J. For. Res. 4: 229–234 (1999)

Cold Acclimation and Photoinhibition of Photosynthesis Accompanied by Needle Color Changes in *Cryptomeria japonica* during the Winter

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This study focused on the physiological changes in *Cryptomeria japonica* accompanied by needle color changes during the winter. The physiological measurements include gas exchange, chlorophyll fluorescence, pigments, active oxygen scavenging enzymes, and several proteins in the photosynthetic apparatus. The light-saturated photosynthesis decreased during the winter. Total chlorophyll content and chlorophyll *a*/*b* ratio decreased; meanwhile xanthophyll cycle pigments and lutein contents increased significantly. These results indicate that cold acclimation had occurred. Photoinhibition was also observed in *C. japonica* which can be determined from the decreases in photochemical efficiency of PS II and the partial closure of PS II reaction centers. Small and large subunits of Rubisco and LHC II in the needles of *C. japonica* showed only a slight decrease during the winter, indicating that photoinhibiton in *C. japonica* probably reflects a protective process to prevent more severe damage to the photosynthetic apparatus during low-temperature stress.

Key words: *Cryptomeria japonica*, low temperature, pigments, photoinhibition, photoprotection

Photoinhibition of photosynthesis results from excessive excitation of the photosynthetic apparatus (Osmond, 1994). The functional consequences of photoinhibition of photosynthesis are inhibition of the quantum yield of PS II photochemistry (Powles, 1984) and a decreased rate of light-saturated photosynthesis. The mechanism of photoinhibition is not yet fully understood (Critchley, 1988; Krause, 1988), but it typically involves inhibition of PS II at the reaction center level (Telfer and Barber, 1994), coupled with increased thermal dissipation of excited chlorophyll (Ögren and Öquist, 1984). Recovery from photoinhibition usually requires protein synthesis since it involves replacement of photodamaged reaction center components, particularly the D 1 protein (Telfer and Barber, 1994). Alternatively, another type of photoinhibition has been proposed to result from the formation of a quencher in the antenna of PS II (Demmig and Björkman, 1987). The quencher is suggested to be a carotenoid, zeaxanthin (Demmig-Adams, 1990), which lowers the yield of photochemistry by competing with the PS II reaction centers for excitation energy. Recovery from this latter type of photoinhibition is not likely to require protein synthesis and is relatively fast.

Cryptomeria japonica, one of the most common evergreen conifer afforestation species in Japan, changes needle color from green to red-brown or orange-yellow in winter. This color change is only observed in sun-exposed needles, and thus it is thought to be caused by the interaction of high light and low-temperature stress. The color change has been demonstrated to result in changes in pigment composition, producing a large amount of the carotenoid rhodoxanthin (Ida, 1981). Moreover, saplings that are able to change needle color from green to brown or orange-yellow in winter are empirically recommended as excellent saplings for planting in silviculture. However, the scientific evidence for this selection has not been characterized physiologically. It is still unknown whether or not the changes in pigment composition are associated with the cold acclimation and photoinhibition of photosynthesis.

In this study, we examined the cold acclimation and photoinhibition of photosynthesis accompanied by color changes in needles of *C. japonica* during the winter. We measured (1) changes in the light dependent responses of photosynthesis to study cold acclimation, (2) chlorophyll fluorescence to determine photoinhibition, (3) pigment composition and active oxygen scavenging enzymes to demonstrate the photoprotective systems, and (4) changes in protein content to clarify whether photoinhibiton protects from further photodamage.

Materials and Methods

1 Plant material

Current-year needles from exposed branches of three 10-year-old trees of *Cryptomeria japonica*, grown in an open field on campus, were collected each month and used for all experiments during the winter season (November 1997–April 1998). The needle color changed in January, and it was only observed in sun-exposed needles. The color change was consistent with the HPLC data, the peak of rhodoxanthin became detectable in the foliage sample in January (Data not shown).

2 CO_2 exchange and chlorophyll fluorescence

Carbon dioxide exchange by detached branches was measured at increasing light intensity using a Mini-Cuvette gas exchange system (CMS-400, Heinz Walz GmbH, Effeltrich, Germany) under ambient or zero CO_2 concentration conditions. An FL-400 lighting unit (Heinz Walz GmbH, Effeltrich, Germany) which uses a halogen lamp, was employed as the light source, and light intensity was changed by neutral filters. Temperatures were controlled to 20, 10, and 5°C. Air-to-leaf vapor pressure deficit was kept at 6.1 ± 0.5 Pa/kPa, by first saturating air with water vapor then condensing it to a known dew point with a gas cooler (MGK-4, Heinz Walz GmbH,

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Effeltrich, Germany). Assuming the non-quadratic hyperbola model adapted from Prioul and Chartier (1977), we calculated the rate of light-saturated photosynthesis (A_{max}) and apparent quantum yield of CO_2 fixation (ϕ) from the light response curves.

