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Photoprotective mechanisms against winter stresses in the needles of *Abies mariesii* grown at the tree line on Mt. Norikura in Central Japan

J.-Y. YAMAZAKI*, S. TSUCHIYA, S. NAGANO, and E. MARUTA

Department of Biology, Faculty of Science, Toho University, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan

Abstract

Evergreen fir *Abies mariesii* growing at the tree line (near 2 500 m altitude) on Mt. Norikura (36°61′N, 137°33′E, 3 026 m altitude) in Central Japan is exposed to harsh winter stresses. To protect against these stresses, the de-epoxidation state of the xanthophyll cycle pigments increased, because the needles contained large amounts of zeaxanthin, which resulted in an increase of non-radiative thermal dissipation from the antenna system. Not only the antenna system but also the inactivated photosystem (PS) 2 reaction centre (RC) might contribute to the heat dissipation of absorbed excess photon energy. In addition, a decrease in the PS2 activity during winter was derived from the degradation of the PS2 RCs. Thus the needles acclimated to the strong sunlight during the harsh winter. Under such conditions, only the abaxial side of *A. mariesii* needles occasionally changed colour from green to reddish-brown in early spring. Since this needle damage was only observed in shoots that protruded from the snow surface, this phenomenon might be caused by the interaction between the strong sunlight reflected from the snow surface and the long period of sub-zero temperatures. We also examined how the photoprotective functions of *A. mariesii* growing at the tree line of a temperate zone mitigate the interactive stresses of high photon flux density and sub-zero temperature in harsh winter.

Additional key words: photosystem 2 reaction centres; tree line; xanthophyll cycle.

Introduction

Although evergreen conifers grown in the boreal zone and in high altitude regions are exposed to stresses caused by extremely low temperatures and high photon flux density (PPFD), they retain their needles for several years. Hence their photosynthetic apparatus must provide a protective mechanism against these stresses to survive severe winters and recover their capacity for photosynthesis in the next spring (Ottander *et al.* 1995, Yamazaki *et al.* 2003). Winter stress causes depression of the photochemical efficiency of photosystem (PS) 2 and the activity of Calvin-Benson cycle enzymes. Excess photons reduce molecular oxygen, resulting in the production of harmful active oxygen species and severe photoinhibition of photosynthesis (Ottander *et al.* 1995, Yamazaki *et al.* 2003). There are some photoprotective mechanisms against the photoinhibition of photosynthesis in over-wintering evergreens such as the degradation of PS2 reaction centres (RC) (Ottander *et al.* 1995) and enhanced cyclic electron transport around PS1 (Ivanov *et al.* 2001). Not only an increased non-photochemical quenching (NPQ) of excitation energy through xanthophyll cycle pigments, *i.e.* violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z), that are connected with the light-harvesting antennae (antenna quenching; *e.g.* Adams and Demmig-Adams 1994, Ottander *et al.* 1995, Verhoeven *et al.* 1999, Gilmore and Ball 2000) but also an increased probability for non-radiative charge recombination within the PS2 RC (RC quenching; Ivanov *et al.* 2003, Öquist and Huner 2003) are very important in the dissipation of excess photon energy.

Mt. Norikura (36°61'N, 137°33'E, 3 026 m altitude) is located at the southernmost tip of the northern Japanese Alps in the central mountainous area of the Japan Archipelago in Nagano Prefecture, which is one of the heaviest snowfall areas in the world, with snow coverage during the winter reaching approximately 3 m. Evergreen

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*Author for correspondence; fax: +81-47-472-5330, e-mail: junya@bio.sci.toho-u.ac.jp
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firs *Abies mariesii* growing at the tree line (2 500 m altitude) on Mt. Norikura are exposed to harsh winter stress, *e.g.* freezing temperatures, strong prevailing winds, and high PPFD. Under such conditions, the abaxial side of *A. mariesii* needles occasionally changes colour from green to reddish-brown in the early spring, which results in the death of the needles. This phenomenon is only observed in shoots that protrude from the snow surface and not in those buried in snow or located at the lower end of the tree line (Fig. 1*A*). Maruta and Nakano (1999) first hypothesized that the needle damage is not caused by desiccation but by the combination of high PPFD reflected from the snow surface and low temperatures at the tree line. Yamazaki *et al.* (2003) demonstrated that although the needle damage might be caused when excess photons flow into the photosynthetic electron transport pathway, the surviving needles of *A. mariesii* show a rapid recovery of photosynthesis.

