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The role of photochemical quenching and antioxidants in photoprotection of *Deschampsia antarctica*

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Abstract. *Deschampsia antarctica* Desv. (Poaceae) is the only grass that grows in the maritime Antarctic. Constant low temperatures and episodes of high light are typical conditions during the growing season at this latitude. These factors enhance the formation of active oxygen species and may cause photoinhibition. Therefore, an efficient mechanism of energy dissipation and / or scavenging of reactive oxygen species (ROS) would contribute to survival in this harsh environment. In this paper, non-acclimated and cold-acclimated *D. antarctica* were subjected to high light and / or low temperature for 24 h. The contribution of non-photochemical dissipation of excitation light energy and the activities of detoxifying enzymes in the development of resistance to chilling induced photoinhibition were studied by monitoring PSII fluorescence, total soluble antioxidants, and pigments contents and measuring variations in activity of 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). The photochemical efficiency of PSII, measured as F_v / F_m, and the yield of PSII electron transport (ΦPSII) both decreased under high light and low temperatures. In contrast, photochemical quenching (qP) in both non-acclimated and cold-acclimated plants remained relatively constant (approximately 0.8) in high-light-treated plants. Unexpectedly, qP was lower (0.55) in cold-acclimated plants exposed to 4°C and low light intensity. Activity of SOD in cold-acclimated plants treated with high light at low temperature showed a sharp peak 2–4 h after the beginning of the experiment. In cold-acclimated plants APX remained high with all treatments. Activity of GR decreased in cold-acclimated plants. Compared with other plants, *D. antarctica* exhibited high levels of SOD and APX activity. Pigment analyses show that the xanthophyll cycle is operative in this plant. We propose that photochemical quenching and particularly the high level of antioxidants help *D. antarctica* to resist photoinhibitory conditions. The relatively high antioxidant capacity of *D. antarctica* may be a determinant for its survival in the harsh Antarctic environment.

Keywords: Antarctic angiosperms, ascorbate peroxidase, glutathione reductase, low temperature, non-photochemical quenching, photochemical quenching, photoinhibition, reactive oxygen species, superoxide dimutase, xanthophyll cycle.

Introduction

The balance between the energy absorbed as light and that photochemically converted and useful for metabolism is critical in photosynthetic organisms (Hüner *et al*. 2002). The alteration of this energy homeostasis may lead to a variety of plant responses that range from short-term down-regulation of the photochemical efficiency and long-term morphological changes in leaves to irreversible damage of the photosynthetic apparatus (Björkman and Demmig-Adams 1995). Rapid dissipation of excess photons as heat is

Abbreviations used: A, antheraxanthin; ABTS, 2,2′-azino-di-[3-ethylbenzotiazoline sulfonate]; APX, ascorbate peroxidase; CATs, catalases; GR, glutathione reducatase; MDHAR, monodehydroascorbate reductase; N, neoxanthin; NPQ, non-photochemical quenching; ROS, reactive oxygen species; SOD, superoxide dismutase; TA, total antioxidants; V, violaxanthin; Z, zeaxanthin.

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widely found in photosynthetic organisms (Adams *et al*. 1995; Niyogi 2000). However, when the absorption of light energy exceeds the capacity of photosynthesis and the photoprotective mechanisms are overwhelmed, reduction of oxygen molecules and formation of ROS may occur (Niyogi 1999; Asada 1999). In addition, several environmental stress conditions (see below) limit the ability of a plant to utilise light energy through photosynthesis so that excessive excitation of the photosystems can occur even at moderate light intensities (Demmig-Adams and Adams 1992). Unfavourable variations in temperature, light intensity, nutrient limitation and water availability, among others, induce severe perturbations of photosynthesis (Hüner *et al*. 2002), thus increasing the production of oxidant substances. Low temperature in combination with high irradiance is especially stressful because these factors may cause increased excitation energy in PSII, which may lead to an overproduction of ROS (Raison and Lyons 1970; Omran 1980; Prasad *et al*. 1994). Enzymes, such as superoxide dismutase, ascorbate peroxidase, catalases, monodehydroascorbate reductase and glutathione reductase, and small molecules, such as ascorbate, glutathione, and α-tocopherol are some of the most important detoxifying enzymes and antioxidants (Cogdell and Frank 1987; Krinsky 1989; Palozza and Krinsky 1992). Reactive oxygen species may be of importance in other processes. For example, O_2^- and H_2O_2, may participate in the signal transduction process during abiotic stress, pathogen defence, programmed cell death and systemic signalling (Inzé and Van Montagu 1995; Bowler and Fluhr 2000; Grant and Loake 2000; Kimura *et al*. 2001; Mittler 2002). Therefore, thermal dissipation (Xu *et al*. 2000) and protective mechanisms against oxidative stress (Kornyeyev *et al*. 2003) are of major importance in plants exposed to low temperature and / or high light.

Deschampsia antarctica is one of the two angiosperms and the only monocotyledonous species naturally adapted to the cold maritime Antarctic climate (Xiong *et al.* 1999, 2000; Alberdi *et al*. 2002; Lewis-Smith 2003). Among the features that may be involved in the success of this plant in this harsh environment are its tolerance to extracellular ice when exposed to freezing and the accumulation of carbohydrates, such as fructans and especially sucrose, which may contribute to the stabilisation of cellular membranes at low temperatures (Bravo *et al*. 2001; Alberdi *et al*. 2002). *D. antarctica* has a robust photosynthetic apparatus, which maintains photosynthesis at approximately 30% of its optimum rate at 0°C (Xiong *et al*. 1999). During the growing season in the Antarctica, this plant experiences low temperatures (usually from –2 to 6°C) and episodes of high irradiance (1000–1500 μmol $m^{-2}s^{-1}$). Therefore, its photosynthetic machinery might be protected from excess radiation and oxidative damage in order to survive the harsh environmental conditions. To test this hypothesis, non-acclimated and cold-acclimated *D. antarctica* were subjected to high light and / or low temperature under laboratory conditions, to study energy dissipating mechanisms, antioxidants and detoxifying enzymes.

