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# Effect of Cold Hardening on Sensitivity of Winter and Spring Wheat Leaves to Short-Term Photoinhibition and Recovery of Photosynthesis[1]

**Vaughan M. Hurry[2] and Norman P. A. Huner***

*Department of Plant Sciences, University of Western Ontario, London, Canada N6A 5B7*

## ABSTRACT

**Photoinhibition of photosynthesis and its recovery were studied in wheat (*Triticum aestivum* L.) leaves grown at nonhardening (20°C) and cold-hardening (5°C) temperatures. Cold-hardened wheat leaves were less susceptible to photoinhibition at 5°C than nonhardened leaves, and the winter cultivars, Kharkov and Monopol, were less susceptible than the spring cultivar, Glenlea. The presence of chloramphenicol, a chloroplastic protein synthesis inhibitor, increased the susceptibility to photoinhibition, but cold-hardened leaves still remained less susceptible to photoinhibition than nonhardened leaves. Recovery at 50 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density and 20°C was at least biphasic, with a fast and a slow phase in all cultivars. Cold-hardened leaves recovered maximum fluorescence and maximum variable fluorescence in the dark-adapted state during the fast phase at a rate of 42% $h^{-1}$ compared with 22% $h^{-1}$ for nonhardened leaves. The slow phase occurred at similar rates (2% $h^{-1}$) in cold-hardened and nonhardened leaves. Full recovery required up to 30 h. Fast-recovery phase was not reduced by either lowering the recovery temperature to 5°C or by the presence of chloramphenicol. Slow-recovery phase was inhibited by both treatments. Hence, the fast phase of recovery does not require de novo chloroplast protein synthesis. In addition, only approximately 60% of the photochemical efficiency lost through photoinhibition at 5°C was associated with lost [$^{14}$C]atrazine binding and, hence, with damage to the secondary quinone electron acceptor for photosystem II-binding site. We conclude that the decrease in susceptibility to photoinhibition exhibited following cold hardening of winter and spring cultivars is not due to an increased capacity for repair of photoinhibitory damage at 5°C but reflects intrinsic properties of the cold-hardened photosynthetic apparatus. A model to account for the fast component of recovery is discussed.**

Cold-tolerant cereals such as winter wheat (*Triticum aestivum* L.) and winter rye (*Secale cereale* L.) acquire maximum freezing tolerance following prolonged growth and development of seedlings at low, nonfreezing temperatures (0–5°C) (18, 21). Hence, adequate photosynthesis is a requirement for the expression of freezing tolerance in cold-tolerant cereals. Usually, however, photosynthesis is one of the first processes adversely affected by exposure to low temperature. Furthermore, damage to the photosynthetic apparatus, typically first expressed as photoinhibition, is exacerbated when high irradiance accompanies the low-temperature exposure (25, 27). Typically, plants with larger antenna, such as shade plants (1), or plants with limited potential to fix $CO_2$, such as plants exposed to low temperatures (25, 28), exhibit increased susceptibility to photoinhibition. Photoinhibition reduces the photochemical efficiency of PSII and it is typically detected as a decrease in $F_V$[3], $F_V/F_M$, or a decrease in the quantum yield of $O_2$ evolution (4, 14, 17).

Greer et al. (10) suggested that the susceptibility of barley (*Hordeum vulgare* L.) to low-temperature-induced photoinhibition is due to an imbalance between rates of damage and repair of PSII reaction center polypeptides through de novo chloroplastic protein synthesis. However, winter rye (20, 23) and spinach (*Spinacia oleracea* L.) (5, 29, 30) exhibit reduced sensitivity to low-temperature-induced photoinhibition when acclimated to cold-hardening conditions. Growth and development at low temperatures (5°C) is a prerequisite for exhibiting this increased resistance to photoinhibition (5, 23). Thus, the reduced sensitivity to low-temperature-induced photoinhibition may reflect an increased capacity to repair photoinhibitory damage to PSII reaction centers following cold hardening.