Although several studies have been done on the photoinhibition caused by the interaction of high PPFD and low temperature at the boreal zone (Ottander *et al.* 1995, Verhoeven *et al.* 1999), only one study tried to elucidate these effects on needles at the tree line of a temperate zone during a harsh winter (Yamazaki *et al.* 2003). The goal of this study was to elucidate how the photoprotective functions of *A. mariesii* growing at the tree line of a temperate zone mitigate the interactive stresses of harsh winter. We limit the discussion to the effect of visible radiation region on the needle photodamage because the combined effects of UV radiation and visible radiation are too complicated.

Materials and methods

Study site and plants: Mt. Norikura (36°61'N, 137°33'E, 3 026 m altitude) is located at the southernmost tip of the northern Japanese Alps in the central mountainous area of the Japan Archipelago in Nagano Prefecture. Shoots of *A. mariesii* were collected at 2 500 m altitude on the eastern gentle slope (5°) and at monthly intervals from March through November 2004, except for August. One shoot was exposed to strong winter stress because it protruded above the snow (more than 2.3 m above the ground), and the abaxial side of the needles occasionally changed to reddish-brown. The other shoot, on the same tree, was stress-protected due to being buried in the snow (less than 2.3 m above the ground) and its needles remained green (Fig. 1*A*). The tree, with some parts above the snow surface and other parts below it, formed an unusual shape because of its exposure to strong prevailing winds (Fig. 1*B*). The vegetation there consisted of a mixed physiognomy with *Betula ermanii*, *Pinus pumila*, and *A. mariesii*. The collected shoots were brought to the laboratory, which was kept dark and below 5 °C, and needles were cut from the shoots for immediate measurement of fluorescence (see below) and stored at –70 °C until biochemical assays were performed.

Maximum and minimum air temperatures were continuously measured with a data-logger (*HOBO*; *Onset Co.*, USA) located 3 m above the ground, which recorded temperatures hourly from 14 October 2003 to 2 June 2004.

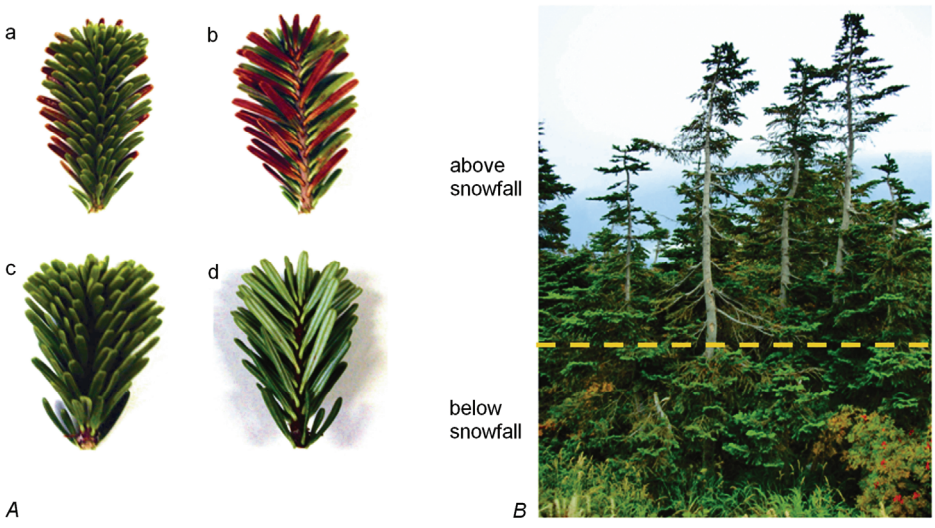

Fig. 1. *A*: Photographs of the shoots collected above (a, b) and below (c, d) the snow surface showing the adaxial (a, c) and abaxial (b, d) sides of needles. *B*: A typical shaped tree on the tree line (altitude *ca.* 2 500 m). *Dashed line* stands for the snow surface.