Materials and methods

Plant material and growth conditions

Deschampsia antarctica Desv. (Poaceae) was collected on Robert Island, maritime Antarctic (62°22′S, 59°43′W). Plants were reproduced vegetatively in plastic pots (three ramets per pot) containing a soil : peat mixture (3 : 1), and fertilised with 0.12 g L^{-1} Phostrogen® (Solaris, Buckinghamshire, UK) once every 2 weeks. Growth conditions were: 100 ± 20 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD) over the range of 400–700 nm, 15°C (100 / 15) at 21 / 3 h light / dark photoperiod. One group of these plants was cold-acclimated with a PPFD of 100 ± 20 μmol $m^{-2}s^{-1}$ and 4°C (100 / 4) for 21 d and then subjected to high PPFD (1600 ± 50 μmol $m^{-2}s^{-1}$) and low temperature (4°C) treatment (1600 / 4). The other group of plants remained in the control growth conditions (100 / 15) for 21 d and then was subjected to high PPFD (1600 ± 50 μmol m^{-2} s^{-1}) and / or low temperature (4°C) treatment. Low and high PPFD were provided by 1000-W halogen lamps (General Electric Co., Fairfield, CT). The photoperiod was always the same (21 / 3 h light / dark, as in the Antarctic summer). Samples were taken from the treatments after 1, 3, 6, 12 and 24 h. Detoxifying enzyme activity of *D. antarctica* was compared with the activity of other *Poaceae* plants, barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), and oat (*Avena sativa* L.) under the same growth conditions (100 ± 20 μmol $m^{-2}s^{-1}$, 15°C). After 2 weeks from the day of sowing one group of plants was cold-acclimated for 21 d under the same conditions described for cold acclimation of *D. antarctica.*

Fluorescence measurements

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 2, Hansatech, Instruments Ltd, Norfolk, UK) according to Schreiber *et al*. (1986). Fully-developed attached leaves from cold-acclimated and non-acclimated plants were dark adapted for 30 min (to obtain open PSII centres) using the instrument leaf-clips to ensure maximum photochemical efficiency. The fibre-optic and its adaptor were fixed to a ring located over the clip at approximately 10 mm from the sample and the different light pulses (see below) were applied following the standard routines programmed within the instrument. Signal recordings and calculations were performed using the data analyses and control software provided with the instrument. Minimal fluorescence (F_0) with all PSII reaction centers in the open state was determined by applying a weak modulated light (0.4 μmol $m^{-2}s^{-1}$). Maximal fluorescence (F_m) with all PSII reaction centers in the closed state was induced by a 0.8-s saturating pulse of white light (9000 μmol $m^{-2}s^{-1}$). After 10 s, the actinic light (180 μmol $m^{-2}s^{-1}$) was turned on and the same saturating pulse described previously was applied every 20 s, until steady-state photosynthesis was reached in order to obtain F_s and F_m'. Finally, F_0' was measured after turning the actinic light off and applying a 2-s far red light pulse. Definitions of fluorescence parameters (qP, NPQ, F_v / F_m and ΦPSII) were used as described by van Kooten and Snel (1990).

Enzyme extraction and determinations

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

Superoxide dismutase (SOD: EC 1.15.1.1)

The extraction phosphate buffer (300 μL, pH 7.8) contained 2 mM EDTA and 1% (w / v) PVP at 4°C. Activity of SOD in the homogenate was determined according to McCord and Fridovich (1969) with modifications by Schöner and Krause (1990) by following the rate of ferricytochrome C reduction 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 (APX: EC 1.11.1.11)

To prepare the homogenate for APX, 400 μL of extraction phosphate buffer (pH 7.5) containing 1 mM EDTA, 1% (w / v) PVP, 0.3% (v / v), Triton X-100, and 5 mM ascorbic acid at 4°C were used. APX activity determination was performed according to Rao *et al*. (1996) by monitoring OD at 290 nm for 2 min in 1 mL solution containing 0.5 mM ascorbate and 200 mM H_2O_2. One unit of APX activity is the amount of enzyme required to oxidise 1 μmol of ascorbate per min, at 25°C.

Glutathion reductase (GR: EC 1.6.4.2)

The extraction phosphate buffer (300 μL, 100 mM, pH 7.6) contained 0.05% Triton X-100, 2% PVP, 1 mM ascorbic acid and 2 mM EDTA. The enzymatic activity of GR 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, catalysed by GR activity present in the sample. One unit of GR is defined as the amount of enzyme that catalyses the reduction of 1 μmol of NADPH per minute at 25°C at pH 7.6.

SOD isozymes

SOD isozymes were determined according to Rao *et al*. (1996). Equal amounts of SOD activity (1 Unit) were subjected to native PAGE (12%) at 80 mV for 6 h at 4°C. Since 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 soluble antioxidants

The homogenate was prepared with phosphate buffer (100 mM, pH 7.4). Soluble total antioxidants were quantified in the centrifuged homogenate using the total antioxidant measurement kit N°615700 (Calbiochem, La Jolla, CA), following the manufacturer instructions. 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 spectrophometrically at 600 nm. This procedure measures soluble antioxidant content.

Pigments

Deschampsia antarctica leaves were cut and placed immediately in a cold mortar. A minimal amount of $CaCO_3$ was added before grinding in 100% (v / v) acetone at 4°C under dim light. The supernatant was filtered through a 0.22-μm syringe filter and samples were stored at –80°C until analysed. Pigments were separated and quantified by HPLC analysis as described previously (Ivanov *et al*. 1995) with some modifications. The HPLC system consisted of a Beckman System Gold programmable solvent module 126, a diode array detector module 168 (Beckman Instruments, San Ramon, CA), CSC-Spherisorb ODS-1 reverse-phase column (5 μm particle size, 25 × 0.46 cm internal diameter) with an Upchurch Perisorb A guard column (both columns from Chromatographic Specialties Inc., Concord, Ontario, Canada). Samples were injected with a Beckman 210A sample injection valve with a 20-μL sample loop. Pigments were eluted isocratically for 6 min with acetonitrile : methanol : 0.1 M Tris–HCl (pH 8.0; 72 : 8 : 3.5, v / v / v), followed by a 2-min linear gradient to 100% methanol : hexane (4 : 1, v / v), which continued isocratically for 4 min. Total run time was 12 min with a flow rate of 2 mL min^{-1}. Absorbance was monitored at 440 nm and peak areas were integrated by a Beckman System Gold software. Retention times and response factors of chl *a*, chl *b*, lutein and β-carotene were determined by injection of known amounts of pure standards purchased from Sigma (St Louis, MO). The retention times of zeaxanthin (Z), antheraxanthin (A), violaxanthin (V) and neoxanthin were determined by using pigments purified by thin layer chromatography as described by Diaz *et al*. (1990). Epoxidation state (EPS) of the xanthophyll pigment pool was estimated as:

