Overwintering Periwinkle (*Vinca minor* L.) Exhibits Increased Photosystem I Activity

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ABSTRACT

The effects of natural, overwintering conditions on photosystem I and photosystem II activity were examined in isolated thylakoids of periwinkle (*Vinca minor* L.), an endemic, cold-tolerant, herbaceous evergreen. DCMU-Insensitive photosystem I activity (ascorbate/dichlorophenolindophenol → methylioologen) exhibited a twofold increase in light-saturated rates upon exposure to low temperature and freezing stress with no effect on the apparent quantum yield of this reaction. DCMU-Sensitive photosystem II activity (H₂O → dichlorophenolindophenol) exhibited only minor fluctuations in light-saturated rates but a 50% decrease in the apparent quantum yield of this reaction upon exposure to overwintering conditions. This was correlated with a decrease in the 77 K fluorescence emission at 694 nanometers. These functional changes occurred with no detectable changes in the relative chlorophyll contents of the chlorophyll-protein complexes or the chlorophyll-thylakoid protein. The chlorophyll a/b varied less than 10% during any single growth year. Analyses of total leaf extracts indicated that all lipid classes exhibited increased levels of linoleic and linolenic acid. Neither the trans-Δ⁹-hexadecenoic acid level nor the ratio of oligomericmonomeric light harvesting of photosystem II was affected by exposure to winter stress. The content of the major chloroplast lipids monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidyl-diacylglycerol, and sulfoquinovosyldiacylglycerol exhibited minor fluctuations, whereas phosphatidylcholine and phosphatidylethanolamine content doubled on a molar percent or chlorophyll basis. We conclude that the previously reported increase in photosystem I activity during controlled, low temperature growth is observed during exposure to natural overwintering conditions. This appears to occur with minimal changes in the structure and composition of the photosynthetic apparatus of periwinkle.

Recently, Huner (7) reported that thylakoids isolated from winter rye (*Secale cereale* L. cv Puma) exposed to growth at low, cold-hardening temperatures (5°C) under controlled environment conditions exhibited a 3.0-fold increase in the uncoupled, light-saturated rate of whole chain electron transport (H₂O → MV). This increase in whole chain activity was attributed totally to a 1.6-fold increase in the light-saturated rate of PSI activity (Asc/DCPIP → MV). Low growth temperature did not affect light-saturated rates of DCMU-sensitive, PSI electron transport measured as either H₂O → DCPP or DPC → DCPIP (7). Krol et al. (9) reported that the assembly pattern for PSI during rye chloroplast biogenesis at 5°C was distinct from the assembly pattern for PSI during chloroplast biogenesis at 20°C. They suggested that this altered assembly pattern at 5°C results in a change in the organization of PSI which, in turn gives rise to the increased capacity for PSI electron transport (9). In contrast, the pattern for development of PSII electron transport was similar at both 5 and 20°C (9). Low temperature growth did not affect the apparent quantum efficiencies of whole chain, PSI, or PSII electron transport (7). Other cold-tolerant species such as wheat, spinach, pea, broad bean, and *Brassica* also exhibit a 1.4- to 2.2-fold increase in light-saturated rates of PSI electron transport after growth at 5°C under controlled environment conditions (NPA Huner, unpublished data). Thus, an increased capacity to process electrons through PSI may be a general characteristic of cold-tolerant plant species grown at low, nonfreezing temperatures under cold environment conditions.

In winter rye, this low temperature-induced increase in PSI activity has been shown to occur with no change in the following thylakoid characteristics: Chl/P₇₀₀ (6, 9), Chl a/b, CP₁/CPₐ, Chl/lipid, Chl/protein, lipid/protein (8-10), polypeptide and lipid complements (8, 10). The only compositional change observed in isolated thylakoids from low temperature-grown rye was a 72% decrease in the *trans*-16:1 content of PG (8). DSC, fluorescence polarization of DPH, and EPR measurements using 16-doxylstearic acid indicated that low temperature growth of winter rye had no significant effect on the fluidity of isolated thylakoid membranes (8). However, it was shown that PG or a certain proportion of rye thylakoid PG was specifically associated with LHCII (11) and that chloroplast biogenesis at low growth temperature modulates the organization of rye LHCII by altering specifically the *trans*-16:1 content of its PG (8, 10, 11).

