

## Seasonal changes in abundance and phosphorylation status of photosynthetic proteins in eastern white pine and balsam fir

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**Summary** During winter, the light-harvesting complexes of evergreen plants change function from energy-harvesting to energy-dissipating centers. The goal of our study was to monitor changes in the composition of the photosynthetic apparatus that accompany these functional changes. Seasonal changes in chlorophyll fluorescence, pigment concentration, and abundance and phosphorylation status of photosynthetic proteins in *Pinus strobus* L. (sun-exposed trees) and *Abies balsamea* (L.) P. Mill. (sun-exposed and shaded trees) were examined in the cold winter climate of Minnesota. Results indicated typical seasonal changes in chlorophyll fluorescence and pigment concentration, with sustained reduced photosystem II (PSII) efficiency during winter, accompanied by retention of zeaxanthin and antheraxanthin, and winter increases in the pool of xanthophyll cycle pigments and lutein. In sun-exposed trees, all photosynthetic proteins that were monitored decreased in relative abundance during winter, although two light-harvesting chlorophyll a/b binding proteins (Lhcb2 and Lhcb5), and the PsbS protein, were enriched in non-summer months, suggesting a role for these proteins in winter acclimation. In contrast, shaded trees maintained most of their protein throughout winter, with reductions occurring in spring. Thylakoid protein phosphorylation data suggest winter increases in the phosphorylation of a PSII core protein, PsbH, in sun-exposed trees, and increases in phosphorylation of all PSII core proteins in shaded trees.

**Keywords:** *conifers, light-harvesting proteins, low temperature stress, sustained energy dissipation, xanthophyll cycle.*

### Introduction

In overwintering evergreen plants, the process of xanthophyll-cycle-mediated energy dissipation changes from one

that responds rapidly to alterations in excess absorbed excitation energy in the summer months to a long-term sustained engagement of energy dissipation that does not respond to a changing light environment during winter (Öquist and Huner 2003, Demmig-Adams and Adams 2006). The winter-induced sustained energy dissipation seems to be critical for maintaining the balance between light absorption and its reduced use as a result of the effects of low temperatures on photosynthetic carbon reduction. This transformation of xanthophyll cycle characteristics appears to involve changes in the composition and characteristics of the photosynthetic apparatus such that there is a functional change from light-harvesting centers to dissipating centers (Öquist and Huner 2003).

The dynamic form of energy dissipation has been well studied and is known to require conversion of the xanthophyll violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z), acidification of the thylakoid lumen and protonation of the PsbS protein (for reviews see Demmig-Adams and Adams 1996, Müller et al. 2001, Szabo et al. 2005). The xanthophyll lutein is also required for this form of energy dissipation (e.g., Pogson et al. 1998). The dynamic form of energy dissipation is a component of non-photochemical quenching (NPQ), termed qE, and is the major and most rapid component of NPQ in plants that are not exposed to extreme stressors (Müller et al. 2001). The sustained form of energy dissipation is observed during severe stress, such as exposure to low temperatures in evergreens, and is less well studied (Müller et al. 2001, Öquist and Huner 2003, Demmig-Adams and Adams 2006). Sustained energy dissipation can also be measured as a component of NPQ, termed qI, and is characterized by being slowly reversible (Müller et al. 2001). In overwintering evergreens, the presence of qI is observed as a persistent reduction in the maximal efficiency of photosystem II (PSII) photochemistry (measured as predawn  $F_v/F_m$ , Ottander et al. 1995, Verhoeven et al. 1998, 1999, 2004, Adams et al. 2001, Demmig-Adams and Adams 2006).

Several studies have demonstrated a correlation between winter-induced sustained low values of maximal PSII efficiency ( $F_v/F_m$ ) and sustained dark-retention of the xanthophyll cycle pigments A and Z (Adams and Demmig-Adams 1994, Ottander et al. 1995, Verhoeven et al. 1996, 1998, 1999, 2004). Additionally, acclimation to winter involves increases in the total pool size of the xanthophyll cycle pigments (Adams and Demmig-Adams 1994, Logan et al. 1998, Verhoeven et al. 1998, 1999, 2004). Such studies have led to the hypothesis that the xanthophyll pigments A and Z are required for the sustained energy dissipation that is observed during winter as sustained reductions in predawn  $F_v/F_m$ . Additional evidence that the winter-induced sustained energy dissipation is dependent on the xanthophyll cycle was obtained by Gilmore and Ball (2000) who examined both spectral characteristics and lifetime distributions of chlorophyll fluorescence in leaves of the evergreen snow gum. They reported changes in both of these parameters that are consistent with dark-sustained xanthophyll-cycle-mediated energy dissipation (see also Matsubara et al. 2002).

Studies examining the recovery kinetics on warming winter-stressed leaves in the laboratory and monitoring increases in  $F_v/F_m$  have demonstrated two phases to the recovery, one that reverses rapidly (hours) and another that reverses slowly (days) on warming (Verhoeven et al. 1998, 1999). Both the rapid and slow components of recovery correlated with reconversion of the xanthophylls Z and A to xanthophyll V. These studies have been interpreted to demonstrate that more than one mechanism may be involved in maintaining xanthophyll-cycle-dependent sustained energy dissipation in overwintering evergreens, with the rapidly reversible component involving a nocturnally retained pH gradient, and the slowly reversible component involving protein synthesis or reorganization, or both (Verhoeven et al. 1998, 1999, Adams et al. 2004).

In addition to alterations in xanthophyll cycle characteristics, there is increasing evidence that sustained energy dissipation involves a major reorganization of the pigment-protein complexes within the thylakoid membrane (Ottander et al. 1995, Savitch et al. 2002, Ensminger et al. 2004, Zarter et al. 2006, Busch et al. 2007, 2008). Studies examining changes in the composition and characteristics of the photosynthetic proteins during winter have consistently found a marked decrease in abundance of the D1 protein (Ottander et al. 1995, Savitch et al. 2002, Ensminger et al. 2004, Zarter et al. 2006, Busch et al. 2007). Both Ottander et al. (1995) and Savitch et al. (2002) observed winter decreases in light-harvesting complex (LHC) proteins and winter increases in the PsbS protein. Zarter et al. (2006) found winter increases in PsbS in shaded, but not in sun-exposed, populations of overwintering *Arctostaphylos uva-ursi* [L.]. However, Ensminger et al. (2004) who studied Scots pine growing in Siberia found no change during winter in LHC concentration until reductions in these proteins occurred during the spring, whereas

winter decreases in PsbS were observed. Busch et al. (2007), who examined the effects of air temperature and photoperiod on the acclimation of jack pine to winter conditions in a chamber study, reported no change in a variety of light-harvesting proteins and decreases in PsbS during the acclimation process. Both Ottander et al. (1995) and Busch et al. (2007, 2008) provide evidence for increases in the aggregation state of LHCs during winter, which accompany sustained energy dissipation.

Thylakoid protein phosphorylation may play a role in the maintenance of sustained energy dissipation during winter. Correlations between sustained energy dissipation and thylakoid protein phosphorylation have been demonstrated in rice leaves under chilling conditions (Kim et al. 1997), in photoinhibited leaves of the shade plant *Monstera deliciosa* Liebm. (Ebbert et al. 2001) and in the evergreen Douglas fir on subfreezing winter nights (Ebbert et al. 2005).

The ability of overwintering evergreens to maintain sustained energy dissipation during winter is clearly of fundamental importance in maintaining the balance between light absorption and its reduced use during winter. The goal of our study was to obtain a more detailed understanding of the changes in individual light-harvesting proteins (their relative abundance and phosphorylation status) during the transition from light-harvesting to dissipating centers which occurs during winter acclimation and the reversal that occurs in the spring. We hypothesized that winter-induced sustained energy dissipation is mediated by certain key light-harvesting proteins (or other proteins associated with the photosystems) and that it may also involve changes in phosphorylation status of thylakoid proteins. We monitored seasonal changes in relative abundance and phosphorylation status of individual light-harvesting and reaction center proteins in two species of overwintering evergreens that are native to Minnesota, eastern white pine (*Pinus strobus* L.) and balsam fir (*Abies balsamea* (L.) P. Mill.). To assess the effects of irradiance on these seasonal changes, both sun and shade populations of balsam fir were monitored.

## Materials and methods

### *Plant material*

We monitored 10–15-year-old trees of balsam fir and white pine trees growing on the grounds of the campus at the University of St. Thomas, St. Paul, MN (44°59'40" N and 93°05'35" W) in full sun and in a shaded site (balsam fir only). The shaded trees were growing on the north side of a building such that the needles on the south side of these trees were effectively shaded throughout the year. Needles were collected for isolation of thylakoids from trees growing in full sun and in the shaded site every 2 months on the following dates: January 15, 2005; March 15, 2005; May 24, 2005; July 6, 2005; September 26, 2005; November 22, 2005 and January 23, 2006. Mean monthly high/low

temperatures (°C) for these months in this location were: January, -5/-14; March, 6/-4; May, 22/9; July, 28/17; September, 22/11 and November 5/-3. Needles were collected on clear days between 1100 and 1300 h to ensure consistency in sampling, although in November 2005 cloudy conditions were persistent. Thus the collection was performed on a day that started clear, but clouds had formed by the time of sampling. Needles were collected from the south-facing side of each tree, such that the sun-exposed needles experienced full sunlight throughout most of the day, and needles collected from the shaded trees were shaded by the building throughout the day. In May 2005, balsam fir had new needles, so both new and old needles were sampled, whereas new needles were not seen until the July sampling date for white pine, at which time both new and old needles were sampled. In subsequent collections for the seasonal analysis, only the newer needles were analyzed. To assess the fresh to dry mass ratios of the needles, they were collected from the field into plastic bags and immediately taken to the laboratory where their fresh mass was determined. They were then dried for 3 days at 80 °C and their dry mass was determined.