$$EPS = (0.5A + V) / (V + A + Z).$$

Statistical analysis

Fluorescence parameters were analysed by a three-way ANOVA. The factors analysed were growth condition (GC: non-acclimated and cold-acclimated), light intensity (L) and exposure temperature (T) with two levels each. In order to simplify these analyses to three factors, the whole series of time were not considered and the statistical analysis was performed at two fixed times, 12-h (middle of time course) and at 24-h (end of the experiment). The activities of detoxifying enzymes and antioxidants in *D. antarctica* were also analysed by a three-way ANOVA. The factors were GC, L and time (t) with six levels (0, 1, 3, 6, 12 and 24 h). Temperature was excluded since the condition 100 / 15 and 1600 / 15 were not performed for cold-acclimated plants. In order to differentiate treatments with the control 100 / 15 the comparison within non-acclimated growth condition was also performed by two-way ANOVAs (L × t and T × t). A three-way ANOVA was also applied to pigment determination, except that the factor time had only three levels (0, 12 and 24 h) for pigments in Table 1 and four levels (0, 4, 12 and 24 h) for xanthophyll cycle pigments. The comparison of the activities of SOD, GR and APX in different species at two growth conditions was performed by two-way ANOVA. A Tukey test was applied to determine individual differences within treatments. Values reported correspond to the mean of three different samples taken from independent pots. Statistical analyses were done with the Sigma Stat 2.03 program (SPSS Inc., Chicago, IL).

Results

Fluorescence measurements

There were significant light effects on all variables at both 12 and 24 h, while temperature effects were significant only for F_v / F_m and Φ PSII, and acclimation treatment affected all but F_v / F_m at 24 h. There was little evidence of a light × temperature interaction (only for NPQ at 24 h). Values of F_v / F_m remained at approximately 0.85 in non-acclimated and cold-acclimated plants at low PPFD. Non-acclimated plants showed a decrease in F_v / F_m after exposure to high light, reaching 0.7 after 12 h at 15°C. At this time, the combination of high light and low temperature changed F_v / F_m from 0.85 to 0.55 (Fig. 1*A*). In cold-acclimated plants, changes in F_v / F_m were generally smaller than in non-acclimated plants after 12 h of treatment, although there was a significant decrease in F_v / F_m at high light and low temperature after 24 h (Fig. 1*B*). Cold acclimation had a significant effect on F_v / F_m after 12 h of treatment ($F_{1,16}$=5.919; P<0.05). There was no T × L interaction in all fluorescence parameters, with the exception of NPQ

Table 1. Effect of cold acclimation and light on pigments content in *D. antarctica*

Non-acclimated and cold-acclimated (21 d at 4°C) plants were subjected to two light intensity treatments. After the specified times, leaf samples from three independent pots were collected for pigment analyses as indicated in the methods section. Control plants (0 h) were grown with a 16 / 8 h photoperiod with low PPFD (100 μmol m^{-2} s^{-1}). Values are the mean of three determinations ± SE. Pigment content (μg g FW^{-1})

	Non-acclimated					Cold-acclimated				
		100 μmol m^{-2} s^{-1}		1600 μmol m^{-2} s^{-1}			100 μmol m^{-2} s^{-1}		1600 μmol m^{-2} s^{-1}	
Pigment	0 h	12 h	24 h	12 h	24 h	0 h	12 h	24 h	12 h	24 h
Chl *a*	1219 ± 13	1715 ± 63	1621 ± 43	1426 ± 73	1446 ± 41	854 ± 16	1281 ± 66	1360 ± 36	1219 ± 84	1147 ± 110
Chl *b*	453 ± 5	674 ± 17	595 ± 8	559 ± 16	551 ± 14	308 ± 7	463 ± 7	497 ± 9	422 ± 10	404 ± 17
Chl *a* / chl *b*	2.7 ± 0.1	2.5 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.9 ± 0.2	2.8 ± 0.2
β-carotene	123 ± 1	181.5 ± 4	161 ± 4	182 ± 4	130 ± 4	90.3 ± 0.8	140 ± 4	139 ± 3	147 ± 14	121 ± 8
Lutein	144 ± 3	200 ± 3	175 ± 1	161 ± 17	185 ± 3	111 ± 2	167 ± 3	170 ± 1	165 ± 2	157 ± 2
Neoxanthin	30 ± 1	49 ± 1	42 ± 1	44 ± 1	43 ± 1	23.1 ± 0.6	35 ± 1	35.3 ± 0.6	37.2 ± 0.5	33 ± 2