Periwinkle (*Vinca minor* L.) is an angiosperm and a cold-tolerant, herbaceous evergreen commonly used as a ground cover. The leaves of this species that develop in the spring and summer are the same leaves that subsequently are exposed to cold-hardening temperatures and are used as a model for cold tolerance.

1 Supported by Operating Grants and Strategic Grants from the Natural Sciences and Engineering Research Council of Canada.

2 Abbreviations: MV, methylioologen; LHCII, light harvesting Chl a/b pigment-protein complex II; CP₁, major Chl a pigment-protein complex associated with the PSI reaction center; CPₐ, Chl a pigment-protein complex associated with the PSII reaction center; FP, free pigment; Asc, ascorbate; DCPIP, oxidized 2,6-dichlorophenol indophenol; F₆₈₅, F₇₃₅, 77 K Chl a fluorescence emission maxima at 685 nm, 694, and 735, respectively; PG, phosphatidyl-diaclylglycerol; SL, sulfoquinovosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOC, deoxycholate; DSC, differential scanning calorimetry; DPH, diphenylhexatriene; EPR, electron paramagnetic resonance.
low temperature and freezing conditions of autumn and winter. The purpose of this investigation was, first, to determine if a native plant species which normally is exposed to natural low temperature and overwintering conditions exhibits the characteristic increase in light-saturated rates of PSI electron observed for plants grown under controlled environment conditions. Second, we wished to establish whether chloroplast biogenesis at low temperature is an absolute requirement for the observed increase in light-saturated rates of PSI electron transport. Finally, we wished to determine if exposure to natural overwintering conditions affected the trans-16:1 content of PG and the LHClI organization of periwinkle thylakoids.

MATERIALS AND METHODS

Plant Material. Two natural stands of periwinkle (Vinca minor L.) in London, Ontario, were used in this study for comparative purposes. Stand 1 was located in north London on the campus of the University of Western Ontario and stand 2 was located in south London about 6 km from the university campus. Both sites had a northwest exposure. Leaf samples were harvested from either stand during various times of the year as specified in the text. In all cases, we examined leaf material produced in the spring, cold-acclimated in the fall, and subsequently subjected to overwintering in the following season. Leaf area was measured with a Li-Cor portable leaf area meter (model LI-3000). Leaf area values represent the averages of at least 8 different samplings ± S.D. Each sampling consisted of the average area of 12 different leaves.

Thylakoid Membrane Isolation. Periwinkle leaves were harvested and immediately ground at 4°C in 50 mM Tricine (pH 7.8) buffer containing 0.4 M sorbitol, 10 mM NaCl, 2% (w/v) PVP, and 0.5% (w/v) BSA with two 10 s bursts of a Waring Blender at high speed. The brei was filtered through two layers of Miracloth (Calbiochem), and the filtrate was centrifuged at 4000×g for 5 min at 4°C. The green pellet containing thylakoid membranes was resuspended and washed in 50 mM Tricine (pH 7.8) containing 10 mM NaCl and 5 mM MgCl₂ by centrifugation at 10,000g for 5 min at 4°C. The thylakoid membranes were resuspended in a minimal volume of 50 mM Tricine (pH 7.8) containing 0.1 M sorbitol, 10 mM NaCl, and 5 mM MgCl₂ and kept on ice in the dark until used. Only freshly isolated thylakoid membranes were used in this study.

Photosynthetic Electron Transport Activities. DCMU-insensitive PSI-associated electron transport activity (Asc-DCPIP → MV) was measured polarographically at 25°C as the rate of O₂ consumption (mg Chl)⁻¹ h⁻¹ using a Clark-type electrode as described in detail elsewhere (7). DCMU-sensitive PSI-associate electron transport activity was measured spectrophotometrically at 590 nm and 25°C in a water-jacketed cell as H₂O → DCPIP (7).