Chlorophyll fluorescence measurements were made simultaneously with the CO_2 exchange measurements using a portable pulse-modulated chlorophyll fluorometer (Mini-Pam, Heinz Walz GmbH, Effeltrich, Germany). The needles were first dark adapted in the Mini-Cuvette for 2 h before the measurements. Actinic light was supplied by the above-mentioned light source. Chlorophyll fluorescence parameters were calculated according to Genty *et al.* (1989), where F_0' was not measured but calculated according to Oxborough and Baker (1997). Three replicates were made for CO_2 exchange and chlorophyll fluorescence measurements. There were almost no differences in gas exchange and chlorophyll fluorescence parameters between measurements made for detached and intact needles (Data not shown).

3 Determination of pigment composition by HPLC

Needle samples (approximately 150 mg fresh weight for each), collected at noon on clean days, were stored in liquid nitrogen until analysis. For pigment analysis, needles were ground in five times volume (v/w) of 100% cold acetone in a mortar under dim green light. Each sample was ground and rinsed several times, and the volume was then made up to 7 ml with 80% acetone. Two hundred microliters was then centrifuged, passed through a 0.22 μm filter, and 10 μl was immediately injected into the HPLC column.

Pigments were separated in an HPLC system (LC-10AD, Shimadzu Corp., Kyoto, Japan) using an ODS-1 C_{18} column (4.6 mm I.D., 250 mm length) and an ODS guard column (Phase Separations, Clwyd, U.K.) following a protocol described by Gilmore and Yamamoto (1991): Elution for 5 min with 75 : 12 : 4 (by vol.) of acetonitrile : methanol : Tris-HCl (100 mM, pH 8.0) was followed by a 0 to 100% gradient of methanol : ethyl acetate (68 : 32; v/v) built up in 10 min. All pigments were recovered from the column within about 18 min at a flow rate of 1.5 ml/min. The eluted pigments were monitored at 440 nm. The peak area was calculated automatically by a chromatopac (C-R6A, Shimadzu Corp., Kyoto, Japan). Temperature was maintained at 20°C. Chlorophyll *a* and *b* were quantified spectrophotometrically for each sample according to Arnon (1949). Three to five replicates were made for the pigments analyzed.

4 Assay of active oxygen scavenging enzymes

All enzyme extractions and centrifugations were carried out at 4°C and the extracts were stored on ice. All assays were made at room temperature. The total protein in enzyme extracts was measured with a modified Lowry assay according to Peterson (1977) using a protein assay kit (Bio-Rad, Hercules, CA, USA).

Glutathione reductase (GR), ascorbate peroxidase (APX), and superoxide dismutase (SOD) were extracted together from needles (approximately 150 mg fresh weight) ground with a mortar and pestle in liquid nitrogen. Equivalent amounts of polyvinylpoly-pyrrolidone (PVPP) and 15 times volume of extraction buffer containing 0.1 M K-Phosphate buffer (pH 7.5), 1 mM EDTA, 5 mM ascorbate, 1 mM (±)-Dithiothreitol, 0.2% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride were added to the resulting powder. The reaction mixture was centrifuged at 12,000 × *g* for 10 min. One milliliter of supernatant was then subjected to ammonium sulfate precipitation for 5% saturation, and kept on ice for 1 h following 10 min centrifugation at 20,000 × *g*. The precipitate was redissolved in 600 μl of 0.1 M K-phosphate buffer (pH 7.5), 0.2 mM ascorbate, and 0.1 mM EDTA.

For the GR assay, the reaction mixture contained 100 μl enzyme extract and 1.9 ml of 0.1 M K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.5 mM GSSG, and 0.2 mM NADPH. The consumption of NADPH was followed as the decrease in absorbance at 340 nm during 5 min of the reaction.