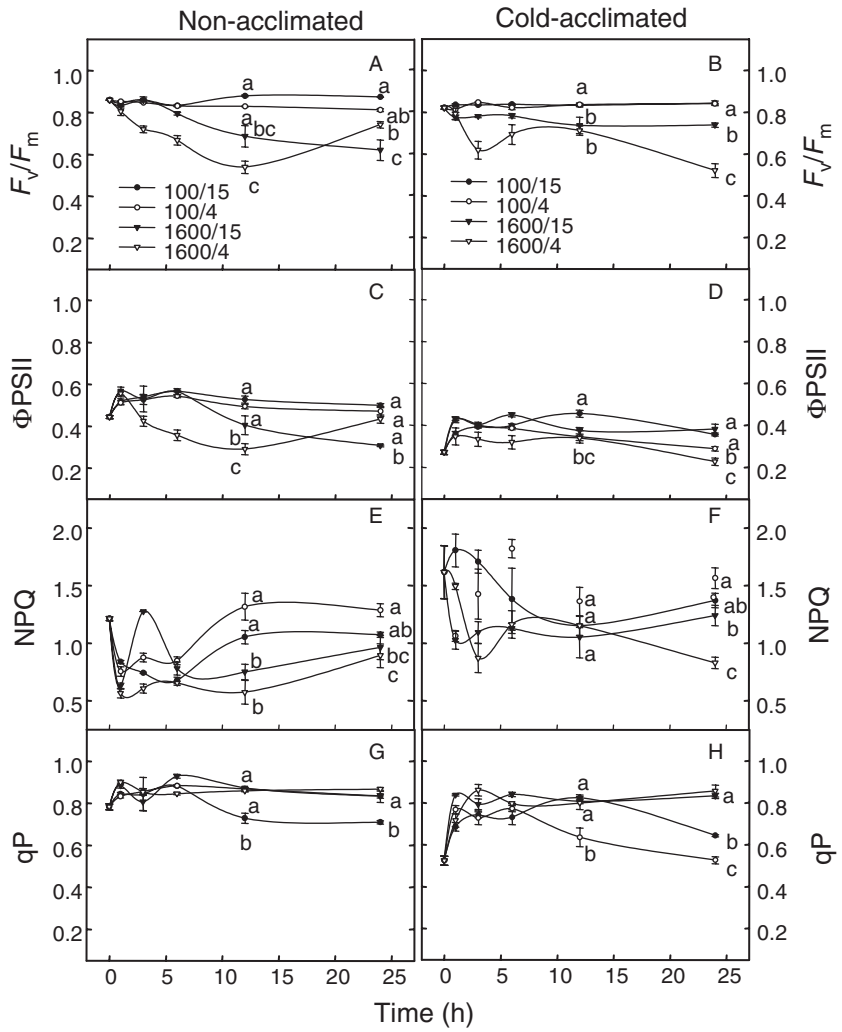

Fig. 1. Chlorophyll fluorescence in *Deschampsia antarctica* after high light and low temperature stress. (*A*, *C*, *E, G*) Non-acclimated plants (21 d at 15 ± 1.5°C and light intensity of approximately 100 μmol $m^{-2}s^{-1}$). (*B*, *D*, *F*, *H*) Cold-acclimated plants (21 d at 4 ± 1.5°C and light intensity of approximately 100 μmol $m^{-2}s^{-1}$). Plants were subjected to 4°C and PPFD of 100 and 1600 μmol $m^{-2}s^{-1}$. Vertical bars indicate SEs of three independent determinations. Different letters on data points within times in both non-acclimated and cold-acclimated treatments indicate significant differences (Tukey test, after three-way ANOVA).

at 24 h of treatment ($F_{1,16}$=16.613; P<0.05). Interactions among factors for F_v/F_m were detected for GC × T ($F_{1,16}$=5.443; P<0.05) and GC × L ($F_{1,16}$=12.177; P<0.05) after 12 h of treatment and for GC × T ($F_{1,16}$=19.498; P<0.05) and GC × T × L ($F_{1,16}$=39.643; P<0.05) after 24 h. Significant changes were observed for ΦPSII under different treatments (Fig. 1*C*, D).Since F_v/F_m and efficiency of PSII (ΦPSII) decreased at high light, it may be assumed that cold-acclimated plants were photoinhibited at low temperature after high light treatment (Fig. 1 *A–D*). There were significant changes in non-photochemical dissipation of energy (NPQ) values in both cold-acclimated and non-acclimated plants (Fig. 1*E–F*). Unexpectedly, NPQ was lower in the treatment with high light than with low light in non-acclimated plants at 12 h. This may be a reflection of the low PPFD of actinic light used in the fluorescence measurements compared with the high intensity used in the treatments. This high-light effect on NPQ was also observed in cold-acclimated plants treated at 4°C after 24 h. Photochemical quenching (qP) fluctuated around 0.7–0.9 after 1 d of exposure to high light in non-acclimated and 0.55–0.85 in cold-acclimated plants (Fig. 1*G–H*), showing a significant effect of cold acclimation, with significant interactions GC × T ($F_{1,16}$=26.838; P<0.05), GC × L ($F_{1,16}$=55.818; P<0.05), GC × T × L ($F_{1,16}$=22.578; P<0.05) at 24 h.

SOD activity

SOD activity in non-acclimated plants after high light treatment (1600/4) increased ($F_{1,48}$=11.058; P<0.05) and leveled off at a higher activity than in the other treatments (100/15 and 100/4) at 24-h (Fig. 2*A*). Acclimation to low light and low temperature (100/4) for 21 d (time 0, Fig. 2*B*) did not change SOD activity with respect to non-acclimated plants (Fig. 1*A*; 100/15). Nonetheless, SOD activity in cold-acclimated plants treated with high light and low temperature (1600/4) showed a transient increase peaking 2–4 h after the beginning of the experiment and returning to a value similar to the control (100/4) by the end of the experiment (Fig. 2*B*).

SOD isozymes

Leaves of non-acclimated (100/15) *D. antarctica* exhibited 2 Cu/ZnSOD isozymes (Fig. 3). A band that corresponded to MnSOD was weak and poorly visualised. After cold-acclimation (100/4) for 21 d, the three isozymes were observed. The MnSOD band was more intense and better visualised in cold-acclimated than in non-acclimated plants.

APX activity

Activity of APX decreased in non-acclimated plants during the low light and low temperature treatment (100/4) for 24 h and increased transiently in high light and low temperature treatment (1600/4, Fig. 2*C*). Cold-acclimated plants at low light (100/4) exhibited 1.5 times more APX activity than control non-acclimated plants (100/15) and remained high during the course of the experiment ($F_{1,48}$=864.468; P<0.05 for GC). No significant changes were observed in cold-acclimated plants at high (1600/4) and low light (100/4) at 12 and 24 h of treatment (Fig. 2*D*).

GR activity

A peak of GR activity was found after 3 h of treatment at low light and low temperature (100/4, Fig. 2*E*). A second increase in activity was found after 24 h of treatment. The exact time of the second peak was not determined, but is clear that it occurred after 24 h of treatment because the activity of cold-acclimated plants (100/4) for 21 d was much lower than that of non-acclimated plants exposed for 24 h to the treatment (100/4). Cold-acclimated plants (100/4) had a lower GR activity than non-acclimated plants (Fig. 2*E*, *F*). High light and low temperature (1600/4) treatment caused a decrease in GR activity in cold-acclimated plants, with respect to the low-light-treated control plants (100/4). Thus, there were significant effects of growth condition ($F_{1,48}$=339.865; P<0.05), light ($F_{1,48}$=70.333; P<0.05) and time ($F_{5,48}$=12.106; P<0.05) on GR activity, as well as interaction among the three factors ($F_{5,48}$=10.772; P<0.05).