#### *Chlorophyll fluorescence and pigment analysis*

Chlorophyll fluorescence was measured with a field portable Fluorescence Monitoring System (FMS, Hansatech, King's Lynn, UK). Needle temperature and light incident on the needles were measured at the same time when the fluorescence measurements were made (both predawn and midday) using the photosynthetically active radiation/temperature sensors on the leafclip (FMS, Hansatech). Midday measurements were performed in ambient light in full sun. Chlorophyll fluorescence measurements and parameters are as described by Demmig-Adams et al. (1996). The maximal efficiency of solar energy conversion was calculated as  $F_v/F_m$ , measured predawn. We used  $\Phi_{PSII}$  ( $(F_m' - F)/F_m'$ ) to estimate the fraction of absorbed light used in PSII photochemistry at midday (Genty et al. 1989). We used  $1 - q_P$  ( $(F_m' - F)/(F_m' - F_o')$ ) to estimate PSII excitation pressure at midday (Huner et al. 1998, Adams et al. 2002). Pigments were extracted according to Adams and Demmig-Adams (1992) and were analyzed by HPLC as described by Gilmore and Yamamoto (1991).

#### *Recovery study*

To monitor the kinetics of recovery from winter-induced reductions in  $F_v/F_m$ , needles were measured in the field on a cold winter morning (January 10, 2006, minimum temperature -7 °C). Following the initial fluorescence measurements, branches were cut and immediately placed in clear plastic bags with moist paper towels and taken to the laboratory. Branches were maintained at room light and temperature (photosynthetic photon flux, PPF = 5–10  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , temperature = 22 °C) and needles were monitored for  $F_v/F_m$  after 1, 8, 25, 53, 74 and 170 h.

At least six branches were monitored from both species and light environments.

#### *Isolation of thylakoids*

For the seasonal analysis, needles were frozen in liquid nitrogen and were taken to the laboratory where they were ground in liquid nitrogen with a mortar and pestle and then homogenized with a polytron (Brinkman, Model 10/35, Metrohm, Westbury, NY) in grinding buffer (50 mM Tricine, pH 7.6, 0.4 M sorbitol, 10 mM NaF, 10 mM  $\text{MgCl}_2$ , 20% polyethyleneglycol (Av. Mol. Wt. 10,000, Sigma-Aldrich, St. Louis, MO), 1 mM benzamidine-HCl and 5 mM 6-amino-*n*-hexanoic acid). Samples were filtered through miracloth and centrifuged at 10,000g for 10 min at 4 °C. The pellet was resuspended in wash buffer (50 mM Tricine, pH 7.6, 10 mM NaCl, 10 mM NaF and 5 mM  $\text{MgCl}_2$ ) and recentrifuged. Thylakoids were resuspended in 50 mM Tricine, pH 7.6, containing 50% glycerol, 5 mM  $\text{MgCl}_2$  and 10 mM NaCl. Protein concentration of thylakoids was determined by the Bio-Rad Protein assay and chlorophyll concentration was determined as described by Porra et al. (1989).

#### *SDS-PAGE and immunoblotting*

Samples of isolated thylakoids were subjected to SDS-PAGE. Twenty micrograms of protein were loaded onto each lane and separated on the 15% reducing gels by standard methods of Laemmli (1970). Gels were stained with Coomassie Blue to visually verify equal loading in all lanes. For immunodetection, proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore) and probed with monospecific antibodies (Lhcb1, 2, 4 and 5; Lhca1, 2 and 4; D1, PsaD, PsbS and PsbH) procured from AgriSera. Secondary antibodies were goat anti-rabbit IgG and rabbit anti-chicken IgY conjugated with horseradish peroxidase (Sigma-Aldrich). Antibodies were detected by chemiluminescence (RPN2109, GE Healthcare) followed by exposure of the membrane to film (Kodak BioMax light film). Band intensity was quantified using Image J (NIH, Bethesda, MD). Before the seasonal analysis, each antibody was tested to verify a linear relationship between protein concentration and antibody signal.

To determine phosphorylation status, thylakoids were solubilized in the presence of 6 M urea and run on 15% gels containing 6 M urea (Rintamäki et al. 1997, Bergo et al. 2002), and probed with rabbit anti-phosphothreonine (71-8200, Zymed, Invitrogen, Carlsbad, CA) using the recommended dilutions (1–2  $\mu\text{g ml}^{-1}$  antibody) and the Zymed membrane blocking solution (00-0105, Invitrogen). To identify the phosphorylated bands, control thylakoids were prepared from pumpkin leaves exposed to low and high light following the protocol described by Rintamäki et al. (1997) and the bands were compared with the published data. Additionally, direct comparisons were performed of LHC (both Lhcb1 and Lhcb4), D1, D2 and

PsbH using purchased antibodies (AgriSera). CP43 was the only protein for which no antibody was available; however, its position and response to light were consistent with the published data. For the conifers, the pumpkin thylakoids were loaded as controls and the phosphorylated bands were identified by comparison with appropriate controls. Because PsbH is not phosphorylated in pumpkin, its position was verified independently in the conifers.

#### Statistical analysis

To test for significant differences between months in chlorophyll fluorescence parameters and in pigment concentration, analysis of variance was applied followed by pairwise comparisons of all means by the Tukey–Kramer HSD comparison (JMP Statistical Software, SAS, Carey, NC).

### Results

Seasonal variations in mean daily temperature from January 2005 until January 2006, collected on the campus of the University of St. Thomas where the study was conducted, are shown in Figure 1. Mean daily temperature remained below zero until mid-March 2005, with mean temperatures dropping below zero again in late November. The low temperatures often coincided with high irradiances, because cold days are often clear in this region (see Table 1). Needles were sampled every 2 months from January of 2005 to January of 2006. Needle temperature at the time of sampling, mean radiation incident on the needles at the time of sampling and the fresh to dry mass ratio of the needle tissue are provided in Table 1. Needle temperature on the day of sampling was consistent with mean monthly temperatures for the region for all months, except January 2006, which was unusually warm (see Materials and methods section for mean monthly temperatures for this location). Although the temperature on the day of sampling in January of 2006 was low, the mean temperature in January 2006 was about 6 °C warmer than in January 2005 (mean high/low was  $-5/-14$  °C in 2005 versus  $1/-5$  °C in 2006). Midday PPF incident on the needles demonstrated that the pine and sun fir were exposed to full sunlight, whereas the shade fir was exposed to a mean PPF of  $40-50 \mu\text{mol m}^{-2} \text{s}^{-1}$ . On November 22, 2005, there was cloud cover at midday, which is reflected by the lower midday PPF values compared with the rest of the year. The ratio of fresh to dry mass of the needles was highest in the young, newly developing needles, and decreased as the needles matured. The ratio was fairly constant over the season in pine and shade fir, although in the sun fir the ratio was significantly lower during the winter.

#### Seasonal changes in chlorophyll fluorescence and needle pigment concentration

Midday chlorophyll fluorescence measurements showed typical values for plants growing in sun or shade (Table 2).

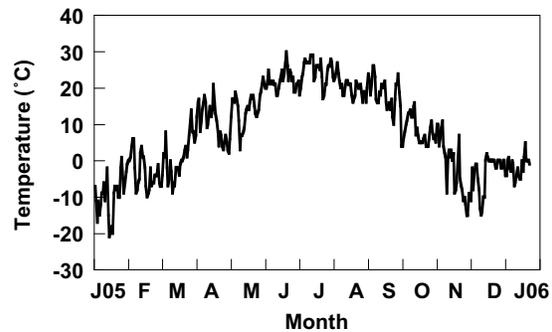


Figure 1. Seasonal variation in mean daily temperature from January 2005 until January 2006, at the study site on the campus at the University of St. Thomas.

In summer, the shaded trees had higher  $\Phi\text{PSII}$  values than the sun-exposed trees, reflecting their ability to use a large fraction of the light absorbed in photochemistry, which is expected at such low irradiances. The shaded trees also maintained a much lower  $1 - q_P$  than the sun-exposed trees, illustrating the low excitation pressure to which they were exposed. All plants showed a significant reduction in  $\Phi\text{PSII}$  during the winter, which is consistent with a reduction in the fraction of absorbed light being used in photochemistry during the winter. Additionally, all plants showed an increase in  $1 - q_P$  during winter (although this increase was not statistically significant in the sun fir needles), reflecting increased excitation pressure on PSII. In the shade fir needles, there was a large increase in  $1 - q_P$  during March, suggesting an increase in excitation pressure during recovery in the spring. Predawn  $F_v/F_m$  values showed typical seasonal responses, with high values (above 0.8) occurring in all species during the summer and significant reductions in predawn  $F_v/F_m$  occurring in all species during the winter (Figure 2A). Pine had the most pronounced reduction in  $F_v/F_m$  in both winters and recovered more slowly in the spring of 2005 compared with balsam fir. Predawn  $F_v/F_m$  of both sun and shade fir were higher in January 2006 than in January of 2005, reflecting the warmer conditions in January 2006.