Chlorophyll (Chl) fluorescence was immediately measured at 20 °C with a pulse-modulated Chl fluorometer (*PAM-2100*; *Walz*, Germany) after the shoots had been dark-adapted for 30 min. The fluorometer was connected to a computer equipped with data-acquisition software (*PamWin 1.93*; *Walz*, Germany). The measurement was carried out at room temperature, and under ambient CO_2 concentration. The fluorescence nomenclature of van Kooten and Snel (1990) was followed. The PS2 activity was monitored as the ratio of the variable

(F_v) to the maximum (F_m) Chl fluorescence, $F_v/F_m = (F_m - F_0)/F_m$, where F_0 is the minimum yield of Chl fluorescence at open PS2 RCs of dark-adapted needles (Schreiber *et al.* 1994).

Traditional fluorescence induction kinetics (O-I-P transient) was measured by a *PAM-2100* in a fast kinetics mode. Needles were adapted in the dark for at least 20 min in order to fully oxidize a primary quinone electron acceptor in PS2 (Q_A). The dark-adapted needles were irradiated with a moderate red radiation [60 μmol (photon) m^{-2} s^{-1}] at a sampling rate of 1 000 μs $point^{-1}$.

Determination of pigment composition by HPLC: To extract the pigments, the shoots were ground with mortar and pestle in cold 85 % aqueous acetone together with quartz sand and 4 % (m/v) *Polyclar AT*. The mortar was then rinsed with a small amount of 100 % acetone. The homogenate was centrifuged at 10 000×*g* for 5 min. The supernatant was placed on ice, and the pellet was re-extracted using a small volume of 85 % acetone and re-centrifugation. The acetone supernatant was combined and filtered through a 0.45-μm syringe filter (*Millex®*; *Millipore*, USA) into vials. After the bubbling of nitrogen to drive off the oxygen in order to prevent the degradation of pigments, the vials were stored on ice in the dark prior to being injected by microsyringe. All extraction procedures were carried out in a dim room. Part of extract (10 mm^3) was immediately injected into an HPLC system (model *600*; *Waters*, USA) using a *TSK ODS-120A* column (4.6 mm I.D., 250 mm length) and a *TSK ODS* guard column (*Toso Co.*, Japan) following a protocol of Yamazaki *et al.* (2003). All pigments were eluted from the column within approximately 30 min at a flow rate of 1.2 cm^3 min^{-1}. The eluted pigments were monitored at 440 nm, and the temperature was maintained at 20 °C. The peak was calculated automatically by a *Waters 740* data module. The authentic pigments were used to construct standard curves.

Western blotting: The needles were frozen in liquid nitrogen and powdered with a chilled pestle and mortar in a homogenation buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM monoiodoacetic acid, 5 mM EDTA, 5 % (m/v) SDS, and 6 M urea. The homogenate was centrifuged at 10 000×*g* for 15 min at 4 °C. The supernatant obtained was denatured at 100 °C for 5 min with 5 % (v/v) 2-mercaptoethanol and 0.01 % (v/v) bromophenol blue, and then subjected to SDS-PAGE using 12.5 % acrylamide gel in accordance with Laemmli (1970). After separation, polypeptides were transferred to a PVDF membrane (*Membrane-P*, 0.2-μm pore size; *ATTO*, Japan). The antisera against the PsbA and PsbS were purchased from *AgriSera* (Sweden), and that against the PsaA/PsaB polypeptides from a cyanobacterium, *Thermosynechococcus elongatus* strain BP-1 was kindly provided by Prof. Enami, Tokyo University of Science. This antiserum raised against the proteins from *T. elongatus* has a high cross-reactivity to the proteins from higher plants (Kashino *et al.* 1990). Goat anti-chicken *IgY* (*Sigma*, USA) conjugated with horseradish peroxidase was used as a secondary antibody for PsbA and PsbS, and goat anti-rabbit *IgG* (*Bio-Rad*, Japan) conjugated with horseradish peroxidase was used as a secondary antibody for PsaA/PsaB. The experimental protocol given in the instruction manual of the *Immun-Blot Kit* (*Bio-Rad*, Japan) was followed.