Low Temperature Fluorescence Emission Spectra. Fluorescence emission spectra (77 K) of isolated periwinkle thylakoid membranes were obtained with a Perkin-Elmer 650-40 fluorescence spectrophotometer. Excitation was at 440 nm through 4 nm slits, and corrected emission spectra were collected through 4 nm slits. The data were plotted using Perkin-Elmer supplied software. Thylakoids were suspended to a final Chl concentration of 10 µg ml⁻¹ in 25 mM Tricine (pH 7.8) containing 50% (w/v) glycerol, 2.5 mM MgCl₂, and 5 mM NaCl. All spectra were normalized to the 686 nm peak.

Lipid and Fatty Acid Analyses. Periwinkle leaves were extracted and analyzed for their lipid content and fatty acid compositions as described in detail elsewhere (22, 23).

Chl-Protein Complexes. Chl-protein complexes were solubilized with DOC and SDS in the ratio of 10:1 (DOC:SDS:Chl) and subsequently incubated in the dark at 4°C on 7.5% (w/v) SDS polyacrylamide slab gels as described elsewhere (8). Gels were scanned at 671 nm with a Shimadzu UV-250 spectrophotometer.

Chl and Protein Analysis. Chl was measured according to Arnon (1). Thylakoid protein was determined according to Bradford (3) after extraction of pigments with 90% acetone.

RESULTS

Growth Characteristics. In stands 1 and 2, flowers appeared early in the spring (mid-April to mid-May) with new leaf material being observed after the onset of flowering in the spring. Leaves reached full expansion by late June. Periwinkle leaves from stand 1 (1984–1985) increased in area from 1.56 cm² on May 2, 1984, to 7.02 cm² by June 24, 1984. The average leaf area from June 24, 1984 to March 18, 1985, was 6.44 ± 0.38 cm². For comparison, periwinkle leaves from stand 2 (August 20, 1986–March 26, 1987) exhibited an average leaf area of 5.54 ± 0.41 cm². After the completion of leaf initiation and expansion, no new leaf material was observed. Thus, leaves that were produced in the spring and summer were the same leaves that subsequently overwintered.

PSI and PSII Activities. The results summarized in Figure 1A and Figure 2A represent PSI and PSII light-saturation curves for thylakoids isolated from periwinkle leaves developed during the spring and summer of 1986 and subsequently exposed to low temperature and overwintering conditions in 1987. Exposure to these natural conditions caused two major effects: first, the light-saturated rate of PSI electron transport exhibited a twofold increase during fall and winter reaching a maximum in February (Fig. 1B). This was followed by a decrease in the light-saturated rate that was irreversible even though no visible signs of leaf damage or senescence were evident. The apparent quantum efficiency for PSI electron transport was independent of exposure to changing climatic conditions (Table I). Second, exposure to fall and overwintering conditions caused a 50% decrease in the apparent quantum efficiency of PSII-associated electron transport (Table I; Fig. 2A) with only minor changes in the calculated F₅₉₃ for this reaction (Fig. 2B). Similar results were obtained for thylakoids of periwinkle leaves harvested from either stand 1 or stand 2. Since periwinkle is typically snow covered during the winter months in Canada, the effect of the absence of snow cover on PSI and PSII activity was examined. In stand 1 and 2, snow cover during January and February varied from 15 to 30 cm during the winters of 1984 to 1987. One section of stand 2 was maintained snow free for 14 d from January 9 to January 23, 1985, by carefully removing snow on a daily basis such that periwinkle leaves were fully exposed to freezing temperatures and sunlight. An adjacent section in stand 2 remained snow covered as a control. The snow-covered winter sample exhibited an 86% higher light-saturated rate of PSI electron transport than the sample harvested the previous summer (Table II). A 14 d absence of snow cover during January 1985 only caused an 11% inhibition of PSI activity and had no effect on the Chl a/b. In contrast, light-saturated rates of PSII activity were inhibited by 38% during the same time period. Clearly, PSII activity appears to be more sensitive to the absence of snow cover than is PSI activity of periwinkle thylakoids.

