dismutase isozyme pattern in *Colobanthus quitensis*. White bands correspond to SOD isozymes that were identified by specific inhibition assays with KCN and hydrogen peroxide; 1 unit of SOD was loaded in each lane. Electrophoresis was performed in a 12% native polyacrylamide gel for 6 h at 4°C (*NA* nonacclimated; *CA* cold-acclimated)

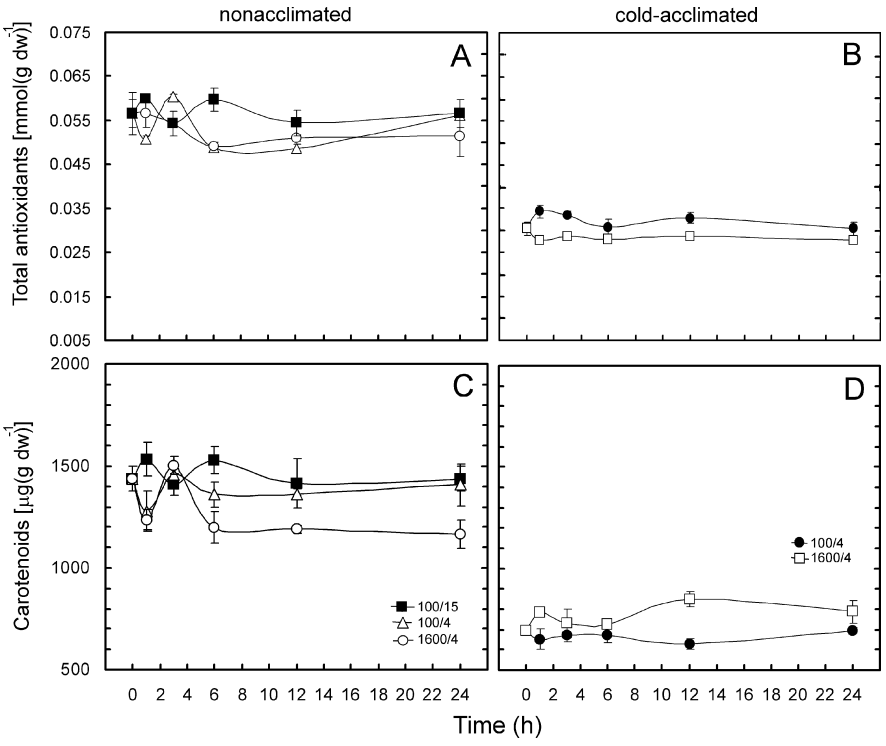

Fig. 3A–D Total antioxidant and carotenoid content in *Colobanthus quitensis*. Graphs **A** and **C** correspond to assays with nonacclimated plants (initial condition 100/15): ■ nonacclimated plants, control treatment (100/15), △ low temperature and low light treatment (100/4), and ○ low temperature, high light treatment (1600/4). Graphs **B** and **D** correspond to assays with cold-acclimated plants (initial condition 100/4): ● cold-acclimated plants, control treatment (100/4), and □ low temperature, high light treatment (1600/4). *Symbols* correspond to the average of three measurements and *bars* to the standard error

significant ($P < 0.05$) increase in their carotenoid content with a peak of 1.4-fold at 12 h of treatment (Fig. 3D). In spite of this, the carotenoid/chlorophyll ratio remained around 0.130 ± 0.005.

Discussion

ROS constitute an unavoidable byproduct of aerobic metabolism and their production is usually increased by environmental stress (Fryer et al. 2002). The extent of ROS damage depends on their steady state concentration. At high concentration, they cause damage by reacting with lipids, proteins, and nucleic acids. At low concentration, they may constitute part of the signaling process of acclimation to stress (Salin 1987; Scandalios 1993; Inzé and Van Montagu 1995; Alscher et al. 1997; Bowler and Fluhr 2000; Grant and Loake 2000; Kimura et al. 2001). Considering this dual role of ROS and the fact that the concentration of oxygen is higher in photosynthetic plant cells compared to animal cells, the regulation of ROS by antioxidants constitutes an important feature in plants exposed to environmental stress.