Assay of APX: The reaction mixture contained 100 μl enzyme extract, 1.9 ml of 0.1 M K-phosphate buffer and 0.5 mM ascorbate (fresh), and 10 μl of 20 mM H_2O_2 (fresh). The decrease in the absorbance at 290 nm (ascorbate consumption) was recorded for 5 min after the reaction was started by adding H_2O_2 (Nakano and Asada, 1981).

The SOD activity was estimated by the inhibition of the nitrobluetetrazolium (NBT) reduction based on the method of Beyer and Fridovich (1987). The absorbance at 560 nm was determined regularly, and the extraction volume causing 100% inhibition of NBT reduction was taken as one unit of activity. Three replicates were made for all scavenging enzymes analyzed.

5 Protein determinations by SDS-PAGE

Proteins were extracted by grinding 100 mg of needles (fresh weight) with 50 mg PVPP in liquid nitrogen. The resulting powder was extracted in an extraction buffer containing 0.1 M Tris (pH 8.0), 28 mM 2-mercaptoethanol, and 1% SDS. The extracts, representing equal fresh weights, were fractionated by SDS-PAGE as described by Laemmli (1970).

Results

1 CO_2 exchange and chlorophyll fluorescence

The rate of light-saturated photosynthesis (A_{max}) measured at 20°C was decreased to the lowest value in February and recovered afterwards (Fig. 1a). At 10 and 5°C, A_{max} increased in December compared to the rate in November, then exhibited the same tendency as the rates measured at 20°C. These results indicate that the photon utilizing capacity by carbon dioxide fixation cycle decreased, and thus excess photons had an increasing potential if the photon absorbing capacity did not change during the winter.

The apparent quantum yield of CO_2 fixation (ϕ) decreased to the lowest value in February at 20°C, and increased again in the following months (Fig. 1b). A similar tendency was also observed in the measurements conducted at 10 and 5°C, except for the initial increase in December at 10°C and in December and January at 5°C.

During the winter in Shizuoka, the photochemical effi-

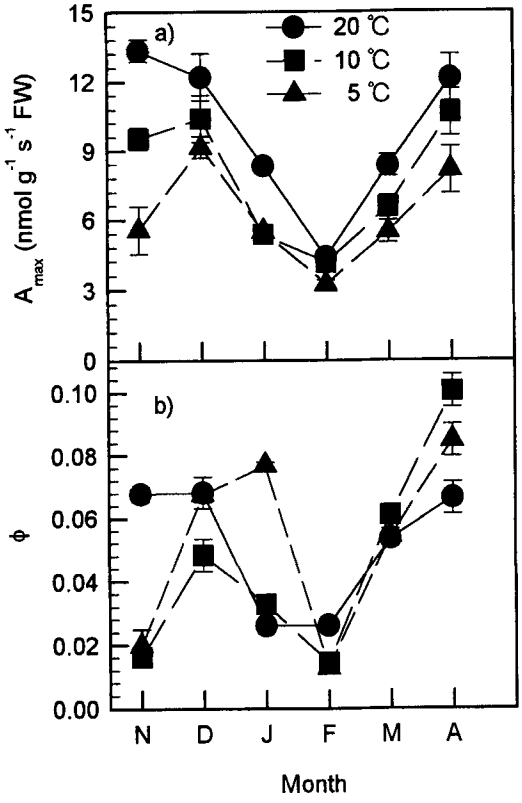

Fig. 1 Changes in a) light saturated photosynthesis (A_{max}) and b) apparent quantum yield of CO_2 fixation (ϕ) in needles of *C. japonica* during the winter, 1997–1998. Values are means ± SD for $n = 3$.