Low Temperature Fluorescence Emission Spectra. The in situ organization and the energy distribution within the photosynthetic apparatus was monitored at 77°K fluorescence emission spectra of Chl a (4). The emission spectra of periwinkle thylakoids isolated at various times during 1986-1987 season from stand 1 exhibited a reduction in the fluorescence intensity at 694 and 735 nm relative to that at 686 nm (Fig. 3) upon exposure to overwintering conditions. This effect appeared to be irreversible since thylakoids isolated in May 1987 that had overwintered exhibited an emission spectrum characteristic of overwintering rather than summer leaves that had never been exposed to winter.
OVERWINTERING OF PERIWINKLE THYLAKOIDS

Fig. 1. A, Light-saturation curves for PSI activity of thylakoids isolated from periwinkle leaves at various times during the 1986-1987 growing season in stand 1. (O) May 23, 1986; (●) August 20, 1986; (△) September 30, 1986; (△) November 18, 1986; (▲) February 2, 1987; (○) March 26, 1987. Each point represents the average ± SD of 3 replicate measurements. Activity is calculated as µmol O₂ consumed (mg Chl⁻¹ h⁻¹). B, Vₘₐₓ, calculated from double reciprocal plots of the data in (A), plotted as a function of time during the 1986-1987 growing season.

![Light-saturation curves for PSI activity](image)

<table>
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<tr>
<th>Table 1. Estimated Quantum Efficiencies for PSI and PSII</th>
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Chl-Protein Complexes. From August 20, 1986 to March 26, 1987, the Chl a/b of isolated thylakoids from periwinkle leaves of stand 1 decreased from 2.59 ± 0.12 to 2.40 ± 0.05. Similarly, from August 16, 1984 to March 18, 1985, the Chl a/b of isolated periwinkle thylakoids decreased from 2.39 ± 0.08 to 2.18 ± 0.10. Thus, minimal variation (8–10%) in the Chl a/b was typically observed over the course of one season.

The Chl of higher plant chloroplasts is associated with specific membrane polypeptides that associate to form the characteristic Chl-protein complexes of thylakoid membranes (2). Figure 4 illustrates comparative scans of Chl-protein complexes separated from isolated thylakoids of periwinkle leaves exposed to summer and winter conditions. Seven major peaks were observed and identified by their electrophoretic migration and characteristic absorption spectra (10). Over the course of 3 years, we observed no major changes in the proportions of the various Chl-protein complexes in thylakoids from spring and summer periwinkle leaves compared to periwinkle leaves exposed to low temperature and overwintering conditions.

Lipid and Fatty Acid Composition. The results summarized in Tables III and IV represent a comparison of the lipid and fatty acid analyses of total leaf extracts from summer and winter

![Light-saturation curves for PSII activity](image)

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<th>Table II. Effect of Snow Cover on PSI and PSII Activity</th>
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<td>All measurements represent the means of 3 determinations ± SD.</td>
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² Light-saturated rates; µmol O₂ consumed (mg Chl⁻¹ h⁻¹) at 25°C. ³ Light-saturated rates; µmol DCPIP reduced (mg Chl⁻¹ h⁻¹) at 25°C. ⁴ Leaf samples were harvested from stand 2 on August 16, 1984. ⁵ Leaf samples were harvested from stand 2 on January 23, 1985, with snow cover. ⁶ Leaf samples were harvested from stand 2 on January 23, 1985, after a 14 d exposure to the absence of snow cover.
periwinkle leaves from stand 1. The summer leaves were developed in the spring of 1987 and were harvested after full expansion in late June and July of 1987. The leaves designated as ‘overwinter’ developed and expanded in the spring and summer of 1986 and subsequently were exposed to overwintering conditions from December 1986 to March 1987. Thus, the former samples were obtained from fully expanded leaves that were never exposed to overwintering conditions. Exposure to low temperatures and overwintering conditions caused significant alterations in both the content and fatty composition of the major phospholipids, PC and PE, primarily associated with nonphotosynthetic membranes (18). A 2-fold increase in the PE content and a 40 to 80% increase in the PC content was observed after overwintering when measured on a Chl basis (Table III) or on a mol % basis (Table IV). In addition, PC exhibited a 1.5-fold and 2-fold increase in the level of 18:3 and 18:2, respectively, whereas PE exhibited about a 1.2-fold increase in 18:3 and a 1.5-fold increase in 18:2. Increases in the levels of these unsaturated fatty acids occurred with a concomitant decrease in the levels of 18:1 in PC and PE.