To date, reduced sensitivity to low-temperature-induced photoinhibition has been established through comparisons of plants grown at high and low temperatures (5, 20, 23, 29, 30). The effects of short-term photoinhibition on cultivars known to differ in cold tolerance have not been evaluated. During fall and winter in the field, plants are exposed to a combination of low to subzero temperatures and high irradiance. The relative susceptibility of plants to photoinhibition may contribute to their ability to cold harden and survive the winter.

We evaluated the hypothesis that cold-hardened winter wheat cultivars exhibit lower susceptibility to short-term photoinhibition at low temperatures than cold-hardened spring wheat. One spring and two winter cultivars were selected that exhibited a range of freezing tolerance. We also tested photoinhibition in the presence or absence of CAP, a 70S protein synthesis inhibitor, to determine the role of de

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[2] Present address: Department of Plant Physiology, University of Umeå, S-901 87 Umeå, Sweden.

[3] Abbreviations: $F_V$, maximum variable fluorescence in the dark-adapted state ($F_M - F_O$); $F_O$, minimum fluorescence in the dark-adapted state; $F_M$, maximum fluorescence in the dark-adapted state; CAP, chloramphenicol.

novo chloroplastic protein synthesis in the repair of photoinhibitory damage.

## MATERIALS AND METHODS

### Plant Material

Three cultivars of wheat (*Triticum aestivum* L.), cv Kharkov, cv Monopol, and cv Glenlea, were grown in coarse vermiculite in 7-cm plastic pots at a density of five plants per pot. Water and nutrients were supplied as required in the form of a modified Hoagland solution as described previously (13, 18). Seeds were germinated under controlled environmental conditions with a day/night temperature regimen of 20/16°C at a PPFD of 250 $\mu$mol $m^{-2}$ $s^{-1}$ and a 16-h photoperiod. After 7 d, some of the winter and spring seedlings were transferred to a growth temperature regimen of 5/5°C with photoperiod and irradiance the same as controls for cold hardening. All fluorescence measurements were made on detached, fully expanded, third and fourth leaves of 75-d-old cold-hardened and 25-d-old nonhardened control plants. At these ages, these leaves were fully expanded, and the seedlings were considered to be at similar physiological stages of development as judged by leaf number, leaf dry weight, leaf fresh weight, and leaf area (14). The freezing temperature that is lethal to 50% of plants of each cold-hardened cultivar was determined by regrowth after exposure to controlled freezing in the dark as described previously (18). The freezing temperatures that were lethal to 50% of plants were −21, −12, and −8°C for cold-hardened Kharkov, Monopol, and Glenlea, respectively.

### Measurements of Room Temperature Chl *a* Fluorescence

Room temperature Chl *a* fluorescence at 695 nm was measured with a PSM Chl fluorometer (Biomonitor S.C.I AB, Umeå, Sweden) as described by Öquist and Wass (24). Instantaneous $F_O$, $F_V$, and $F_M$ were determined and the $F_V/F_M$ ratio, expressing the maximum photochemical yield of PSII, was calculated. The actinic light source had a PPFD of 400 $\mu$mol $m^{-2}$ $s^{-1}$, with a peak wavelength of 500 nm. Exposure was for 2 s. Before fluorescence was measured, all leaves were dark adapted for 30 min at room temperature.

### Photoinhibition and Recovery Treatments

For photoinhibition and recovery experiments, 10-cm-long leaf segments were placed on moist filter paper with the adaxial side face up and the cut ends of the leaf segments covered with moist filter paper. Paper and segments were placed in trays in a 5°C cold room with light supplied through a 10-cm deep continuous-flow water bath by a bank of three Lumalux LU-400 high-pressure sodium lamps. The PPFD incident on the leaves was 1200 $\mu$mol $m^{-2}$ $s^{-1}$ (PAR). Constant leaf temperature (5°C) was maintained with air circulation by two oscillating 15-cm fans. The change in the $F_V/F_M$ ratio relative to control was used to quantify photoinhibition. Recovery after a photoinhibitory treatment at 5°C for 12 h was evaluated at 5 or 20°C at an irradiance of 50 $\mu$mol $m^{-2}$ $s^{-1}$ (PAR).