C. quitensis lives in regions with episodic high light and stable low temperature, such as high elevation on the Andes Mountains and the maritime Antarctic. Among the adaptations to cold environments, it has been determined that *C. quitensis* avoids ice formation and consequent tissue damage by supercooling (Bravo et al. 2001), and maintains a considerable photosynthetic rate at 0°C (~30% of the optimum). When exposed to low temperature in the field, this plant is reversibly photoinhibited (Xiong et al. 1999). We hypothesized that an elevated antioxidant capacity might be determinant in the survival of this plant to environments that promote oxidative stress. Taking this into account, we analyzed the antioxidant response of this plant to low temperature and high light, conditions that impose an energy imbalance by increasing the excitation pressure of PSII (Huner et al. 2002).

Although high light and low temperature treatment for 24 h caused a considerable decrease in photosynthetic efficiency and a significant increase in PSII excitation pressure, it did not induce sustained variations in the antioxidant parameters measured. High light and low temperature treatments in nonacclimated plants increased APx activity 40% at 24 h (Fig. 3A); meanwhile the other antioxidants measured remained constant or decreased. Cold-acclimated plants exposed to high light and low temperature diminished or maintained their antioxidant levels relatively constant, with the exception of the carotenoid content, which increased. Changes in the activity of antioxidant enzymes in plants under stress are thought to be a consequence of the accumulation of ROS, as evidenced for FeSOD in tobacco (Tsang et al. 1991; Mishra et al. 1993). Therefore, *C. quitensis* should scavenge or avoid ROS formation by a different mechanism. However, heat dissipation, as evidenced by the increase in qN, may be the major mechanism that balances the excess of energy at high light. Nonetheless, qN was significantly reduced at low temperature (Table 1). It is well established that the xanthophyll cycle is an important part of nonphotochemical quenching (Müller at al. 2001). The de-epoxidation of violaxanthin to zeaxanthin is dependent on the de-epoxidase activity, which is known to be strongly suppressed at low temperature

(Eskling et al. 2001). The xanthophyll cycle also depends on the availability of ascorbate as an electron donor for the de-epoxidation reaction. The higher activity of APx at low temperature and high irradiance could compete for ascorbate and decrease de-epoxidase activity (Eskling et al. 2001). Based on the above evidence, we hypothesized that the difference in qN observed between plants treated at 15°C and 4°C at high irradiance could be due to xanthophyll-cycle impairment at low temperature. Further research should be conducted to address this hypothesis.

Cold acclimation (21 days at 4°C) increased only SOD activity; other antioxidant parameters measured remained unaltered or decreased. A lower carotenoid content observed after cold acclimation did not change the carotenoid/chlorophyll ratio in nonacclimated and cold-acclimated plants, which implies a similar degree of protection against singlet oxygen.

SOD isozymes are generally associated with specific organelles: MnSOD with the mitochondrion, Cu/ZnSOD with the chloroplast and cytosol, and FeSOD with the chloroplast. In *C. quitensis*, SOD zymograms revealed one MnSOD and two FeSODs (Fig. 2). Stress treatments that affect the cytoplasm, the mitochondrion or the chloroplast usually lead to increases in all SOD isozymes (Perl et al. 1993). These responses probably reflect a tight coordination between different SOD isozymes in the cell. A phylogenetic approach of SOD isozymes in *C. quitensis* may be interesting considering that this plant has FeSOD, the most uncommon of all SOD isozymes in plants, and lacks detectable activity of Cu/ZnSOD, the most usual and abundant SOD isozyme in plants (Alscher et al. 1997; Fink and Scandalios 2002).

SOD activity in *C. quitensis* when compared with other plants such as *Arabidopsis thaliana*, *Lupinus angustifolius* (Yu and Rengel 1999), *Allium fistolosum* (Stajner et al. 1998), or *Lycopersicum esculentum* (Un-Haing and Jung-O 2000) has similar or higher values. APx activity is several fold higher than in *Gossipium hirsutum* (Rajguru et al. 1999) or *Pinus strobus* L. (Anderson et al. 1992). The same is observed for GR activity when compared to *Lycopersicum esculentum* (Un-Haing and Jung-O 2000) and *Arabidopsis thaliana*. Our results indicate that, in general, exposure of *C. quitensis* to high light and low temperature stress does not involve a significant antioxidant response. The evidence shows that other photoprotective mechanisms (probably nonphotochemical quenching) rather than antioxidants are responsible for the survival of this plant in the Antarctic environment.

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