In contrast, minimal changes were observed in the content of the major chloroplast lipids SL, DGDG, PG, and MGDG measured on a Chl basis or as mol % (Tables III and IV). However, the fatty acid compositions of these chloroplast lipids did exhibit a trend to greater levels of unsaturation. Again, this was primarily due to increases in 18:2 and 18:3 with concomitant decreases in 18:1.

PG of periwinkle exhibited significant levels of the trans-$\Delta^3$ hexadecenoic acid (trans-16:1) (Table IV). However, the content of this fatty acid was not affected significantly by exposure to low temperature and overwintering conditions.

**DISCUSSION**

Exposure to natural low temperature and overwintering conditions induced major functional changes in isolated periwinkle thylakoids. First, an approximate doubling in light-saturated rates of PSI was observed (Fig. 1, A and B) with no effect on the apparent quantum efficiency (Table I). The 77°F fluorescence emission spectra indicate that this increased PSI activity is not associated with a shift in energy distribution in favor of PSI since the ratio of $F_{700}/F_{680}$ appeared to decrease during the winter months (Fig. 3). Winter rye (7) and other cold-tolerant species such as wheat, spinach, pea, broad bean, and Brassica exhibit a similar increased capacity for light-saturated electron transport through PSI upon development at cold-hardening temperatures under controlled environment conditions (NPA Huner, unpublished data). Thus, the increased capacity for PSI electron transport under cold-hardening conditions appears to be a general phenomenon observed under natural and controlled environment conditions. Krol et al. (9) recently suggested that the increased PSI activity observed after growth of winter rye, an annual, at cold-hardening conditions was a consequence of thylakoid biogenesis at low temperature. Although periwinkle thylakoids exhibit a twofold increase in PSI activity upon exposure to winter conditions, the overwintering leaves had developed and became fully expanded during the warm spring and summer months. Thus, leaf development was complete prior to exposure to overwintering conditions. We conclude that chloroplast biogenesis at low temperature is not necessary to observe the increased rates of light-saturated PSI activity in periwinkle, a dicotyledonous evergreen.

Second, the apparent quantum efficiency for PSII electron transport ($H_2O \rightarrow DCPIP$) decreased by 50% from September to
March while light-saturated rates exhibited minor changes (Fig. 2, A and B). In addition, we reported a decrease in 77 K fluorescence at 694 nm in periwinkle thylakoids during the winter. The fluorescence band at 694 nm originates from the Chl a of the core antennae of PSII (4). The Chl a fluorescence associated with PSII core antennae exhibited an irreversible decrease during the winter. This may reflect a disorganization of the PSII core antennae which would cause the reduced quantum efficiency observed for PSII electron transport. Clearly, further experimentation is required to substantiate this hypothesis.

Isolated thylakoids from *Peltiseris silvestris* exhibited seasonal variations in the light-saturated rates of photosynthetic electron transport (13-17). PSI and PSII electron transport exhibited a decrease after the first frosts of September and October with maximum inhibition (60-70%) occurring in February and March. Senser and Beck (20) also reported that Hill activity in isolated thylakoids from *Picea abies* was lowest during the fall and winter months. These results are in sharp contrast to those presented for periwinkle in this report. Under continuous, natural, snow-covered conditions, periwinkle thylakoids exhibited a doubling in the calculated $V_{\text{max}}$ for PSI electron transport (Fig. 1B) and only minor variation in the calculated $V_{\text{max}}$ for PSII activity (Fig. 2B). Thus, not all plant species exhibit an inhibition of *in vitro* PSI and PSII electron transport activity upon exposure to natural overwintering conditions. As discussed by Oquist and Martin (17), the study of the effects of winter stress on thylakoids from conifers exposed to natural conditions is confounded by the interaction of light, extreme desiccation, and low temperature. However, periwinkle is typically snow-covered during exposure to overwintering conditions in Canada which should minimize the possible damaging effects of high light intensity and extreme desiccation coupled with freezing temperatures. This may, in part, account for the differences between conifers and periwinkle with respect to inhibition of PSI and PSII activity upon exposure to overwintering conditions. This is supported by the fact that periwinkle thylakoids exhibited significant inhibition of light-saturated PSII activity only when exposed to overwintering conditions in the absence of snow cover (Table II).