No significant seasonal changes in total chlorophyll concentration (Figure 3A, expressed per unit fresh mass), or chlorophyll a/b ratio (Figure 3E) occurred in either pine or fir. There was no seasonal change in the concentration of neoxanthin (Figure 3B) in pine or shade fir; however, there was a significant increase in neoxanthin concentration in sun fir needles collected in winter relative to summer (significant difference between both January and July). Additionally, there were no significant seasonal changes in the concentration of  $\beta$ -carotene (Figure 3D) in sun or shade fir, whereas in pine there was a significant increase in  $\beta$ -carotene in November relative to the rest of the year. The concentration of  $\alpha$ -carotene (Figure 3H) decreased significantly during winter in all trees studied, although the total amount of  $\alpha$ -carotene was generally less than that of the other

Table 1. Predawn and midday temperatures, PPF incident on the needles at time of collection and fresh mass/dry mass ratios of needle samples of sun-exposed *P. strobus* trees and *A. balsamea* trees growing in both sunny (sun fir) and shaded (Shade fir) environments on all sampling days. Abbreviations: PD, predawn; MD, midday; FW, fresh mass; DW, dry mass; and y1 and y2 represent needles produced the previous summer and new growth, respectively. Data are mean  $\pm$  SD,  $n = 6$ . For FW/DW, significant differences between mean values are indicated by different letters.

	Jan. 22, 2005	Mar. 19	May 24	Jul. 6	Sep. 26	Nov. 22	Jan. 23, 2006
<i>Pine</i>							
PD temperature ( $^{\circ}\text{C}$ )	$-12.2 \pm 0.3$	$-0.9 \pm 1.3$	$11.8 \pm 0.2$	$21.5 \pm 0.2$	$11.8 \pm 0.2$	$-10 \pm 1$	$-6 \pm 1$
MD temperature ( $^{\circ}\text{C}$ )	$3.2 \pm 0.5$	$-0.4 \pm 0.2$	$22 \pm 0.2$	$24.8 \pm 1.2$	$20.3 \pm 0.5$	$1.4 \pm 0.8$	$-5.5 \pm 2/5$
MD PPF ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$1250 \pm 50$	$875 \pm 75$	$1850 \pm 150$	$1860 \pm 125$	$1455 \pm 145$	$250 \pm 60$	$1310 \pm 110$
FW/DW	$2.2 \pm 0.05$ c		$1.9 \pm 0.03$ d	$2.1 \pm 0.02$ (y1) c $2.5 \pm 0.03$ (y2) a	$2.3 \pm 0.06$ b	$2.2 \pm 0.03$ bc	$2.2 \pm 0.09$ c
<i>Sun fir</i>							
PD temperature ( $^{\circ}\text{C}$ )	$-12.2 \pm 0.3$	$0.2 \pm 1$	$11.6 \pm 0.2$	$21.3 \pm 0.1$	$11.5 \pm 0.2$	$-10.7 \pm 0.7$	$-6.7 \pm 0.05$
MD temperature ( $^{\circ}\text{C}$ )	$-1 \pm 1$	$-0.6 \pm 0.5$	$22.5 \pm 0.3$	$22.8 \pm 0.6$	$21 \pm 0.5$	$1.9 \pm 0.5$	$-1 \pm 2$
MD PPF ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$1150 \pm 100$	$970 \pm 30$	$1645 \pm 205$	$1725 \pm 295$	$1400 \pm 105$	$220 \pm 65$	$1325 \pm 100$
FW/DW	$2.0 \pm 0.04$ c		$2.0 \pm 0.02$ (y1) c $3.3 \pm 0.13$ (y2) a	$2.4 \pm 0.04$ b	$2.2 \pm 0.02$ b	$2.1 \pm 0.10$ c	$2.0 \pm 0.03$ c
<i>Shade fir</i>							
PD temperature ( $^{\circ}\text{C}$ )	$-12.1 \pm 0.2$	$-0.8 \pm 0.3$	$10.8 \pm 0.1$	$21.4 \pm 0.1$	$10.6 \pm 0.3$	$-10.9 \pm 0.3$	$-6 \pm 1$
MD temperature ( $^{\circ}\text{C}$ )	$-1 \pm 0.4$	$-0.2 \pm 0.1$	$21.9 \pm 2.1$	$20.4 \pm 0.2$	$15.2 \pm 0.3$	$1.6 \pm 0.2$	$-2.8 \pm 0.2$
MD PPF ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$45 \pm 40$	$55 \pm 40$	$43 \pm 20$	$41 \pm 23$	$44 \pm 30$	$20 \pm 10$	$25 \pm 10$
FW/DW	$2.0 \pm 0.09$ c		$2.1 \pm 0.03$ (y1) c $3.8 \pm 0.10$ (y2) a	$2.2 \pm 0.13$ bc	$2.3 \pm 0.21$ b	$2.1 \pm 0.16$ bc	$2.2 \pm 0.02$ bc

Table 2. Midday values of the fraction of absorbed light used in photosystem II photochemistry ( $\Phi\text{PSII}$ ) and PSII excitation pressure ( $1 - q_P$ ) in needles of sun-exposed *P. strobus* trees and *A. balsamea* trees growing in both sunny (sun fir) and shaded (shade fir) environments on all sampling days. Values are mean  $\pm$  SD,  $n = 6$ . Abbreviations: y1 and y2 represent needles from the current-year and new growth, respectively. Significant differences between means are indicated by different letters.

	Jan. 22, 2005	Mar. 19	May 24	Jul. 6	Sep. 26	Nov. 22	Jan. 23, 2006
<i>Pine</i>							
$\Phi\text{PSII}$	$0.08 \pm 0.08$ c	$0.08 \pm 0.04$ c	$0.37 \pm 0.09$ ab	$0.37 \pm 0.55$ (y1) ab $0.28 \pm 0.08$ (y2) b	$0.40 \pm 0.12$ ab	$0.43 \pm 0.13$ a	$0.03 \pm 0.02$ c
$1 - q_P$	$0.77 \pm 0.15$ b	$0.75 \pm 0.15$ b	$0.31 \pm 0.10$ a	$0.39 \pm 0.10$ (y1) a $0.38 \pm 0.15$ (y2) a	$0.34 \pm 0.12$ a	$0.21 \pm 0.12$ a	$0.77 \pm 0.22$ b
<i>Sun fir</i>							
$\Phi\text{PSII}$	$0.14 \pm 0.09$ d	$0.09 \pm 0.07$ d	$0.32 \pm 0.07$ (y1) b $0.16 \pm 0.03$ (y2) cd	$0.31 \pm 0.06$ b	$0.26 \pm 0.07$ bc	$0.58 \pm 0.03$ a	$0.08 \pm 0.04$ d
$1 - q_P$	$0.56 \pm 0.20$ b	$0.66 \pm 0.23$ bc	$0.43 \pm 0.12$ (y1) a $0.51 \pm 0.08$ (y2) bcc	$0.41 \pm 0.09$ a	$0.54 \pm 0.14$ a	$0.11 \pm 0.05$ a	$0.61 \pm 0.15$ c
<i>Shade fir</i>							
$\Phi\text{PSII}$	$0.22 \pm 0.04$ d	$0.08 \pm 0.03$ e	$0.79 \pm 0.02$ (y1) ab $0.74 \pm 0.01$ (y2) c	$0.81 \pm 0.01$ ab	$0.83 \pm 0.01$ a	$0.77 \pm 0.03$ bc	$0.23 \pm 0.32$ d
$1 - q_P$	$0.28 \pm 0.06$ c	$0.61 \pm 0.12$ d	$0.02 \pm 0.01$ (y1) ab $0.05 \pm 0.02$ (y2) b	$0.02 \pm 0.01$ ab	$0.02 \pm 0.02$ a	$0.02 \pm 0.02$ ab	$0.15 \pm 0.05$ c

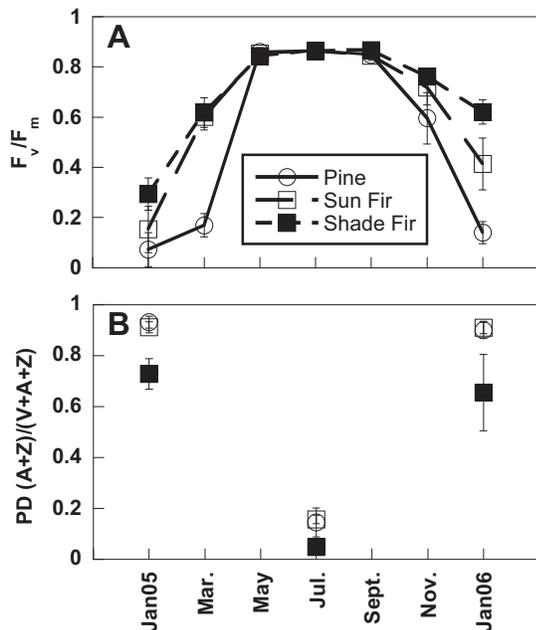


Figure 2. Seasonal changes in predawn (PD) maximal PSII efficiency (A,  $F_v/F_m$ ) and predawn de-epoxidation state of the xanthophyll cycle pigments (B,  $(A + Z)/(V + A + Z)$ ) in needles of sun-exposed *P. strobus* trees (open circles) and *A. balsamea* trees growing in both sunny (open squares) and shaded (black squares) environments. Values are mean  $\pm$  SD,  $n = 6$ .