### Inhibitor Studies

Leaf segments, 10 cm in length, were treated with the 70S protein synthesis inhibitor CAP (Sigma Chemical Co., St. Louis, MO). Segments were treated either before photoinhibition or before recovery. CAP was applied to the upper leaf surface at a concentration of 300 $\mu$g $mL^{-1}$ (1 mM) in a 1% (v/v) Tween 20 solution by vigorous brushing with a soft brush (10). Treated leaf segments were placed in the dark at 20°C for 30 min to allow the CAP to penetrate the cuticle and diffuse through the leaf. Segments treated with a 1% (v/v) Tween 20 solution were used as controls in both photoinhibition and recovery experiments.

Penetration of CAP and its effect on in vivo protein synthesis was assessed by incorporation of L-[$^{35}$S]methionine. The [$^{35}$S]methionine, specific activity of 4 MBq $mL^{-1}$, was applied to the leaves along with CAP in a 1% (v/v) Tween 20 solution. Control leaves were treated with [$^{35}$S]methionine in a 1% (v/v) Tween 20 solution, but no CAP was added. CAP-treated and control leaves were incubated in the dark at 20°C for 30 min, followed by a 3-h incorporation period at 20°C and 50 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD.

Thylakoids were extracted using the method of Huner (12). Leaf segments were macerated in a Waring blender by two 5-s bursts in a 50 mM Tricine grinding buffer (pH 7.8) containing 0.4 M sorbitol, 10 mM NaCl. The brei was filtered through two layers of Miracloth and the filtrate centrifuged at 4000*g* for 3 min at 4°C. The pellet was resuspended in a 50 mM Tricine wash buffer (pH 7.8) containing 10 mM NaCl and 5 mM $MgCl_2$ and centrifuged at 10,000*g* for 5 min at 4°C. The pellet was then resuspended in a 50 mM Tricine resuspension buffer (pH 7.8) containing 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and stored on ice in the dark. The effect of CAP was assessed by $^{35}$S cpm $\mu g^{-1}$ of Chl in extracted thylakoids and by fluorographic analysis of denaturing SDS-PAGE gels (6).

### Atrazine Binding

Binding of the PSII herbicide atrazine to isolated thylakoids following photoinhibition was measured according to the method of Tischer and Strotman (31) as described in detail by Lapointe et al. (20). Leaf segments, 10 cm long, were placed on moist filter paper with the adaxial side face up and with the cut ends of the leaf segments covered with moist filter paper. Segments were exposed to 1200 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD at 5°C for periods up to 12 h. Following exposure, thylakoids were isolated using the method of Huner (12) described above. Chl concentration was determined according to the method of Arnon (2). No loss in Chl was observed during the high-light treatment. Thylakoids were diluted with the resuspension buffer to give a Chl concentration of 150 $\mu$g $mL^{-1}$.

The suspended thylakoids were mixed with 1.2 $\mu$Ci $mL^{-1}$ of [ethyl-1-$^{14}$C]atrazine and resuspension buffer to give a 1-mL reaction mixture containing a fixed Chl concentration of 20 $\mu$g $mL^{-1}$ and a 1 $\mu$M concentration of [$^{14}$C]atrazine, the latter being optimal based on binding kinetics of [$^{14}$C]atrazine to isolated thylakoids (20). The reaction mixture was incubated for 10 min at room temperature and 2 $\mu$mol $m^{-2}$ $s^{-1}$