In general, the fatty acid composition of overwintering periwinkle leaves tended toward greater unsaturation with the accumulation of 18:2 and 18:3 apparently at the expense of 18:1 (Table IV). This is consistent with reports on overwintering spruce and white pine (5, 19, 21), but it contrasts the results for *Peltiseris silvestris* which exhibited a decrease in the level of saturation specifically in MGDG upon exposure to winter conditions (15). Whether these changes in the fatty acid composition of periwinkle thylakoids are sufficient to alter the membrane fluidity will have to await further experimentation.

In contrast to the major thylakoid lipids (MGDG, DGDG, PG, SL) (18), the major lipids of nonphotosynthetic membranes (PE and PC) exhibited a twofold increase in lipid content (Table III). These results are consistent with recent published reports of increased membrane content and lipid unsaturation in nonphotosynthetic membranes during low temperature acclimation (12).

Recent results with winter rye indicated a specific 70% decrease in the *trans*-16:1 content in thylakoid PG upon growth and development at low, cold-hardening temperatures (18). Concomitantly, the organization of LHII was affected such that oligomeric LHII predominated during development at high temperature whereas the monomeric or some intermediate form of LHII predominated upon development at cold-hardening temperatures. It was concluded that low, developmental temperature modulates the organization of LHII specifically by altering the *trans*-16:1 content of PG (8, 10, 11). In contrast, the results of this report clearly show that the organization of LHII (oligomeric LHII:monomeric LHII) in periwinkle thylakoids is stable to environmental changes and that the *trans*-16:1 content of PG in periwinkle thylakoids is not significantly affected by exposure to overwintering conditions. This may reflect species differences and/or differences in the pattern of leaf development between a cold-tolerant, dicotyledonous, herbaceous evergreen and a cold-tolerant, annual grass species.

In summary, the increased rate of *in vitro*, light-saturated electron transport through PSI observed upon exposure of periwinkle, an endemic species, to natural, overwintering conditions is consistent with the results reported for winter rye developed at low temperature under controlled environment conditions (7). The increased PSI activity occurred with minimal change in the structure of the photosynthetic apparatus. This may be a general phenomenon in cold-tolerant, herbaceous plants. Since the overwintering periwinkle leaves examined in this study were fully developed prior to winter, chloroplast biogenesis at low temperature does not appear to be an absolute requirement to develop an increased capacity for PSI electron transport. Prolonged exposure of fully expanded leaves to low temperature may accomplish the same effect in certain species. The regulation and the possible physiological importance of this unique phenomenon to plant cold-tolerance is forthcoming (our manuscript in preparation).

**Acknowledgments**—We thank Dr. A. Maun for the use of his Li-Cor leaf area meter and Dr. W. Ware for the use of his fluorescence spectrophotometer. NPAH is grateful to Erin Huner for her assistance in the snow cover experiment.

**LITERATURE CITED**


![Table IV. Lipid and Fatty Acid Composition of Total Leaf Extracts from Summer and Overwintering Periwinkle](image-url)

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<thead>
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<th>Lipid (mol %)</th>
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<td>MGDG</td>
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</table>

*Leaf samples were harvested during late June and early July 1987 from stand 1; n = 3. a Leaf samples were harvested during March 1987 from stand 1; n = 3.*
5. DyoE DR, GN Brown 1979 Glycerolipid and fatty acid changes in Eastern white pine chloroplast lamellae during the onset of winter. Plant Physiol 64: 924–929