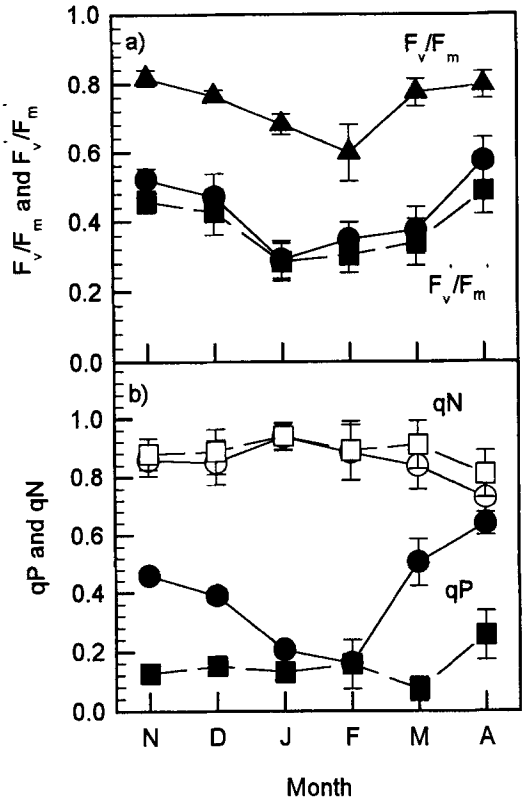

Fig. 2 Changes in a) photochemical efficiency of PS II in the dark (F_v/F_m, triangles) and actinic light (F_v'/F_m', circles and squares), b) photochemical quenching (*qP*, closed symbols) and nonphotochemical quenching (*qN*, open symbols) coefficients in needles of *C. japonica* during the winter, 1997–1998. Circles and squares indicate values measured at 20 and 5°C respectively. Light intensity was 1,100 μmol m^{-2} s^{-1} for F_v'/F_m', *qP* and *qN*. Values are means ± SD for $n = 3$.

ciency of PS II was inhibited, as shown by a drop in F_v/F_m (Fig. 2a). The reductions in F_v/F_m imply the closure of PS II reaction centers, which may be due either to damage or down-regulation of PS II (Lovelock and Winter, 1996). Before the excitation energy reaches the PS II reaction center, it also dissipates in the antenna. This dissipation process competes with photochemistry for excitation energy. It is thus associated with a decrease in the intrinsic PS II efficiency (Kitajima and Butler, 1975) that can be measured from chlorophyll fluorescence F_v'/F_m'. At both 20 and 5°C, F_v'/F_m' decreased to the lowest value in January and gradually recovered afterwards (Fig. 2a). A slight increasing tendency in nonphotochemical quenching (*qN*), which is one means of estimating the level of energy dissipation (Demmig-Adams, 1990), was observed in these needles at both 20 and 5°C (Fig. 2b). Following the recovery of PS II efficiency, the dissipation of excitation energy in the antenna decreased again. At 20°C, *C. japonica* showed the lowest value of *qP* in February, which means that the fraction of closed PS II reaction centers increased ($1 - qP$). When measurements were conducted at 5°C, *C. japonica* exhibited a relatively low value of *qP* in November, and no further significant decreases were observed.

2 Pigment component

Chlorophyll loss started in December, with a substantial drop in January and February (Fig. 3). Net chlorophyll accumulation began in March but the accumulation rate was far slower than the loss process. There was a slight decrease in chlorophyll *a/b* ratio during chlorophyll loss. *Cryptomeria japonica* accumulated a substantial amount of xanthophyll cycle pigments and lutein (Fig. 4). The content of lutein and xanthophyll cycle pigments increased about two-fold during the winter. Neoxanthin and β-carotene, which is a precursor of zeaxanthin, stayed fairly constant during the winter.

3 Photorespiration and active oxygen scavenging enzymes

Photorespiration now can be considered as inevitable but essential inefficiencies of photosynthesis which help preserve photosynthetic competence in bright light (Osmond and Grace, 1995). To evaluate the role of photorespiration in the protection of the photosynthesis apparatus, maximum photorespiration (R_{max}) measured under zero CO_2 concentrations is shown in Fig. 5a, and the ratio of R_{max}/A_{max} in Fig. 5b for comparison to the values measured under ambient CO_2 concentrations (Fig. 1a). At 20°C, R_{max} increased two-fold in December in comparison to the value in November, then decreased to the lowest value in February. The ratio of R_{max}/A_{max} was higher from December to February than in November, which means that photorespiratory O_2 uptake decreased less than photosynthetic CO_2 uptake *via* Rubisco during the winter. When the measurements were conducted at

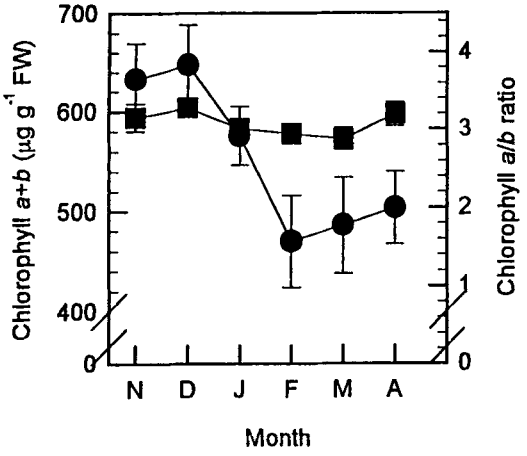

Fig. 3 Changes in chlorophyll $a + b$ contents (circles) and chlorophyll a/b ratios (squares) in needles of *C. japonica* during the winter, 1997–1998. Values are means ± SD for $n = 3$.