pigments. In both conifers growing in the sun, concentrations of lutein (Figure 3F) and the total pool of xanthophyll cycle pigments (Figure 3C,  $V + A + Z$ ) increased significantly during winter, to values about double the summer values, although these increases were less pronounced in January 2006 than in January 2005. In shade needles, there was no increase in the xanthophyll cycle pool; however, there was a significant increase in lutein concentration during the winter. The midday de-epoxidation state of the xanthophyll cycle pigments (Figure 3G,  $(A + Z)/(V + A + Z)$ ) was significantly higher during winter in all trees studied. This value was unusually low in sun fir needles in November, which correlated with a collection on a cloudy day (Table 1). During winter, the predawn de-epoxidation state (measured only in January and July) remained high in all trees that were analyzed (Figure 2B) and was low in all trees during the summer. The shade fir needles typically had lower concentrations of the photoprotective pigments,  $\beta$ -carotene,  $V + A + Z$  and lutein and higher concentrations of  $\alpha$ -carotene than the sun fir needles.

#### Seasonal changes in relative abundance of photosynthetic proteins

To probe for seasonal changes in the abundance of individual photosynthetic proteins, thylakoids were run on gels on an equal protein basis. Thylakoid fractions were also assessed for chlorophyll concentration, and the ratio of chlorophyll to protein in the thylakoid fractions varied

across the season (Table 3), with higher concentrations of chlorophyll to protein occurring during the summer months in pine and sun fir. The shade fir needles generally had higher chlorophyll to protein ratios; however, there was a reduction in these values during the spring months. Examples of Western blots (loaded on an equal protein basis) showing seasonal changes in abundance of four light-harvesting proteins in white pine are depicted in Figure 4. The compiled data of all blots from both species and light environments are summarized in Figure 5 where columns indicate the relative abundance of each protein normalized against a July signal equated to 100%. The data represent mean values of at least three blots.

To probe for changes in the concentration of the PSII core, the D1 protein was monitored (Figure 5A). The relative concentration of D1 protein decreased about 50% in winter, relative to summer in all trees that were studied, although a more pronounced reduction occurred in January 2006 in sun fir. Presence of the photosystem I (PSI) core was monitored by probing for the protein product of *PsaD*, a stromal subunit of the PSI core (Figure 5G; Fromme et al. 2003). In the sun-exposed trees, the PSI protein showed a pronounced decline during winter, with a reduction to 15–20% of summer values. The shaded trees maintained high concentrations of PSI protein during winter, but showed reductions during the spring months.

Of the LHCs associated with PSII, two of the major peripheral LHCII proteins (Lhcb1 and Lhcb2, Figure 5B and C) and two of the minor peripheral antenna complexes (CP29 (product of the *Lhcb4* gene) and CP26 (product of the *Lhcb5* gene), Figure 5D and E) were monitored. The major LHCII protein (Lhcb1) and the minor antennae CP29 showed similar seasonal changes in the sun needles, with dramatic reductions in relative abundance during winter, to around 10–15% of summer values. The major LHCII protein (Lhcb2) and the minor antennae CP26 also showed similar seasonal changes in the sun needles, with a smaller reduction during winter (maximum reduction of about 50% in January 2005). The shade fir needles maintained high values of both of the major LHCII antennae (Lhcb1 and Lhcb2) during winter, with smaller reductions in the abundance of these proteins occurring in the spring. For the shade needles, data from CP29 and CP26 produced inconsistent results and so were excluded from the analysis.

Three of the four PSI light-harvesting antennae were monitored (Lhca1, Lhca2 and Lhca4, Figure 5H–J). The Lhca proteins all showed pronounced reductions (6–40%) in protein concentration during winter in the sun needles, although in winter 2006 these reductions were less pronounced (13–75%). Shade needles maintained high values of all of these proteins throughout the winter, again with decreases in relative abundance of these proteins occurring in the spring.

The PsbS protein was maintained at higher concentrations all year (Figure 5F), with a maximum reduction to about 50% of summer values occurring in sun needles in

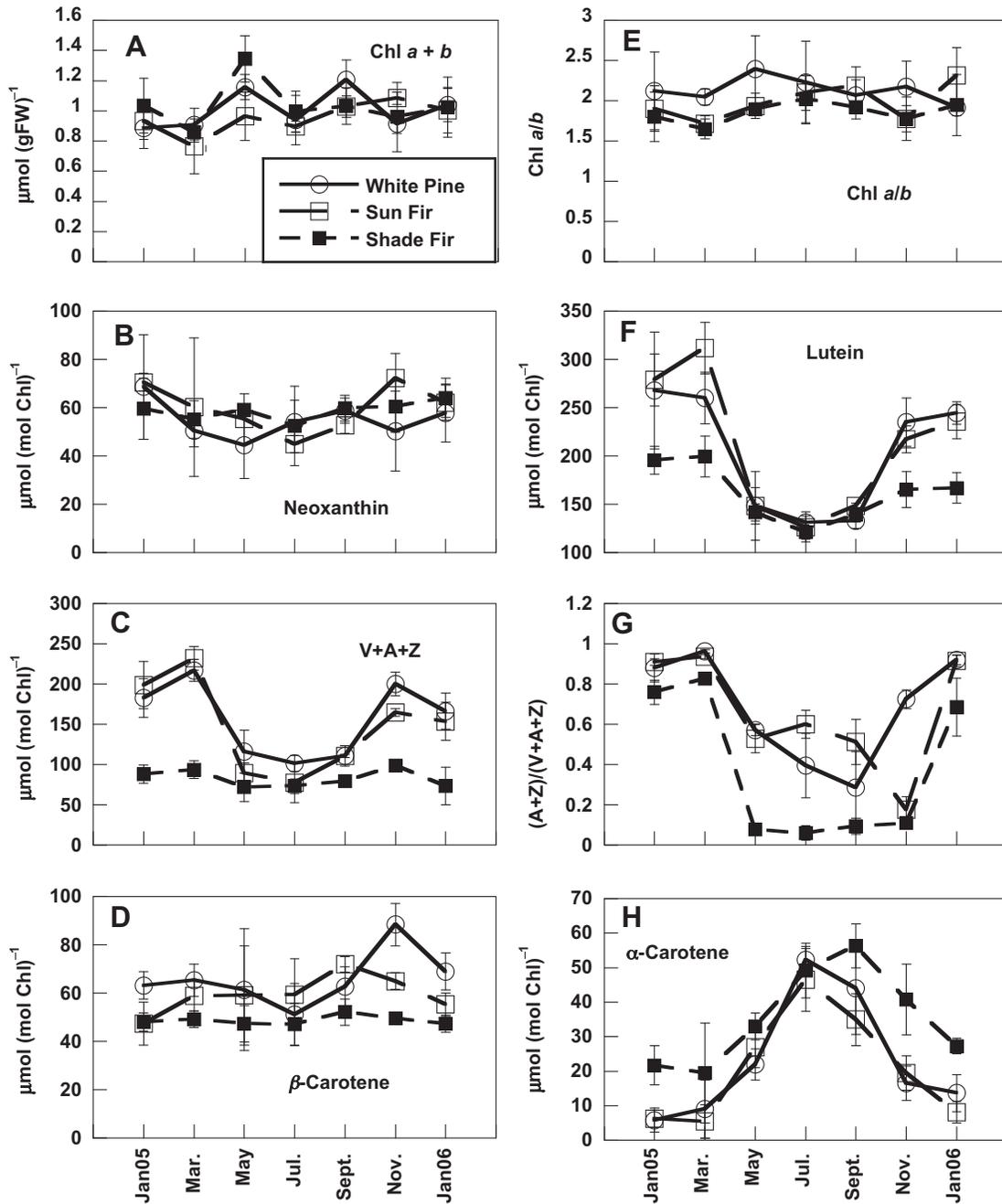


Figure 3. Seasonal changes in chlorophyll and carotenoid composition in needles of sun-exposed *P. strobus* trees (open circles) and *A. balsamea* trees growing in both sunny (open squares) and shaded (black squares) environments. Needles were collected midday at the PPFs indicated in Table 1. Values are mean  $\pm$  SD,  $n = 3-6$ .

January 2005. The PsbH protein showed winter reductions of the order of 20–30% of summer values in the sun needles (Figure 5K), whereas the shade needles maintained high concentrations of this protein.