Comparative enzyme activities in several grasses

Antioxidant enzyme activities were measured in several cold-acclimated and non-acclimated Poaceae plants (Fig. 4). SOD activity was 14–83 times higher in *D. antarctica* than in the other cereals ($F_{3,16}$=196.771, P<0.05). Activity of SOD was not significantly affected by cold acclimation in any of the studied species ($F_{3,16}$=0.323, P>0.05, Fig. 4*A*). Activity of APX was 1.5–6.7 and 2–5.2 times higher in non-acclimated and cold-acclimated plants of *D. antarctica*, respectively, than in other Poaceae (Fig. 4*B*). In non-acclimated plants, GR activity was also 2–3.2 times higher in *D. antarctica* than in the other Poaceae (Fig. 4*C*). In cold-acclimated plants, however, GR activity decreased by 2.3 times in *D. antarctica,* reaching a level similar to that of other cold-acclimated Poaceae. Thus, there was a significant effect of cold acclimation on APX ($F_{1,16}$=19.154; P<0.05 for GC) and GR ($F_{1,16}$=16.771; P<0.05) activities.

Total antioxidants

Cold acclimation had a significant negative effect on total antioxidant accumulation ($F_{1,48}$=485.087; P<0.05). Non-acclimated plants exposed to low light and low temperature (100/4) exhibited two peaks of antioxidant accumulation at 1 and 6 h, which subsequently decreased to nearly control values after 12 h (Fig. 5*A*). Total antioxidant levels increased at high light and low temperature (1600/4) during the assay. In contrast, total antioxidant content in cold-acclimated plants exposed to high light

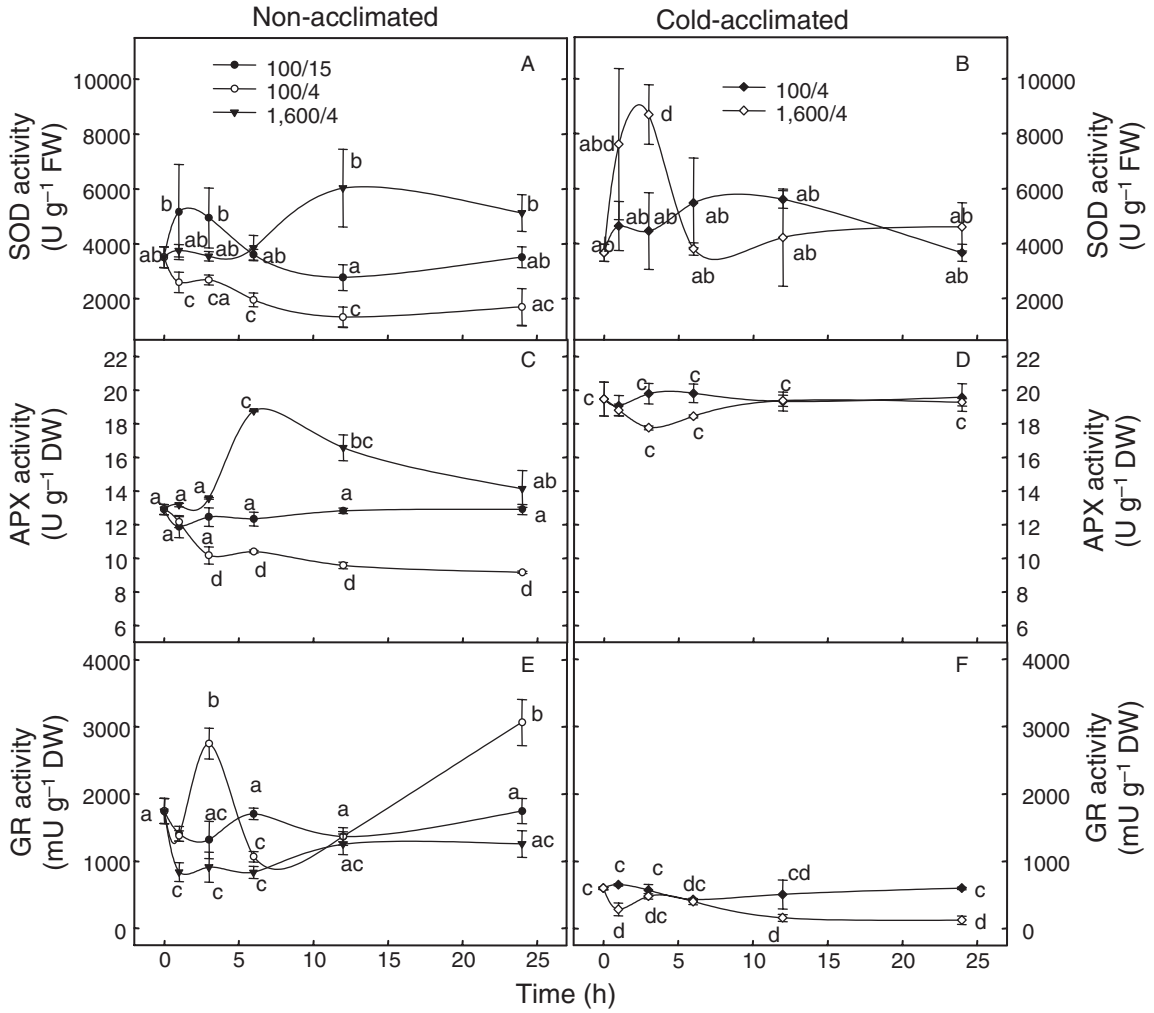

Fig. 2. Activity of SOD, APX and GR in *Deschampsia antarctica*. (*A*, *C*, *E*) Assays with non-acclimated plants (initial condition 100 / 15 light / temperature). Non-acclimated plants control (100 / 15), low light and low temperature treatment (100 / 4), high light and low temperature treatment (1600 / 4). (*B*, *D*, *E*) Assays with cold-acclimated plants (initial condition 100 / 4). Cold-acclimated plants control (100 / 4) and high light and low temperature treatment (1600 / 4). Values represent the mean of three measurements; vertical bars indicate SEs. Different letters on data points within times indicate significant differences (Tukey test, after three-way ANOVA).

and low temperature (1600 / 4) and low light and low temperature (100 / 4) did not differ significantly ($P<0.05$, Fig. 5*B*). Thus, significant increases in total antioxidants were observed only at early times of stress in non-acclimated plants. The significant effect of light on total antioxidants ($F_{1,48}=44.095$; $P<0.05$) could be explained by its positive effect in non-acclimated plants only.