Lipid peroxidation was analyzed by the method of Heath and Packer (1968). Needles were frozen in liquid nitrogen and powdered with a chilled pestle and mortar. The homogenized tissue powder was suspended in 5 % (m/v) trichloroacetic acid (TCA) on ice, and the residue of the suspension was rinsed into a centrifuge tube with an extract 1 cm^3 of 5 % (m/v) TCA. Supernatant of 1.5 cm^3 was added to the same volume of 35 mM thiobarbituric acid (TBA) dissolved in 20 % (m/v) TCA, and the mixture was heated at 95 °C for 30 min. The coloured mixture obtained was measured at 440 nm in addition to 535 and 600 nm, and each sample had a reference without TBA. The TBA-reactive substance (TBARS) concentration was calculated from the equation of Hodges *et al.* (1999), and the millimolar extinction coefficient used was 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Results

Climate: Seasonal changes in air temperature from 14 October 2003 to 2 June 2004 above the snow are shown in Fig. 2. The air temperature was continuously below 0 °C from late November 2003 until early-April 2004 and above 0 °C from late April on. The minimum temperature was –23 °C on 22 January 2004. The ground was covered with snow from late November to May with a maximum snow depth of approximately 3 m, and the melting of the snow was complete in early June. The maximum PPFD between April and May was instantaneously over 2 000 μmol(photon) m^{-2} s^{-1} at subzero temperatures. The maximum reflectance from the snow surface to the sensor (more than 2.3 m above ground) was *ca.* 1 800 μmol(photon) m^{-2} s^{-1} on a clear day in early April (Yamazaki *et al.* 2003). Furthermore, the penetration of radiation into the snow decreased exponentially, being 0.06 % of the surface value at a depth of 25 cm (Yamazaki *et al.* 2003).

Effect of winter stress on PS2 photochemistry: The maximal quantum yield of PS2 (F_v/F_m) is usually used as a good indicator of PS2 activities. As judged from the seasonal patterns of F_v/F_m, the PS2 activity dropped below 0.2 in both samples in March, and the recovery of

the PS2 activity from photoinhibition occurred rapidly during May–June (Fig. 3).

Nevertheless, the F_v/F_m ratio gives no direct information on the heterogeneity of the PS2 RC (Lavergne 1982, Melis 1991). In order to examine whether harsh winter stress induces changes in the heterogeneity of the PS2 RC, we measured the fluorescence induction kinetics. When the dark-adapted needles were irradiated with a moderate red radiation [60 μmol(photon) m^{-2} s^{-1}], a typical Kautsky curve was observed that displays a rapid rise of Chl fluorescence from the minimum level (O) to an intermediate level (I) followed by a very fast rise to the maximum level (P) (Fig. 4). The O–I phase has been attributed to Q_A reduction in the secondary quinone electron acceptor in the PS2 (Q_B) non-reducing PS2 RCs, in which the electron transfer from Q_A^- to Q_B is suppressed, and phase I–P reflects the accumulation of Q_A^- in the active PS2 RCs with efficient electron transfer to the plastoquinone pool (Cao and Govindjee 1989, Melis 1991). The $(F_I - F_O)/(F_P - F_O)$ ratio can thus be considered a measure of the percentage of Q_B-non-reducing PS2 RCs (Cao and Govindjee 1989, Melis 1991). Neither needles above or below snowfall showed a rise of the induction curve in March, suggesting that the water-splitting complexes were inactivated in the period (Fig. 4). In June, the percentage of Q_B-non-reducing PS2 RCs was 51 % in needles above snowfall and 67 % in needles below snowfall. In July, the values decreased to 31 and 35 %, respectively, indicating the recovery of the PS2 primary photochemistry.

Seasonal changes in pigment compositions: Although the Chl contents were lower in needles above snowfall than in the below snowfall ones during sub-zero periods,

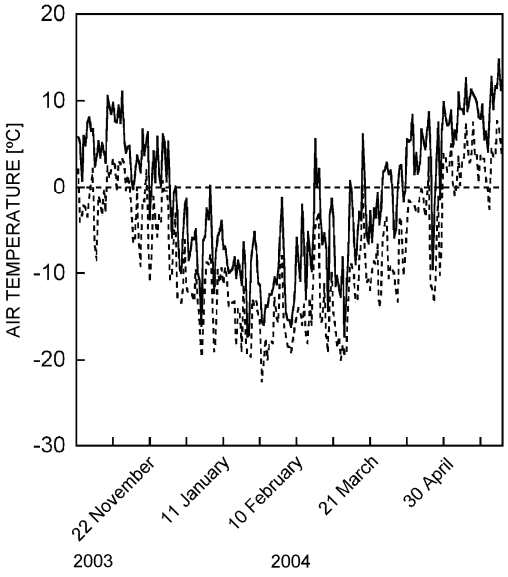

Fig. 2. Seasonal changes in air temperature. *Solid and closed lines* represent maximum and minimum air temperatures, respectively.