#### Seasonal changes in midday phosphorylation status of photosynthetic proteins

Examples of Western blots from pine and the sun and shade fir, showing seasonal profiles of thylakoids collected

at midday and probed with anti-phosphothreonine, are depicted in Figure 6A. Five bands were identified (CP43, D2, D1, LHC and PsbH). The data collected from these blots were normalized by summing the optical density of a given band across the seasons and then dividing the optical density for a particular band for an individual month by the total combined signal for that band across the seasons (Figure 6B). Thus, if there were no seasonal changes in band intensity, the relative signal would be about equal for each month. These data must be considered within

Table 3. The ratio of total chlorophyll to total protein ( $\mu\text{g Chl per } \mu\text{g protein}$ ) in thylakoids isolated from needles of sun-exposed *P. strobus* trees and *A. balsamea* trees growing in both sunny (sun fir) and shaded (Shade fir) environments. Abbreviations: y1 and y2 represent needles from the current-year and new growth, respectively. Ratios were calculated from means of three replicates per thylakoid for both the protein and the chlorophyll measurements. For each, the mean values had a standard deviation of not more than 10% of the mean.

	Jan. 22, 2005	Mar. 19	May 24	Jul. 6	Sep. 26	Nov. 22	Jan. 23, 2006
Pine	2.7	4.9	4.1	9.2 (y1) 15.3 (y2)	6.8	8.0	5.5
Sun fir	2.7	4.7	7.5 (y1) 4.3 (y2)	11.4	10.6	5.7	4.8
Shade fir	13.3	5.2	3.6 (y1) 3.7 (y2)	14.8	15.3	14.1	14.7

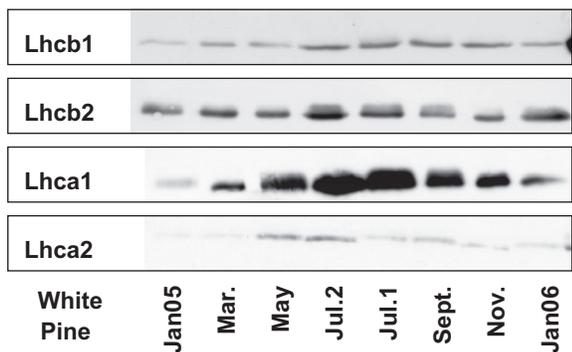


Figure 4. Examples of Western blots showing seasonal changes in relative abundance of individual light-harvesting proteins from thylakoids isolated from *P. strobus* needles, with each lane loaded on an equal protein basis. Jul.1 and Jul.2 are the thylakoids isolated from current-year's needles and new growth, respectively.

the context of changes in relative abundance of each protein (Figure 5).

Seasonal changes in the phosphorylation profile of the two sun populations (pine and sun fir) demonstrate a fairly consistent phosphorylation pattern from July until January, with LHCII and the PSII core proteins (D1, D2 and CP43) being phosphorylated at midday. There were declines in the intensities of the bands during autumn and winter, which correlates in all cases with a decrease in protein abundance (Figure 5). The relative abundance of the D2 protein was probed and was similar to that of D1 (Figure 5A, D2 data not shown). There is evidence for an increase in the relative phosphorylation of PsbH in winter and spring in pine and possibly also in sun fir, particularly given the decrease in relative abundance of these proteins that occurred during winter (Figure 5A and K).

Some differences were observed in the phosphorylation profile of sun-exposed trees of both conifers during recovery in the spring. In early spring (March), when  $F_v/F_m$  values were still low (particularly in pine, Figure 2A), D1 remained phosphorylated at a high level, whereas there were no signals for D2 and CP43. Additionally, there was an increase in phosphorylation of the PsbH protein (particularly in pine) during March. By late spring (May), the

phosphorylation profiles of all proteins were similar to those in July, although with reduced intensities.

In the shade fir needles, July measurements showed phosphorylation of LHCII and showed a small amount of signal for the PSII core protein CP43, with no signal for D1 or D2. The LHCII signal remained constant throughout the year, whereas there were dramatic changes in the PSII core phosphoproteins. During autumn, there was a small increase in the phosphorylation signal of the core proteins (D1, D2 and CP43), whereas there was a dramatic increase in phosphorylation of all core proteins, including PsbH in winter and early spring. By May, the phosphorylation profile was similar to that of summer.

#### Rate of recovery of $F_v/F_m$ on warming needles

Recovery of  $F_v/F_m$  on warming needles was monitored to probe for the presence of the rapidly reversible and slowly reversible forms of energy dissipation (Figure 7). Predawn  $F_v/F_m$  values measured in the field were similar for sun fir and pine ( $0.23 \pm 0.05$  and  $0.22 \pm 0.03$ , respectively) and were somewhat higher for shade fir ( $0.38 \pm 0.07$ ). Both sun and shade fir had a component of their recovery that occurred very rapidly (within 1 h) after which the recovery occurred more slowly, such that by Day 6 (170 h) the  $F_v/F_m$  values were  $0.79 \pm 0.01$  and  $0.75 \pm 0.04$  for the shade and sun needles, respectively. The shade needles recovered more rapidly than the sun needles and had a larger component of the rapidly reversible form of energy dissipation. In pine, there was no recovery after 1 h of warming, and overall, the recovery was much slower than in balsam fir, with values only reaching  $0.58 \pm 0.07$  after 6 days in these conditions. The slower recovery of pine needles in the laboratory is consistent with the slower recovery in the field in the spring (Figure 2A).

## Discussion

We found typical seasonal changes in chlorophyll fluorescence and pigment composition in overwintering sun and shade evergreens. All needles showed a sustained reduction in predawn  $F_v/F_m$  during winter and a correlated sustained

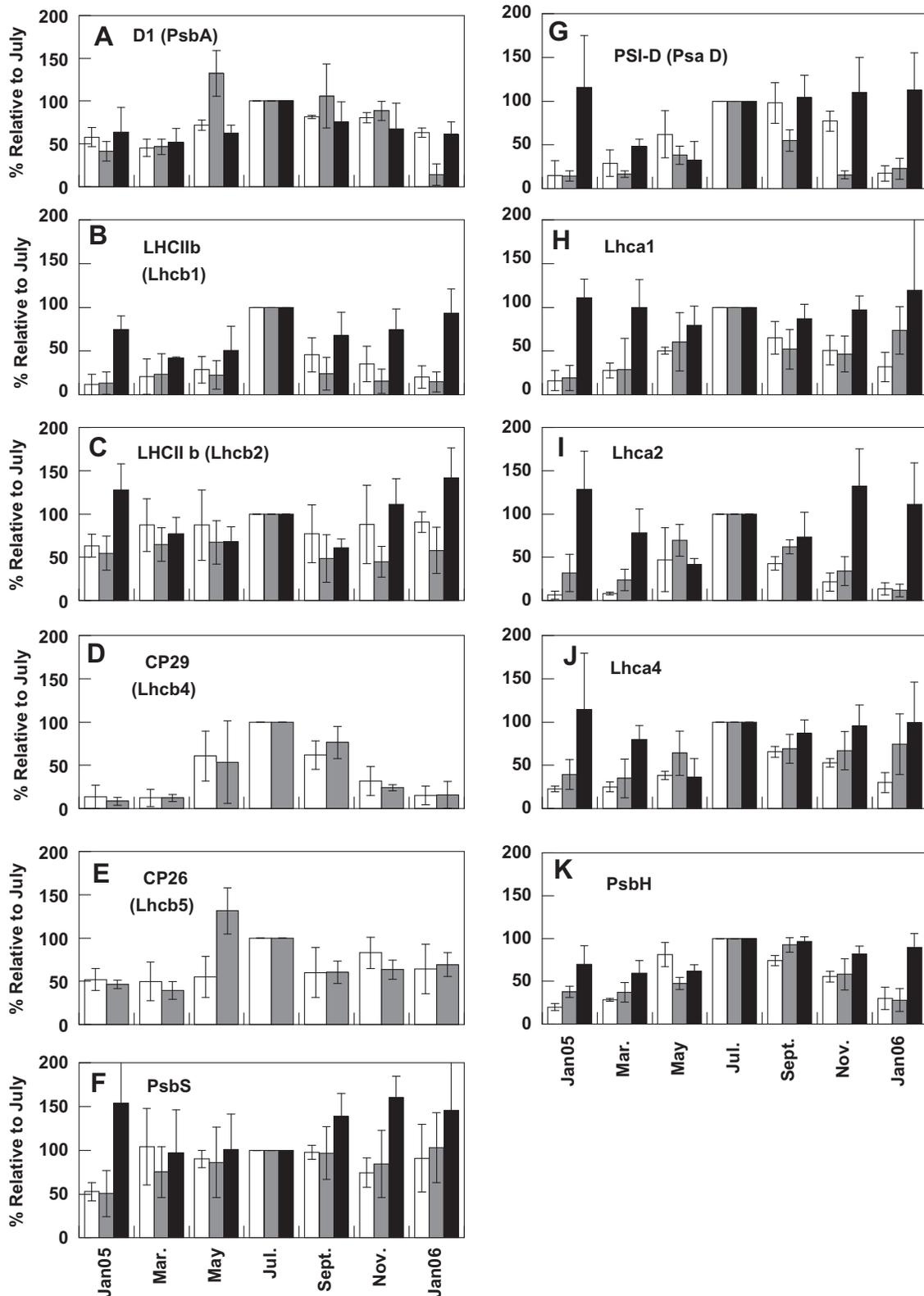


Figure 5. Seasonal changes in relative abundance of individual photosynthetic proteins in needles of sun-exposed *P. strobus* trees (open bars) and *A. balsamea* trees growing in both sunny (shaded bars) and shaded (black bars) environments. Western blots of thylakoids, loaded on an equal protein basis, were scanned and band intensity was quantified with Image J (NIH). Individual blots were normalized such that July samples were equated to 100%, and all other months are expressed as relative optical density compared with July. Values are mean  $\pm$  SD of at least three separate blots.