Pigments

All pigment contents were lower in cold-acclimated plants than in non-acclimated plants ($P<0.05$; Table 1). Light treatments produced small but significant increases in all pigments measured, with the exception of neoxanthine ($F_{2,24}=1.569$, $P>0.05$ for factor L). In spite of the statistically significant effect of cold acclimation on chl *a* / chl *b* ratio, it remained nearly constant in all treatments. Thus, there were interactions between growth condition, light, and time to determine pigment content (for chl *a* $F_{2,24}=1.605$, $P<0.05$; chl *b*, $F_{2,24}=190.017$, $P<0.05$; β-carotene $F_{2,24}=4.858$, $P<0.05$). The xanthophyll cycle is clearly operative in this species. In contrast to low-light treatment, high-light treatment in cold-acclimated and non-acclimated plants produced the expected inverse effects on zeaxanthin and violaxanthin contents, respectively [significant effect ($P<0.05$) of GC, L and t for V, A, Z], decreasing the epoxidation state (Fig. 6*A–D*).

Discussion

The overall performance of PSII photochemistry, as determined by chlorophyll fluorescence, was stable after low

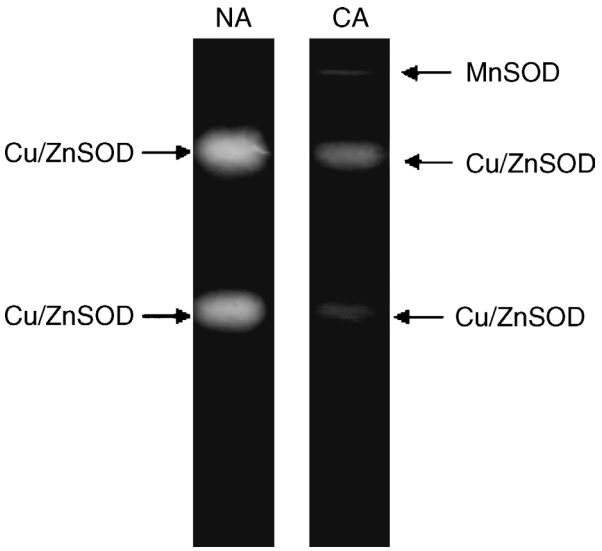

Fig. 3. Superoxide dismutase isozyme pattern in *Deschampsia antarctica*. NA, non-acclimated *D. antarctica* (100 / 15 light / temperature); CA, cold-acclimated *D. antarctica* (100 / 4). White bands correspond to SOD isozymes that were identified by specific inhibition assays with KCN and hydrogen peroxide. The same SOD activity was loaded in each lane to improve visualisation. Electrophoresis was performed in a 12% native polyacrylamide gel for 6 h at 4°C.

light and low temperature treatments in both non-acclimated and cold-acclimated *D. antarctica* plants. However, at high light treatments there were significant decreases in F_v / F_m of up to 35%, suggesting some degree of photoinhibition. These results apparently disagree with an earlier report demonstrating that this plant appears to be quite resistant to photoinhibition in the field (Xiong *et al*. 1999). In our experiments, plants were exposed to high light for period longer than those usually found in the Antarctic. Thus, *D. antarctica* may be partially photoinhibited in the field only on the more luminous days. This suggestion is also supported by the fact that there were no dramatic changes in ΦPSII during the various treatments. Non-photochemical quenching did not reach high values in cold-acclimated and non-acclimated plants being approximately 1.0 and 1.5, respectively, and remained relatively low after high light treatments (Fig. 1*E*, *F*). This indicates that these conditions do not induce significant changes in the dissipation of excess energy as heat in *D. antarctica*. Moreover, retaining high photochemical quenching even in high-light-treated leaves (Fig. 1*G*, *H*) indicated that a high proportion of the absorbed energy was being directed towards photochemistry. These results suggest that *D. antarctica* relies mainly on photochemical reactions for photoprotection rather than on dissipation of excess energy as heat. In support of this assumption, high photosynthetic rates have been found in *D. antarctica* at low temperatures (Xiong *et al*. 1999). Furthermore, it has also been reported, that increased photochemistry effectively

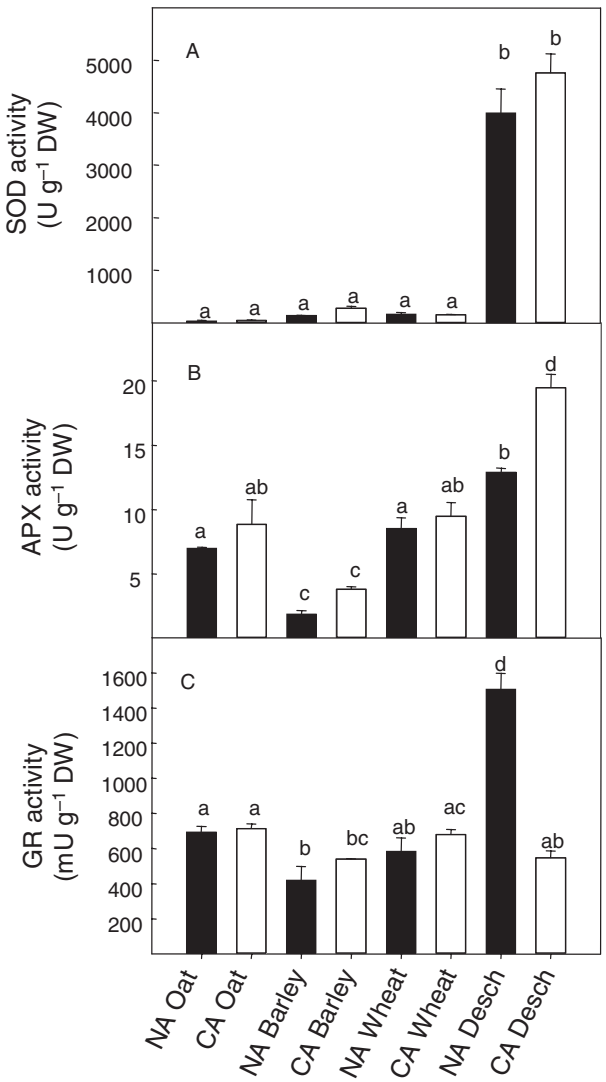

Fig. 4. Detoxifying enzyme activity in different Poaceae. *Deschampsia antarctica*, oat (*Avena sativa)*, wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*) were grown under the same laboratory conditions. Activity of SOD, APX and GR were assayed in leaf tissues of each species. NA, non-acclimated; CA, cold-acclimated. Values represent the mean of three independent measurements; vertical bars indicate SEs. Different letters indicate significant differences among species and treatments ($P<0.05$, Tukey test after two-way ANOVA).

decreases chilling-induced photoinhibition (Kornyeyev *et al*. 2003).