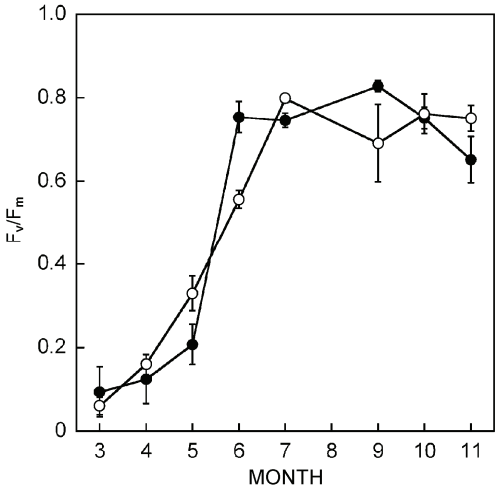

Fig. 3. Seasonal changes in the maximal quantum yield of photosystem 2 (F_v/F_m) of samples above (●) and below (○) snowfall. *Bars* indicate SD (n = 4–5).

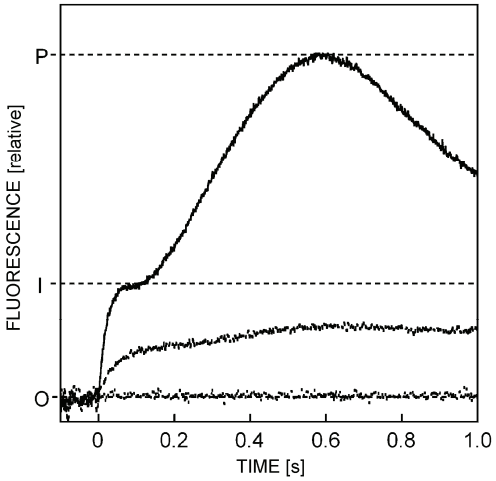

Fig. 4. A typical Kautsky curve which displays a rapid rise of Chl fluorescence from the minimum level (O) to an intermediate level (I) followed by a very fast rise to the maximum level (P). For symbols see Fig. 3. *Curves from the bottom*: needles collected in March, June, and July 2004, respectively.

they gradually increased from June to November (Fig. 5*A*). The Chl contents in both samples transiently decreased from March to May. It is possible that the energy was consumed for the sake of the recovery of the metabolic pathway rather than for the Chl biosynthesis. Seasonal changes in the Chl *a*/*b* ratios gradually decreased from March to June and remained constant from June to November, indicating that the amount of Chl *b* was relatively low in early spring (Fig. 5*B*).

β-carotene content was relatively higher in summer than in winter and slightly higher in above snowfall needles than in the below snowfall ones (Fig. 5*C*). Contents of lutein and the xanthophyll cycle pigments (V+A+Z) showed a similar trend because they decreased

from May to July: the lutein content was 1.5 times higher in both samples, and the V+A+Z pools were over double and triple the contents in the compared samples in early spring, respectively (Fig. 5*D*,*E*). The de-epoxidation state of the xanthophyll cycle pigments (DPS), expressed as (A+Z)/(V+A+Z), indicates the enzymatic conversion of