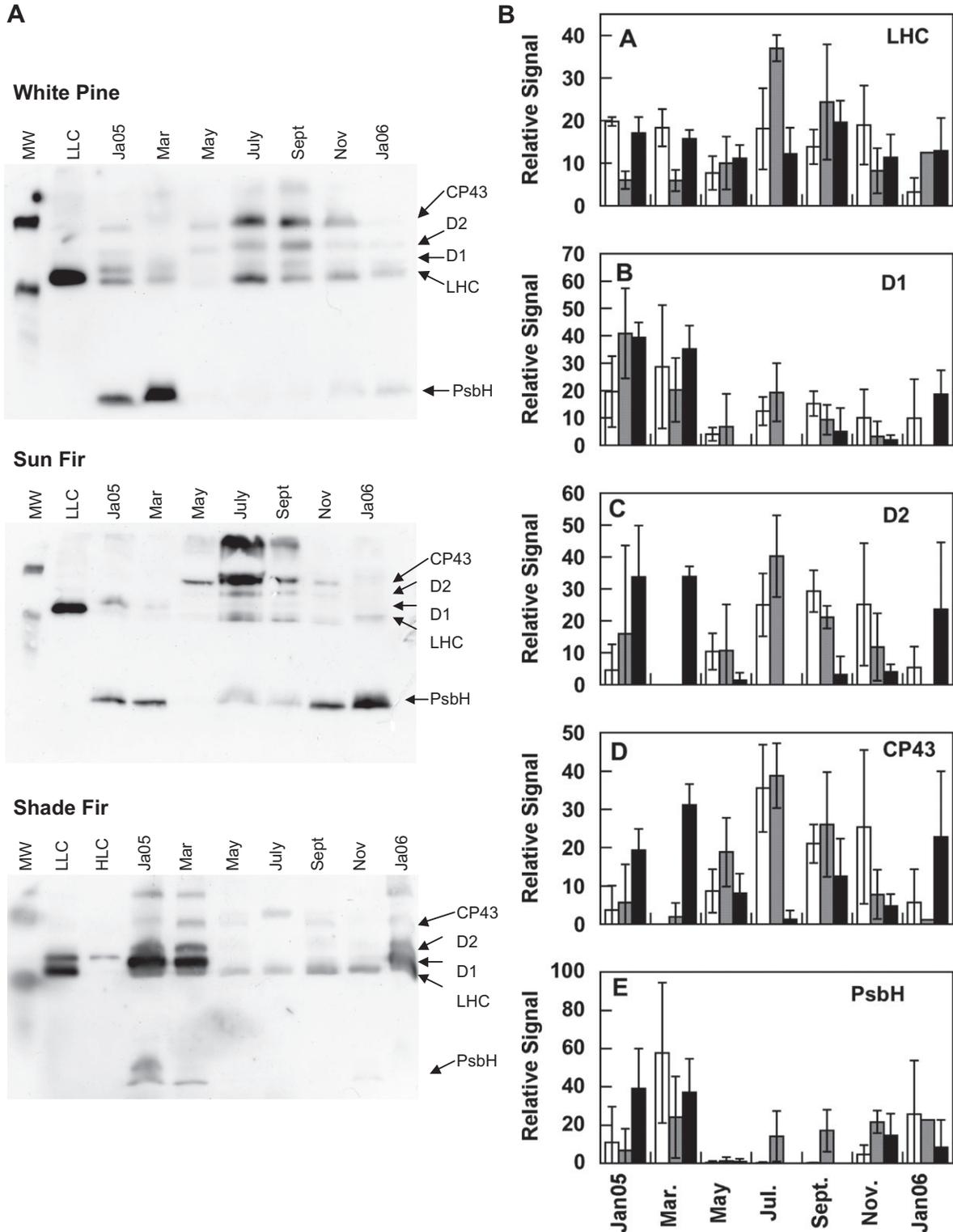


Figure 6. Panel A depicts blots of thylakoids isolated from needles collected at midday from sun-exposed *P. strobus* trees and *A. balsamea* trees growing in both sunny and shaded environments and probed with anti-phosphothreonine. Molecular mass standards (MW) and thylakoids from pumpkin isolated after exposure to low light (LLC) or high light (HLC) were included as controls. Samples were loaded on an equal protein basis (20  $\mu$ g protein per lane). Blots were scanned and band intensity was quantified with Image J (NIH). Individual blots were normalized such that the total signal for a given protein (across the season) was summed and equated to 100%. (B) Data from each month were then expressed as a fraction of that total pool, such that relative differences between months could be observed. Open bars represent pine and shaded and black bars represent sun fir and shade fir, respectively. Values are mean  $\pm$  SD from three blots.

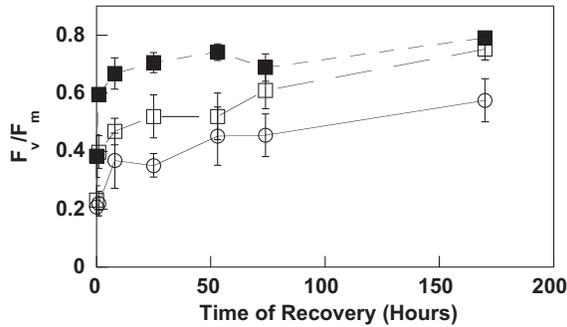


Figure 7. Recovery of  $F_v/F_m$  in needles of sun-exposed *P. strobus* trees (open circles) and *A. balsamea* trees growing in both sunny (open squares) and shaded (black squares) environments; the needles were collected on January 10, 2006 (temperature =  $-7^\circ\text{C}$ ) and taken them to the laboratory in which they were maintained at room temperature ( $22^\circ\text{C}$ ) and light ( $5\text{--}10\text{ mmol m}^{-2}\text{ s}^{-1}$ ) for 170 h. Time zero measurements were taken at predawn in the field.

dark-retention of  $Z + A$  (Figure 2), as has been well established in overwintering evergreens (Ottander et al. 1995, Verhoeven et al. 1996, 1998, 1999, 2004, Adams et al. 2001, 2004), demonstrating the presence of sustained energy dissipation. Additionally, winter needles showed reduced  $\Phi\text{PSII}$ , elevated  $1 - q_p$  (Table 2), and increased de-epoxidation of the xanthophyll cycle pigments at midday (Figure 3G), illustrating marked increases in the absorption of excess excitation energy during winter even in the shaded trees that were exposed to low irradiances (Table 1). Winter increases in lutein concentration and the pool of xanthophyll cycle pigments ( $V + A + Z$ ) were observed (Figure 3C and F), which is consistent with previous results (Logan et al. 1998, Verhoeven et al. 1998, 1999, 2004, Adams et al. 2001, 2002) demonstrating the importance of increasing pool sizes of these photoprotective pigments in the acclimation of evergreens to winter stress.

A novel aspect of our study was the examination of changes in relative abundance of most of the individual LHCs of PSI and PSII during the acclimation to winter conditions, and on recovery in the spring. Variation in protein concentration was analyzed as a function of thylakoid protein concentration, and a general observation was that there were reductions in most of the photosynthetic proteins during winter in sun-exposed trees (Figure 5). There were no significant changes in total chlorophyll concentration (assessed on the basis of needle fresh mass). The analysis of thylakoid fractions demonstrated a decrease in the concentration of chlorophyll per protein during the non-summer months in sun needles and during the spring months in shade needles. Therefore, it is possible that winter increases in other non-chlorophyll binding proteins, not monitored in this study, may have occurred. Despite this, it is possible to gain insight into seasonal changes in relative abundance of the individual proteins by comparing the responses of the proteins with each other. For example,

Table 4. Protein abundance relative to the D1 protein in January 2005 in thylakoids isolated from needles of sun-exposed *P. strobus* trees and *A. balsamea* trees growing in both sunny (sun fir) and shaded (shade fir) environments.