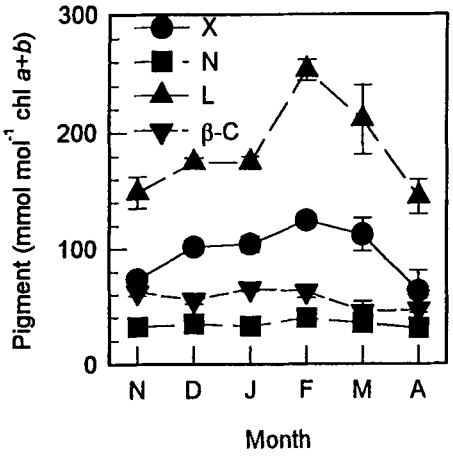

Fig. 4 Pigment changes in needles of *C. japonica* sampled at noon on clear days during the winter, 1997–1998. X, xanthophyll cycle pigments; N, neoxanthin; L, lutein; β-C, β-carotene. Values are means ± SD for $n = 3$–5.

lower temperatures of 10 and 5°C, only a slight decrease in R_{max} was observed during the winter, and the ratio of R_{max}/A_{max} increased. Some decrease of the ratio of R_{max}/A_{max} in December at 5°C was caused by an increase in photosynthetic CO_2 uptake (Fig. 1a). These results indicate that photorespiration intensively protects the photosynthetic apparatus from photoinhibition.

Reactive oxygen species appear to be involved in aerobic photoinhibition, and active oxygen scavenging enzymes protect chloroplasts from attack by activated oxygen species (Asada, 1994). We analyzed changes in the activities of active oxygen scavenging enzymes in needles of *C. japonica* during the winter. As shown in Fig. 6, the activities of superoxide dismutase (SOD) and glutathione reductase (GR) increased about two-fold, and ascorbate peroxidase (APX) also exhibited an increasing tendency.

4 Protein content

From the above results, photoinhibition occurred in the needles of *C. japonica* during the winter, and several protec-

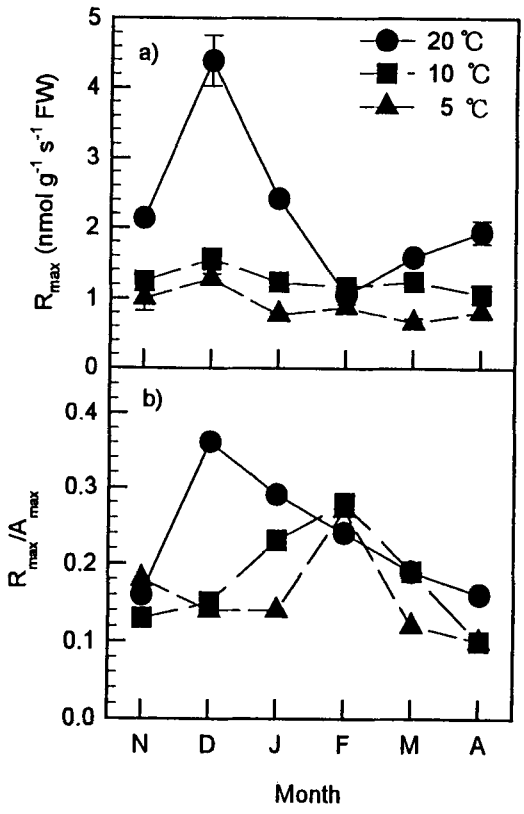

Fig. 5 Changes in a) maximum photorespiration (R_{max}) and b) R_{max}/A_{max} in needles of *C. japonica* during the winter, 1997–1998.