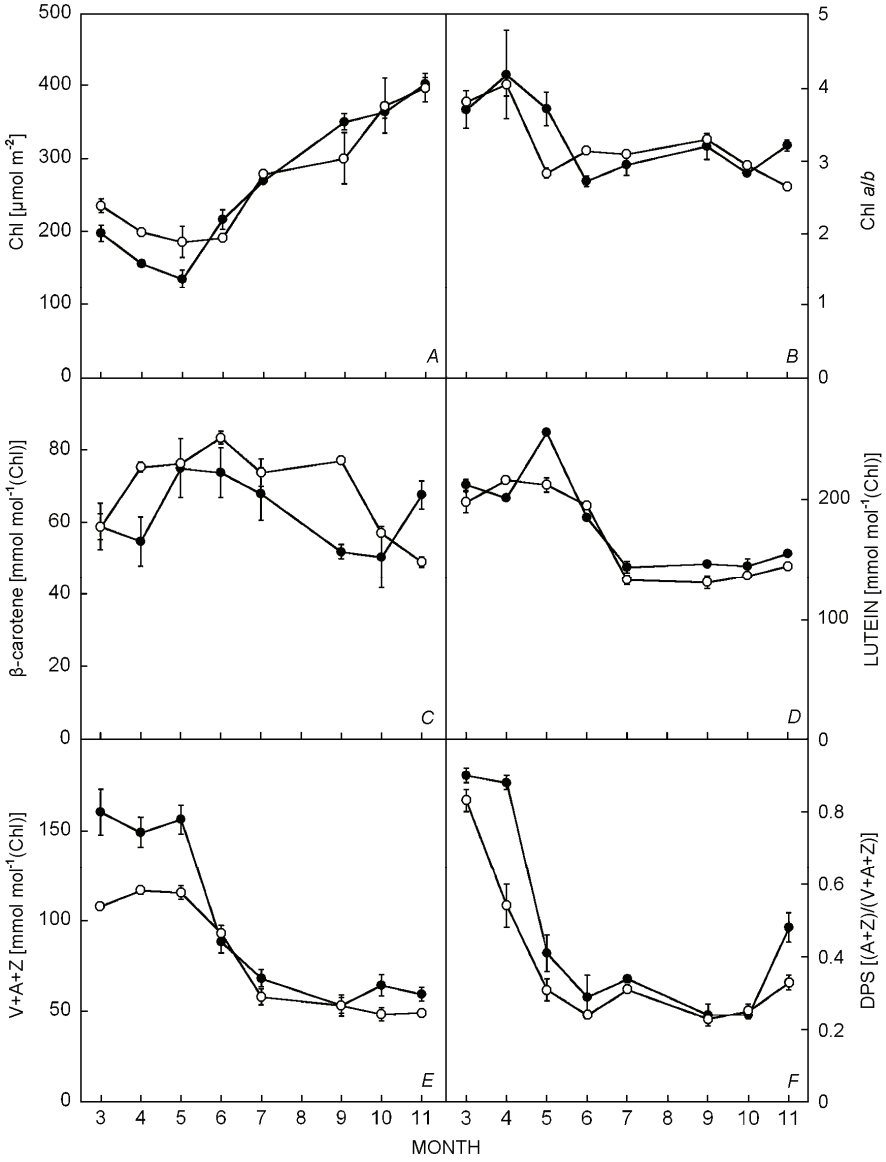

Fig. 5. Seasonal changes in pigment contents (*A, C, D*), chlorophyll (Chl) *a/b* ratio (*B*), and de-epoxidation state of xanthophyll cycle pigments (*E* – Chl-based xanthophyll cycle pool size; *F* – de-epoxidation state of xanthophyll cycle). *Bars* indicate SD (n = 4–5).

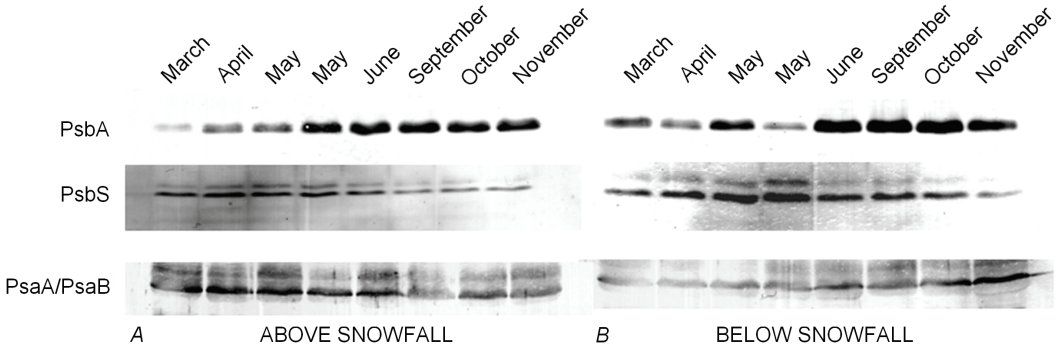

Fig. 6. Seasonal changes in the amounts of PsbA, PsbS, and PsaA/PsaB in needles above (*A*) or below (*B*) snowfall detected with Western blotting. Each lane was loaded on an equal leaf area (10 mm^3 of the leaf extracts).