The activity of SOD increased after high-light treatment in both non-acclimated and cold-acclimated *D. antarctica* plants (Fig. 2*A*, *B*), although this effect was transient in cold-acclimated leaves. Non-acclimated plants also exhibited considerably higher transient activity of APX as a result of high light treatment (Fig. 2*C*). In cold-acclimated plants no effect was registered. However, it should be noted that APX activity was significantly higher in cold-acclimated compared to non-acclimated leaves (Fig. 2*C*, *D*), which is

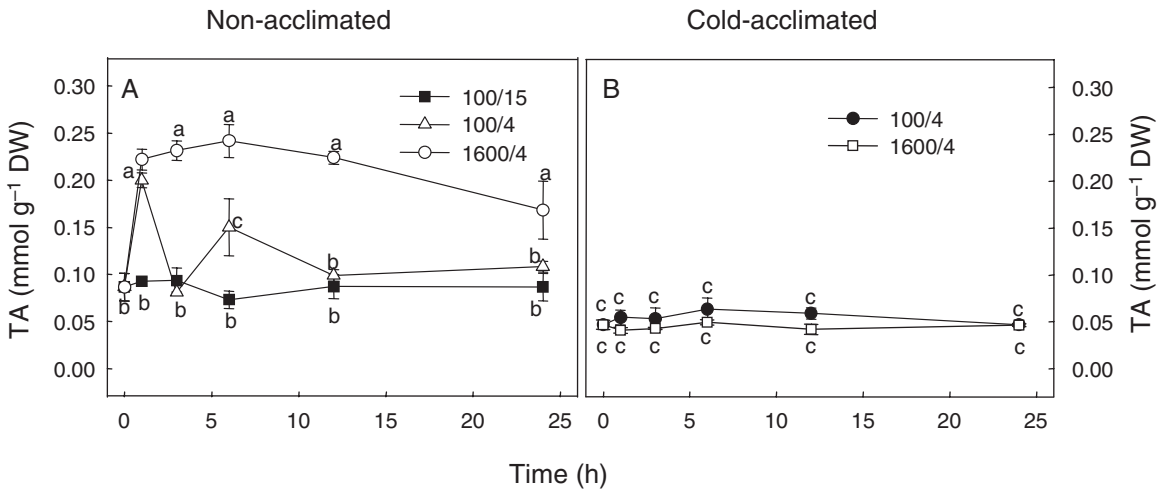

Fig. 5. Total antioxidant (TA) content in *Deschampsia antarctica*. (*A*) Non-acclimated plants (initial condition 100 / 15 light / temperature): non-acclimated control plants (100 / 15), low light and low temperature treatment (100 / 4) and high light treatment and low temperature (1600 / 4). (*B*) Cold-acclimated plants (initial condition 100 / 4): cold-acclimated control plants (100 / 4) and high light and low temperature treatment (1600 / 4). Values represent the mean of three independent measurements; vertical bars indicate SEs. Different letters within times indicate significant differences (Tukey test, after three-way ANOVA).

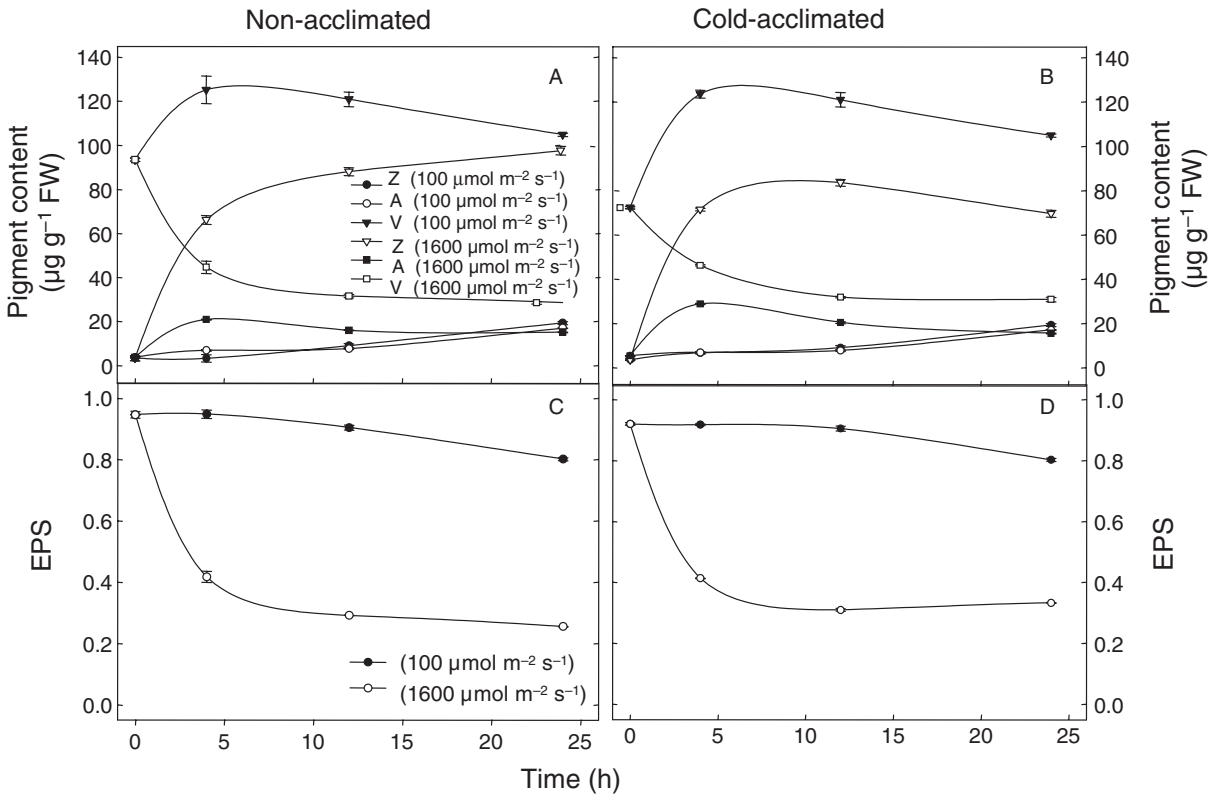

Fig. 6. Xanthophyll cycle pigments in *Deschampsia antarctica.* Non-acclimated and cold-acclimated plants were exposed to 4°C at low (100 µmol $m^{-2}s^{-1}$) and high light (1600 µmol $m^{-2}s^{-1}$). After the indicated time leaf samples from three independent pots were extracted in 100% acetone and pigments were analysed by HPLC. EPS, epoxidation state = (0.5A + V) / (V + A + Z), A, antheraxathin; V, violaxanthin; Z, zeaxanthin. Bars represent SEs of three determinations.