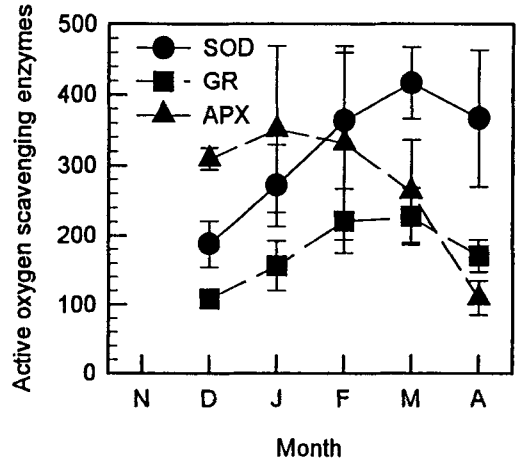

Fig. 6 Changes in active oxygen scavenging enzyme activities in needles of *C. japonica* during the winter, 1997–1998. SOD: superoxide dismutase, U/min/mg protein; GR: glutathione reductase, nmol/min/mg protein; APX: ascorbate perosidase, nmol/min/mg protein. Values are means ± SD for $n = 3$.

tive systems were found during this period. It was of interest to learn whether photodamage occurred or not. For this purpose, we determined the changes in the light-harvesting chlorophyll a/b binding protein of PS II (LHC II) and small and large subunits of Rubisco (SSU and LSU, respectively) (Fig. 7). A slight decrease in the bands corresponding to LHC II and SSU and LSU was observed from December to February in the needles of *C. japonica* during the winter,

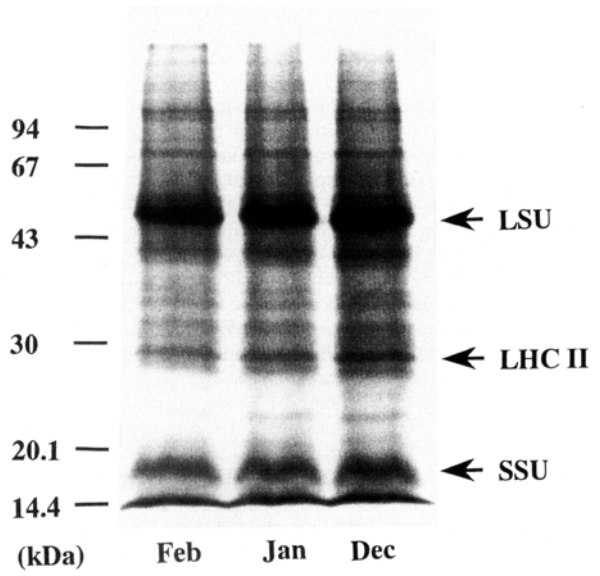

Fig. 7 Changes in small and large subunits of Rubisco (SSU and LSU, respectively) and LHC II in needles of *C. japonica* during the winter, 1997–1998.

which probably indicates that photodamage did not occur and thus photoinhibition was photoprotective.

Discussion

Low temperature during the winter resulted in decreases in the rate of light-saturated photosynthesis (A_{max}) in *C. japonica* (Fig. 1a). Such decreases in A_{max} in conifers have frequently been observed in winter months, and have usually been ascribed to a combination of temperature stress and light stress (Strand and Öquist, 1985; Ottander and Öquist, 1991; Weger *et al.*, 1993). Low temperature can produce a decrease in the maximum photosynthetic rate due to the decreases in the activities of Benson-Calvin cycle enzymes, while light stress will result in the photoinhibition of PS II (indicated by a decrease in F_v/F_m or by a decrease in quantum yield). In *C. japonica*, photoinhibition is seen probably as a reflection of a protective process to prevent more severe damage to the photosynthetic apparatus during low-temperature stress, *via* the following mechanisms: chlorophyll loss, the xanthophyll cycle pigments dependent energy dissipation process, increases in the fraction of closed PS II centers, photorespiration, and active oxygen scavenging enzymes.

1 Cold acclimation

The decreases in A_{max} were correlated with chlorophyll loss (Figs. 1a, 3), in agreement with the results of Ottander *et al.* (1995). This may be part of a cold acclimation process. Given that the fixation of CO_2 by the Benson-Calvin cycle is limited by low temperature, sufficient light energy can most likely be captured with fewer chlorophyll molecules during the winter months. Decreases in chlorophyll content could thereby lead to a decrease in the amount of excess energy absorbed under such conditions as well as contribute to an increase in the ratio of the xanthophyll cycle pigments (responsible for dissipation) to the chlorophyll molecules (responsible for absorbing the light) (Fig. 4). The apparent quantum yield of CO_2 fixation and A_{max} increased in early winter when measurements were conducted at 10 and 5°C (Fig. 1b). This increase was concomitant with a slight decrease in the chlorophyll *a/b* ratio during chlorophyll loss. This indicates another part of the cold acclimation process in *C. japonica* during early winter which can maintain a relatively high assimilation rate even at decreased light absorption.