xanthophylls into the de-epoxidized form, with Z being completely de-epoxidized. DPS was above 0.9 early in spring, indicating sustained conversion of the V+A+Z pool into Z (Fig. 5*F*).

Seasonal changes in pigment compositions: Western blotting analysis of seasonal changes in the RC subunits of PS2 and PS1 (Fig. 6) showed that the contents of PsbA proteins, encoding D1-protein in the PS2 RC complex, were suppressed until May in above snowfall needles and until June in the below snowfall ones, reflecting counteraction against the harsh winter stress, and recovered rapidly from May to June. This was caused by both subzero temperatures and increased PPFD during winter, indicating that the PS2 activity as a measure of F_v/F_m (Fig. 3) was regulated by the loss of PsbA. The contents of PsaA/PsaB of PS1 of the heterodimer in below snowfall needles were also suppressed during the winter and then gradually increased in the summer, whereas those in the other sample were enhanced during the winter and then gradually decreased in the summer. The contents of PsbS, which is possibly involved in the non-radiative heat dissipation process, were high from March to June in above snowfall needles. In contrast, in the below snowfall needles, PsbS contents were remarkably high in May and June. We considered that the needles under snow were rapidly exposed to strong sunlight when the snow melted.

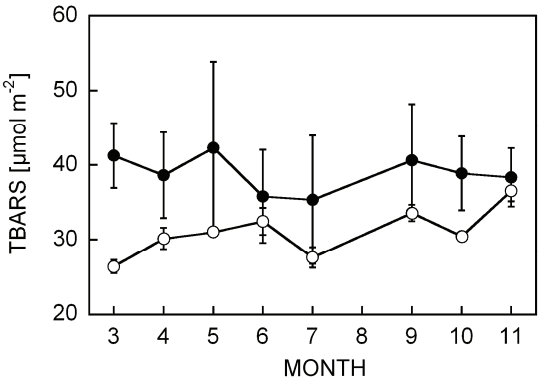

Fig. 7. Seasonal changes in thiobarbituric acid (TBA) reactive substance (TBARS) as an index of lipid peroxidation. For symbols see Fig. 3. *Bars* indicate SD (n = 4–5).

Seasonal changes in lipid peroxidation: The TBA reaction is a useful technique for detecting the lipid peroxidation as TBARS. TBARS contents were higher in above snowfall needles than in the below snowfall ones from March to November (Fig. 7). Hence the above snowfall needles always encountered the environmental stresses.

Discussion

During a harsh winter, evergreen conifers growing at a high altitude are exposed to severe winter stresses. Under such conditions, the primary photochemistry which is not affected by temperature proceeds, whereas enzyme reactions of the Calvin-Benson cycle are affected by temperature and stop or slow down. This results in the production of active oxygen species and a degradation of functional components (*e.g.* lipid peroxidation, Chl bleaching, malfunction of enzymes). In early spring, since the ground was still snow covered, above snowfall needles were exposed to both strong sunlight reflected from the snow surface and low temperatures, and the lower needles were protected from these stresses due to the snow coverage.

Therefore, the above snowfall needles exhibited a higher TBARS value, which is an index of lipid peroxidation, than the below snowfall ones (Fig. 7), suggesting where the active oxygen species are easily produced. However, attacks of the active oxygen species are mitigated by photoprotective mechanisms such as the xanthophyll cycle, which is involved in the dissipation of absorbed excess energy through the antenna system (Adams and Demmig-Adams 1994).