consistent with earlier observations for other plants (Alscher *et al*. 1997). Activity of GR was lower in cold-acclimated than in non-acclimated plants. This enzyme is capable of decreasing chilling-induced photoinhibition in *Gossypium hirsutum* (Kornyeyev *et al*. 2003). This suggests that, in *D. antarctica*, GR has a reduced role in ascorbate regeneration in long-term cold-acclimated plants, compared with the first hours of light and cold stress. Alternative mechanisms for regeneration of ascorbate may be more prevalent in *D. antarctica* under these conditions. Reduced ferredoxin or high monodehydroascorbate reductase could fulfill this role, as suggested by Asada (1999). Nonetheless, this remains to be clarified in *D. antarctica.*

Multiple forms of SOD are present in all plants examined. Perhaps the existence of multigene SOD families is reflective of the various roles played by these enzymes in plants, which must cope with a variety of environmental stress factors (Alscher *et al*. 2002; Fink and Scandalios 2002). SOD zymograms of non-acclimated *D. antarctica* plants presented two clear bands corresponding to two Cu / ZnSOD isozymes. An MnSOD band was weak and poorly visualised. Cold-acclimated plants presented the three types of isozymes, including a clear band for MnSOD. Thus, it appears that cold-acclimated plants have a higher activity of MnSOD than non-acclimated plants. This isozyme is resistant to high levels of hydrogen peroxide, unlike Cu / ZnSOD and FeSOD (Bowler *et al*. 1992). This feature could determine a better performance in the detoxification of superoxide at high levels of ROS in this species under stressful conditions.

The comparative enzymatic activity study has demonstrated that SOD activity in non-acclimated and cold-acclimated plants of *D. antarctica* is much higher than in other Poaceae plants (Fig. 4*A*). This activity was also higher than SOD activity reported for *Lupinus angustifolius* (Yu and Rengel 1999), *Allium fistolosum* (Stajner *et al*. 1998), and *Lycopersicum esculentum* (Un-Haing and Jung-O 2000). The activity of APX is also higher compared with other cereals (Fig. 4*B*), as well as in *G. hirsutum* (Rajguru *et al*. 1999) or *Pinus strobus* (Anderson *et al*. 1992). Furthermore, GR activity in *D. antarctica* was also greater than in other plants, but only in the non-acclimated state, when compared with other grasses, *Arabidopsis* and *Lycopersicum esculentum* (Un-Haing and Jung-O 2000). Thus, it appears that the higher constitutive antioxidant activity in *D. antarctica* compared with other plant species may be a specific adaptation for survival in the harsh Antarctic environment. A transient increase in total soluble antioxidants (possibly glutathione or ascorbate) was observed in non-acclimated plants subjected to low temperature and to a higher extent in high light-treated leaves (Fig. 5*A*). The high initial increase in these antioxidants may reflect a rapidly induced protective mechanism to low temperature and high light stress in non-acclimated plants. Cold-acclimated plants, however, did not show these variations. A high antioxidant capacity and efficient energy dissipation through accessory pigments may be partially responsible for protecting against photoinhibition, because they maintain a low ROS pool (Gould *et al*. 2002; Neill *et al*. 2002).

The xanthophyll cycle is operative in *D. antarctica*. Carotenoids of the xanthophyll cycle and the formation of zeaxanthin are intimately related to the control of singlet oxygen production by chlorophylls when the photosynthetic electron chain is saturated (Foyer *et al*. 1994). In spite of this, NPQ did not reach high values during high light treatments. Non-photochemical quenching has at least three components (Müller *et al*. 2001). The relative importance and contribution of these components in *D. antarctica* is presently unknown. Non-photochemical quenching changes could be low, even if the xanthophyll cycle is operative, as shown for the NPQ-deficient mutant npq4 in *Arabidopsis* (Li *et al*. 2000; Niyogi *et al.* 2001). It has been suggested that a component of non-photochemical quenching independent of the xanthophyll cycle also exists for dissipation of excess light energy (Niyogi *et al*. 1998).

In summary, the experimental results indicate that the xanthophyll cycle is functional in *D. antarctica* in both cold-acclimated and non-acclimated plants (Fig. 6). However, it appears that high light induced formation of zeaxanthin is poorly related to dissipation of excess light through the NPQ. Although somewhat unexpected, this could be explained by an earlier report indicating that an increase in light-dependent zeaxanthin formation is not required for the development of NPQ (Hurry *et al*. 1997). It has been also demonstrated that NPQ related to xanthophyll-dependent dissipation of excess light is not required for the protection of PSII against high-light-induced photo-destruction in *Arabidopsis* (Grasses *et al*. 2002). Moreover, only a few molecules of zeaxanthin per PSII may be involved in effective non-photochemical dissipation of light energy (Gilmore 1997; Hurry *et al*. 1997; Ruban *et al*. 2002). Most likely, the predominant energy dissipation mechanisms are related to photochemical quenching as reported for over-wintering cereals (Hüner *et al*. 1993). Photochemical photoprotection is a result of the combination of the Calvin cycle, photorespiration, cyclic electron flow through PSI, chlororespiration, and the Mehler reaction (Bendall and Manasse 1995; Asada 1999; Niyogi 1999; Badger *et al*. 2000). *D. antarctica* is able to maintain a high CO_2 fixation rates at low temperature (Xiong *et al*. 1999), thus contributing significantly to qP. In addition, a high antioxidant capacity in *D. antarctica* may provide an environment in which the reduction of oxygen to superoxide (Mehler reaction) is safe and may become an important alternative electron sink. High levels of SOD and APX in this plant may efficiently detoxify most active oxygen species formed during high light stress, especially in cold-acclimated plants. We suggest that photochemical quenching makes important contributions

to the photoprotection of *D. antarctica* in the Antarctic environment.

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