2 Protection by quenching of excited chlorophyll

Although the chlorophyll loss process in needles of *C. japonica* in the winter decreased the amount of excess energy absorptance, the value of F_v'/F_m' decreased (Fig. 2a), and *qN* seemed to increased slightly (Fig. 2b). This indicates that the dissipation of excitation energy in the antenna before it reaches the PS II reaction center increased in the winter. This increase was correlated with a pronounced increase in the levels of xanthophyll cycle pigments and the lutein pool (Fig. 4), in accordance with reports for other conifers (Adams and Demmig-Adams, 1994; Ottander *et al.*, 1995). For needles of *C. japonica* that are experiencing high-light stress in addition to low-temperature stress, PS II efficiency is down-regulated through xanthophyll cycle dependent energy dissipation to match the absorbed energy utilization. However, the down-regulation and protection of the photosynthetic apparatus can not be solely explained through the xanthophyll cycle, as reported for other conifers (Adams and Demmig-Adams, 1994; Ottander *et al.*, 1995). Substantial accumulation of rhodoxanthin may serve to protect the photosynthetic apparatus, in consequence of intercepting some of the incident light and decreasing the effective light intensity reaching the photosynthetic apparatus (Weger *et al.*, 1993). Although the quantitative rhodoxanthin could not be determined, the peak of rhodoxanthin became detectable in the foliage sample in January according to the HPLC data (Data not shown). This result was consistent with the decrease in F_v'/F_m' (Fig. 2a). Xanthophyll cycle-dependent energy dissipation seemed insufficient in *C. japonica*, which can be proved by the increased fraction of closed PS II reaction centers in the winter, especially at 20°C ($1 - qP$, Fig. 2b).

3 Protection by photorespiration and active oxygen scavenging enzymes

The decreases in A_{max} were consistent with the increases in electrons transported to photorespiratory O_2 uptake (Fig. 5b). This indicates that photorespiration plays an important role in protecting the photosynthetic apparatus against photoinhibition in *C. japonica* in agreement with earlier studies by Wu *et al.* (1991).

Cryptomeria japonica showed higher activities of SOD, APX, and GR (Fig. 6) are in agreement with the findings from Scots pine (Krivosheeva *et al.*, 1996). This indicates that the SOD, APX, and GR systems protect chloroplasts from oxygen radicals formed upon excessive excitation, and also allow O_2 to function as an electron acceptor, thus opening a fraction of the PS II reaction centers and supporting electron transport in excess of CO_2 fixation (*qP*, Fig. 2b).

The pigment contents on a basis of fresh weight decreased

about 30% from December to February in chlorophyll (Fig. 3), 13% in xanthophyll cycle components and increased 7% in lutein (calculated from Figs. 3 and 4). These changes of pigments as well as the protection by photorespiration and active oxygen scavenging enzymes resulted in a slight decreases in LHC II (Fig. 7), and protected the photosynthetic apparatus from further photodamage.

4 Recovery from photoinhibition

A_{max} was almost fully recovered in April to the level observed in November (Fig. 1a). However, chlorophyll content in April was significantly lower than that in November (Fig. 3). The photochemical efficiency of opened PS II centers (F_v'/F_m') was also fully recovered to the level of preinhibition (Fig. 2a). This means that the dissipation of excited energy through the xanthophyll cycle decreased to match the high utilizing potential of the absorbed energy under partial chlorophyll recovery conditions. The decrease of *qN* in April supports this conclusion (Fig. 2b). The conclusion is also supported by the decreases of the xanthophyll cycle pool (Fig. 4). In addition, the fraction of opened PS II centers recovered to a much higher level in April than the value of preinhibition in November at both 20 and 5°C (*qP*, Fig. 2b). This may compensate for the low accumulation rate of chlorophyll. The quick recovery of the photochemical efficiency of PSII and the low accumulation process of chlorophyll may help the needles to respond to environmental factors more quickly and efficiently.

As a conclusion, *C. japonica* had acclimating ability to the low temperature and high light environment during the winter. The acclimation included changes in pigment component and other protective mechanisms and accompanied by needle color changes. These findings provide physiological evidence for sapling selection in silviculture.

This research was supported by the Bio-oriented Technology Research Advancement Institute (BRAIN), Japan.

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(Accepted April 15, 1999)