The contents of V+A+Z and the de-epoxidation state (DPS) were highest (Fig. 5*E,F*) and F_v/F_m decreased to about 0.2 (Fig. 3) between March and April due to increased PPFD and low temperatures in both samples. The DPS values were about 0.8 for both samples in March (Fig. 5*F*). The DPS of other severely photoinhibited conifer species is 0.8–0.9 (Adams and Demmig-Adams 1994, Verhoeven *et al.* 1999). Both the xanthophyll cycle and the protonation of PsbS play pivotal roles in modulating non-radiative energy dissipation from the light-harvesting antennae (Li *et al.* 2000). In early spring, although Z was highly accumulated, PsbS contents were low, indicating that contents of PsbS are not in parallel with Z contents (Figs. 5*F* and 6). It is inferred from this result that Z acts as a quencher of the excess excitation energy from the triplet state of Chl *a* during a severe winter (Frank *et al*. 1994), rather than as an element of the mechanism for inducible NPQ, and that the Z-dependent and PsbS-dependent NPQ arises from an alternative mechanism (Crouchman *et al.* 2006). The content of PsbS during winter in above snowfall needles (Fig. 6) contradicts earlier data showing increased contents of PsbS in pine in winter when the content of PsbA drops (Ottander *et al.* 1995). This contradiction may be explained by the fact that our study was conducted in different climatic conditions, at the tree line of the temperate zone in Japan rather than in the lowland of the boreal zone (Ottander *et al.* 1995).

When warm temperatures and moderate PPFD occurred in early May, the xanthophyll cycle pigments and DPS decreased (Fig. 5*E,F*) and F_v/F_m increased to 0.7 in June (Fig. 3). These changes indicate that the recovery from winter photoinhibition started under an air tempera-

ture of 0–5 °C from May to June (Fig. 2).

In contrast to deciduous trees, evergreen conifers that retain Chl during winter (Öquist and Martin 1980) require an increase in carotenoid content to elevate non-radiative dissipation of excess energy to protect the remaining PS2 RCs when photosynthesis is inhibited (Gilmore and Ball 2000). Accumulation of lutein has also been reported in over-wintering plants (Tausz *et al.* 2001). Contents of lutein were higher during winter and lower during summer compared to contents of Chl (Fig. 5*D*), perhaps suggesting that lutein is a quencher of excess energy (Niyogi 2000). The amount of light-harvesting Chl *a*/*b*-protein complexes is probably relatively resistant to winter stress in pine needles (Öquist and Martin 1980), facilitating rapid recovery of photosynthesis in spring (Ottander *et al.* 1995, Ensminger *et al.* 2004).

The F_v/F_m and the traditional fluorescence induction kinetics (O–I–P) indicated that winter stress had direct effects on the primary photochemistry of PS2 (Figs. 3 and 4). In particular, winter stress induced a substantial increase in the proportion of the Q_B-non-reducing PS2 RC, and this increase was higher in above snowfall needles than in the below-snow ones (Fig. 4). The Q_B-non-reducing PS2 RC represents a suppression of the electron transfer from Q_A to Q_B (Cao and Govindjee 1990, Melis 1991). From the viewpoint of the heterogeneity of the PS2 RC we can say that the Q_B-non-reducing PS2 RC is regarded as the inactivated PS2 RC. Despite some findings about the inactivated PS2 RC (Melis 1991, Öquist *et al.* 1992, Yamazaki *et al.* 1999), its physiological significance remains unclear. Öquist *et al.* (1992) proposed that the inactivated PS2 RC dissipates excess energy in order to avoid photoinhibition. We found that the abundance of the inactivated PS2 RC was greater in June than in July (Fig. 4) and therefore hypothesized that since the needles have not yet completely built up their photosynthetic machinery in spring, the inactivated PS2 RCs might dissipate the heat from the absorbed excess photon energy, supplementing antenna-based quenching during winter, owing to a rapid recovery of photosynthesis.

Thus, although the needles protruding from the snow surface are exposed to harsh winter stresses, these needles are protected from the stresses by several photoprotective mechanisms such as the degradation of the PS2 RC and heat dissipation through both the inactivated PS2 RC and the light-harvesting antennae. However, when the excess photons flow into the photosynthetic pathway and exceed the capacity of the photoprotective mechanisms, the needle colour occasionally changes from green to reddish-brown, resulting in the death of the needles. As a consequence, these mechanisms alone are not able to protect against photoinhibition under harsh winter, and the surviving needles prepare for a rapid recovery of photosynthetic capacity when warmer temperatures return the next spring.

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