	Pine	Sun fir	Shade fir
Lhcb1	0.20	0.32	1.17
Lhcb2	1.09	1.32	2.00
Lhcb4	0.23	0.20	
Lhcb5	0.90	1.12	
PsaD	0.26	0.35	1.81
Lhca1	0.28	0.46	1.74
Lhca2	0.11	0.76	2.01
Lhca4	0.39	0.95	1.79
PsbS	0.91	1.22	2.42
PsbH	0.34	0.91	1.09

in January 2005, if each of the individual light-harvesting proteins is expressed as a function of the relative abundance of the D1 protein (Table 4), there were changes in relative abundance of the proteins compared with July, when all proteins were normalized to 100%. For the individual light-harvesting proteins associated with PSII, there were relative increases in Lhcb2 compared with Lhcb1 in all trees studied, with the Lhcb2 protein being enriched about four-fold in sun needles and about twofold in shade needles compared with summer values. Similarly, the Lhcb5 protein (CP26) was present in winter sun needles in 4–5 times greater abundance relative to the Lhcb4 proteins (CP29) compared with summer values. These data are the first showing variations in the seasonal responses of individual light-harvesting proteins, and suggest roles for Lhcb2 and CP26 in acclimation to non-summer conditions. It is possible that Lhcb2 or CP26, or both, are important in facilitating the sustained energy dissipation that occurs during the winter months. Winter reductions in D1 and LHCs have been reported previously (Ottander et al. 1995, Savitch et al. 2002), although individual LHCs were not monitored in these studies. In Scots pine growing in Siberia, Ensminger et al. (2004) did not observe reductions in LHCs until spring recovery, when reductions in Lhcb1 and Lhcb4 were noted.

An examination of the protein concentrations of PSI and its light-harvesting proteins, in sun needles, indicated that PSI values were reduced to about one-third of the PSII values during winter (Figure 5; Table 4), with the concentrations of its light-harvesting proteins being reduced as well. In shade needles, these proteins were maintained at summer concentrations during winter (about twice that of D1), with reductions occurring during spring (Figure 5). No previous field studies, to our knowledge, have examined seasonal responses of the PSI core, but two chamber studies of winter acclimation have provided conflicting data, with Savitch et al. (2002) showing no change in PSI core or its antennae, and Busch et al. (2007) showing a 50% reduction in PSI core with no change in antennae abundance. The reduction

in PSI and its antennae observed in our study is counter to the hypothesis of Öquist and Huner (2003) that PSI and its antennae remain largely intact during winter.

Seasonal changes in protein abundance differed between sun and shade needles. Although there was a similar winter decline in the abundance of the D1 protein (about a 50% reduction in both sun-exposed and shaded trees), the shaded trees maintained high summer concentrations of all other proteins monitored during autumn and winter. During recovery in the spring, there were marked decreases in some of the photosynthetic proteins, particularly the concentrations of the PsdD protein of PSI and the Lhcb1 protein, both of which were reduced by 50% or more in the March and May collections. These general reductions in protein concentration observed in the spring correspond to the very high  $1 - q_P$  values measured in March. Similarly, Ensminger et al. (2004) reported winter reductions in D1, whereas Lhcb1, Lhcb4 and Lhca1 concentrations were maintained throughout the winter with concentrations declining dramatically in the spring. Although the study by Ensminger et al. (2004) did not focus on shaded trees, it is possible that a low overall light environment in the boreal Scots pine forest during winter accounts for the similarity between the Scots pine data and our data from shade needles.

Of the proteins that we monitored, the PsbS protein showed the smallest change in relative abundance across the seasons (Figure 5F). This protein was reduced to about 50% of summer values in January 2005, but otherwise was maintained at near-summer concentrations. This indicates an enrichment of PsbS, relative to other photosynthetic proteins, during non-summer seasons (Table 3). There have been conflicting reports on seasonal changes in PsbS concentration, possibly because of differences in the temperature or light environment, or both. PsbS is critical for the qE component of NPQ, which responds rapidly to changes in light environment (Li et al. 2000, Müller et al. 2001). Following acclimation to low temperatures, the resulting increase in excess excitation pressure would require increased capacity for qE, which is consistent with the enrichment in PsbS and the increase in xanthophyll cycle pool size that we observed. Presumably, this increased capacity for qE is critical in autumn and spring when temperatures are low but do not preclude carbon fixation. However, the sustained energy dissipation that occurs during winter is not well understood mechanistically and this form of energy dissipation may not require PsbS. Although our data demonstrate the importance of the PsbS protein in seasonal acclimation, the importance of this protein in the sustained energy dissipation observed during the coldest months remains to be determined.

Another novel aspect of our study was the examination of the midday thylakoid protein phosphorylation profiles from needles of pine and fir (sun and shade) throughout the course of a year. The thylakoid phosphorylation profiles of sun-exposed needles, collected at midday in July,

demonstrated strong phosphorylation of the PSII core proteins (D1, D2 and CP43), which was largely absent in the shade needles, in agreement with previous reports showing maximal phosphorylation of PSII core proteins at saturating light and above (Rintamäki et al. 1997, Pursiheimo et al. 1998). Our sun-exposed needles had a fairly strong midday phosphorylation of LHCII, which was unexpected because previous studies have shown that LHCII phosphorylation peaks at limiting light and disappears at higher irradiances in both angiosperms and a variety of seedless plants (Rintamäki et al. 1997, Pursiheimo et al. 1998, 2003).

No pronounced seasonal effects on the phosphorylation profile of the two sun populations were observed during the transition from summer to winter. There appeared to be an increase in the phosphorylation of the PsbH protein during winter and early spring in pine, and possibly also in sun fir when considering the reduction in abundance of this protein during winter (Figure 5K). During recovery from winter stress in early spring (March), D1 remained phosphorylated in sun needles of both pine and fir; however, there was no phosphorylation signal for either D2 or CP43. The function of the reversible phosphorylation of the PSII core proteins is reported to be associated with the PSII repair cycle (see review by Aro and Ohad 2003, Vener 2007). The core proteins are phosphorylated under high light conditions, and after damage there is partial dephosphorylation associated with lateral migration of the core complex to the stromal region of the thylakoid membrane, followed by disassembly and repair (Baena-Gonzalez et al. 1999, Vener 2007). There is evidence for sequential dephosphorylation of the core proteins, with CP43 being dephosphorylated first, followed by D2 and finally D1 (Baena-Gonzalez et al. 1999). Our data suggest that, during winter, and moving into the spring, the increased phosphorylation of D1 reflects this sequential dephosphorylation, with D1 remaining phosphorylated after CP43 and D2 have been dephosphorylated. The role of phosphorylation of the PsbH protein is less well defined, although this protein is generally thought to be important in assembly of the PSII reaction centers (Komenda et al. 2003). It is possible that the phosphorylated form of PsbH plays a role in maintaining or facilitating the winter arrangement of the photosystems.

The effect of season on the phosphorylation profiles of photosynthetic proteins was highly dependent on light environment, with the shade needles undergoing dramatic increases in phosphorylation of all PSII core proteins (D1, D2, CP43 and PsbH) during winter and early spring. High concentrations of PSII core phosphorylated proteins are consistent with increased excitation pressure on PSII in response to low temperatures (Table 2; Pursiheimo et al. 2003). It is possible that the phosphorylated PSII core proteins are involved in maintaining the sustained energy dissipation in these plants, because phosphorylation of these proteins was observed only during winter months when shade needles maintained reduced predawn  $F_v/F_m$

(Figure 2A). This would be consistent with a role for sustained phosphorylation of the D1 protein in maintaining sustained energy dissipation suggested by Ebbert et al. (2001).

An examination of the recovery kinetics of the sun and shade evergreens, after artificially warming the needles in the laboratory, demonstrated the presence of both the rapidly reversible and slowly reversible forms of sustained energy dissipation in balsam fir, with the shade needles having a much more pronounced rapid component to their recovery (Figure 6). This is consistent with previous studies (Verhoeven et al. 1998) and is suggestive of different mechanisms maintaining the sustained energy dissipation that reverses rapidly versus slowly. In contrast to fir, pine showed no recovery after 1 h of warming, suggesting that pine lacks this rapidly reversible component of sustained energy dissipation. We speculate that the ability of balsam fir to use the rapidly reversible component of sustained energy dissipation allows for its more rapid recovery, which was observed both during the spring and in the artificial recovery experiment in the laboratory. The overall ability of shade needles to recover more rapidly than sun needles is related to the ability of shade needles to maintain most of their photosynthetic proteins during winter, whereas the sun needles exhibited reductions in winter in all of the photosynthetic proteins monitored.

In conclusion, our results are consistent with the hypothesis that a structural reorganization occurs in the LHCs of the photosynthetic apparatus during acclimation to winter stress that accompanies the functional change from light-harvesting to energy-dissipating centers (Öquist and Huner 2003). The characteristics of this reorganization vary depending on the light environment to which the needles are exposed. In sun needles, changes in the relative abundance of the photosynthetic proteins were observed such that, during non-summer months, there was a pronounced relative enrichment of Lhcb2, CP26 and PsbS. Additionally, an increase in the phosphorylation of the PsbH protein was observed during winter months. These changes may be important for facilitating the slowly reversible sustained energy dissipation observed in winter-stressed needles. In shade needles, most proteins were retained at concentrations similar to summer values throughout the year, with some reductions in protein concentration occurring during the spring. However, shade needles exhibited a dramatic increase in the phosphorylation of all PSII core proteins during winter that may facilitate the more rapidly reversible sustained energy dissipation observed in these needles.

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#### References

- Adams III, W.W. and B. Demmig-Adams. 1992. Operation of the xanthophyll cycle in higher plants in response to diurnal changes in incident sunlight. *Planta* 186:390–398.
- Adams III, W.W. and B. Demmig-Adams. 1994. Carotenoid composition and down regulation of photosystem II in three conifer species during the winter. *Physiol. Plant.* 92:451–458.
- Adams III, W.W., B. Demmig-Adams, T.N. Rosenstiel and V. Ebbert. 2001. Dependence of photosynthesis and energy dissipation activity upon growth form and light environment during the winter. *Photosynth. Res.* 67:51–62.
- Adams III, W.W., B. Demmig-Adams, T.N. Rosenstiel, A.K. Brightwell and V. Ebbert. 2002. Photosynthesis and photoprotection in overwintering plants. *Plant Biol.* 4:545–557.
- Adams III, W.W., C.R. Zarter, V. Ebbert and B. Demmig-Adams. 2004. Photoprotective strategies of overwintering evergreens. *Bioscience* 54:41–50.
- Aro, E.-M. and I. Ohad. 2003. Redox regulation of thylakoid protein phosphorylation. *Antioxid. Redox Signal.* 5:55–67.
- Baena-Gonzalez, E., R. Barbata and E.-M. Aro. 1999. Role of phosphorylation in the repair cycle and oligomeric structure of photosystem II. *Planta* 208:196–204.
- Bergo, E., S. Pursiheimo, V. Paakkariinen, G.M. Giacometti, A. Donella-Deana, F. Andreucci, R. Barbato and E.-M. Aro. 2002. Rapid and highly specific monitoring of reversible thylakoid protein phosphorylation by polyclonal antibody to phosphothreonine-containing proteins. *J. Plant Physiol.* 159:371–377.
- Busch, F., N.P.A. Hüner and I. Ensminger. 2007. Increased air temperature during simulated autumn conditions does not increase photosynthetic carbon gain but affects the dissipation of excess energy in seedlings of the evergreen conifer Jack pine. *Plant Physiol.* 143:1242–1251.
- Busch, F., N.P.A. Hüner and I. Ensminger. 2008. Increased air temperature during simulated autumn conditions impairs photosynthetic electron transport between photosystem II and photosystem I. *Plant Physiol.* 147:402–414.
- Demmig-Adams, B. and W.W. Adams III. 1996. How to use solar energy without getting burnt: the role of the xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends Plant Sci.* 1:21–26.
- Demmig-Adams, B. and W.W. Adams III. 2006. Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytol.* 172:11–21.
- Demmig-Adams, B., W.W. Adams III, D.H. Barker, B.A. Logan, D.R. Bowling and A.S. Verhoeven. 1996. Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal energy dissipation of excess excitation. *Physiol. Plant.* 98:254–264.
- Ebbert, V., B. Demmig-Adams, W.W. Adams III, K.E. Mueh and L.A. Staehelin. 2001. Correlation between persistent forms of zeaxanthin-dependent energy dissipation and thylakoid protein phosphorylation. *Photosynth. Res.* 67:63–78.
- Ebbert, V., W.W. Adams III, A.K. Matoo, A. Sokolenko and B. Demmig-Adams. 2005. Up-regulation of a photosystem II core protein phosphatase inhibitor and sustained D1 phosphorylation in zeaxanthin-retaining, photoinhibited needles of overwintering Douglas fir. *Plant Cell Environ.* 28:232–240.
- Ensminger, I., D. Sveshnikov, D.A. Campbell, C. Funk, S. Jansson, J. Lloyd, O. Shibistova and G. Öquist. 2004. Intermittent low temperatures constrain spring recovery of photosynthesis in boreal Scots pine forests. *Glob. Change Biol.* 10:995–1008.

- Fromme, P., E. Schlodder and S. Jansson. 2003. Structure and function of the antenna system in photosystem I. *In* Light-Harvesting Antennas in Photosynthesis. Eds. B.R. Green and W.W. Parson. Kluwer Academic Publishers, Netherlands, pp 253–279.
- Genty, B., J.M. Briantais and N.R. Baker. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990:87–92.
- Gilmore, A.M. and M.C. Ball. 2000. Protection and storage of chlorophyll in overwintering evergreens. *Proc. Natl. Acad. Sci. USA* 97:11098–11101.
- Gilmore, A.M. and H.Y. Yamamoto. 1991. Resolution of lutein and zeaxanthin using a nonendcapped, lightly carbon-loaded C-18 high-performance liquid chromatographic column. *J. Chromatogr.* 543:137–145.
- Huner, N.P.A., G. Öquist and F. Sarhan. 1998. Energy balance and acclimation to light and cold. *Trends Plant Sci.* 3:224–230.
- Kim, J.-H., H.J. Hwang, H.-S. Park, C.B. Lee, K.Y. Myung and C.-H. Lee. 1997. Differences in the rate of dephosphorylation of thylakoid proteins during dark incubation after chilling in the light between two rice (*Oryza sativa* L.) varieties. *Plant Sci.* 128:159–168.
- Komenda, J., D. Stys and L. Lupinkova. 2003. The PsbH protein of photosystem 2. *Photosynthetica* 41:1–7.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680–685.
- Li, X.-P., O. Björkman, C. Shih, A.R. Grossman, M. Rosenquist, S. Jansson and K.K. Niyogi. 2000. A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* 403:391–395.
- Logan, B.A., S.D. Grace, W.W. Adams III and B. Demmig-Adams. 1998. Seasonal differences in xanthophyll cycle characteristics and antioxidants in *Mahonia repens* growing in different light environments. *Oecologia* 116:9–17.
- Matsubara, S., A.M. Gilmore, M.C. Ball, J.M. Anderson and B. Osmond. 2002. Sustained downregulation of photosystem II in mistletoes during winter depression of photosynthesis. *Funct. Plant Biol.* 29:1157–1169.
- Müller, P., X.-P. Li and K.K. Niyogi. 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiol.* 125:1558–1566.
- Öquist, G. and N.P.A. Huner. 2003. Photosynthesis of overwintering evergreen plants. *Annu. Rev. Plant Biol.* 54:329–355.
- Ottander, C., D. Campbell and G. Öquist. 1995. Seasonal changes in photosystem II organization and pigment composition in *Pinus sylvestris*. *Planta* 197:176–183.
- Pogson, B.J., K.K. Niyogi, O. Björkman and D. DellaPenna. 1998. Altered xanthophyll compositions adversely affect chlorophyll accumulation and nonphotochemical quenching in *Arabidopsis mutants*. *Proc. Natl. Acad. Sci. USA* 95:13324–13329.
- Porra, R.J., W.A. Thompson and P.E. Kriedemann. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* 975:384–394.
- Pursiheimo, S., E. Rintamäki, E. Baena-Gonzalez and E.-M. Aro. 1998. Thylakoid protein phosphorylation in evolutionally divergent species with oxygenic photosynthesis. *FEBS Lett.* 42:178–182.
- Pursiheimo, S., P. Martinsuo, E. Rintamäki and E.-M. Aro. 2003. Photosystem II protein phosphorylation follows four distinctly different regulatory patterns induced by environmental cues. *Plant Cell Environ.* 26:1995–2003.
- Rintamäki, E., M. Salonen, U.-M. Suoranta, I. Carlberg, B. Andersson and E.-M. Aro. 1997. Phosphorylation of light-harvesting complex II and photosystem II core proteins shows different irradiance-dependent regulation in vivo. *J. Biol. Chem.* 272:30476–30482.
- Savitch, L.V., E.D. Leonardos, M. Krol, S. Jansson, B. Grodzinski, N.P.A. Huner and G. Öquist. 2002. Two different strategies for light utilization in photosynthesis in relation to growth and cold acclimation. *Plant Cell Environ.* 25:761–771.
- Szabo, I., E. Bergantino and G.M. Giacometti. 2005. Light and oxygenic photosynthesis: energy dissipation as a protection mechanism against photo-oxidation. *EMBO Rep.* 6:629–634.
- Vener, A.V. 2007. Environmentally modulated phosphorylation and dynamics of proteins in photosynthetic membranes. *Biochim. Biophys. Acta* 1767:449–457.
- Verhoeven, A.S., W.W. Adams III and B. Demmig-Adams. 1996. Close relationship between the state of the xanthophyll cycle pigments and photosystem II efficiency during recovery from winter stress. *Physiol. Plant.* 96:567–576.
- Verhoeven, A.S., W.W. Adams III and B. Demmig-Adams. 1998. Photoinhibition during winter stress in sun and shade *Euonymus kiautschovicus* leaves; the xanthophyll cycle and leaf adenylate status. *Plant Cell Environ.* 21:893–903.
- Verhoeven, A.S., W.W. Adams III and B. Demmig-Adams. 1999. The xanthophyll cycle and acclimation of *Pinus ponderosa* and *Malva neglecta* to winter stress. *Oecologia* 118:277–287.
- Verhoeven, A.S., A. Swanberg, M. Thao and J. Whiteman. 2004. Seasonal changes in leaf antioxidant systems and xanthophyll cycle characteristics in *Taxus × media* growing in sun and shade environments. *Physiol. Plant.* 123:428–434.
- Zarter, C.R., W.W. Adams III, V. Ebbert, I. Adamska, S. Jansson and B. Demmig-Adams. 2006. Winter acclimation of PsbS and related proteins in the evergreen *Arctostaphylos uva-ursi* as influenced by altitude and light environment. *Plant Cell Environ.* 29:869–878.