**Table I.** *Fluorescence Characteristics and Chl Concentration of Winter and Spring Wheat Leaves Grown at Cold-Hardening and Nonhardening Temperatures*

Measurements were made on detached, fully expanded third and fourth leaves from 25-d-old nonhardened and 75-d-old cold-hardened plants. Data are the means ± SE, $n = 4$.

| Cultivar | Growth Temperature | $F_O$ | $F_V$ | $F_M$ | $F_V/F_M$ | Chl a + b |
|---|---|---|---|---|---|---|
| | °C | | *Relative fluorescence units* | | | *mg m$^{-2}$* |
| Spring (Glenlea) | 20/16 | 1.5 ± 0.1 | 6.2 ± 0.2 | 7.7 ± 0.2 | 0.80 ± 0.01 | 320 ± 27 |
| | 5/5 | 1.8 ± 0.1 | 5.8 ± 0.4 | 7.6 ± 0.3 | 0.76 ± 0.01 | 340 ± 37 |
| Winter (Monopol) | 20/16 | 1.4 ± 0.1 | 6.4 ± 0.1 | 7.8 ± 0.1 | 0.82 ± 0.01 | 320 ± 32 |
| | 5/5 | 2.0 ± 0.2 | 7.2 ± 0.4 | 9.2 ± 0.4 | 0.78 ± 0.01 | 510 ± 47 |
| Winter (Kharkov) | 20/16 | 1.7 ± 0.2 | 6.0 ± 0.3 | 7.7 ± 0.3 | 0.78 ± 0.01 | 410 ± 14 |
| | 5/5 | 2.6 ± 0.1 | 7.0 ± 0.2 | 9.6 ± 0.4 | 0.73 ± 0.01 | 596 ± 23 |

PPFD and then centrifuged at 4°C for 3 min at 16,000*g* to pellet the thylakoids. The supernatant (0.7 mL) was added to 10 mL of ECOSCINT (DiaMed, Mississauga, Ontario, Canada) and counted in a Beckman LS6000IC scintillation counter. The loss of atrazine binding during photoinhibition was calculated from the difference between bound and unbound [$^{14}$C]atrazine in the reaction mixtures with and without thylakoids (7, 31). The effects of photoinhibition were determined by comparing the loss of [$^{14}$C]atrazine binding in treated and untreated control leaf segments. The extent of photoinhibition was assayed on thylakoids isolated from leaf tissue with and without high-light treatment by measuring the $F_V/F_M$ with a PSM Chl fluorometer.

## RESULTS

### Effect of Cold Hardening on Room Temperature Chl *a* Fluorescence

Spring and winter wheat grown at 5°C had a 6% lower $F_V/F_M$ relative to nonhardened seedlings (Table I). Thus, cold hardening caused a minor change in photochemical efficiency. The reduction in $F_V/F_M$ for the two winter cultivars was due to a 50 to 60% increase in $F_O$ coupled with a 20 to 25% increase in $F_M$ (Table I). This increase in $F_O$ can, for the most part, be accounted for by the 45 to 60% higher Chl per unit area in both winter cultivars (Table I). In the spring wheat, $F_O$ increased with no concomitant increase in $F_M$, thus causing a net decrease in $F_V/F_M$.

### Effect of Cold Hardening on Sensitivity to Photoinhibition

Growth of the winter cv Monopol at cold-hardening temperatures resulted in lower susceptibility to photoinhibition at 5°C as indicated by a smaller decline in $F_V/F_M$ (Fig. 1). The reduction in $F_V/F_M$ during photoinhibition was due specifically to the quenching of $F_V$. $F_O$ was not affected by the photoinhibitory treatment (Fig. 1), suggesting that the response was associated with a loss in the yield of electron transport through PSII rather than quenching in the antennas. The latter would tend to reduce $F_O$ as well as $F_V$.

Similar sensitivities to photoinhibition, measured as a reduction in $F_V/F_M$, are shown in Figure 2 for all three cultivars

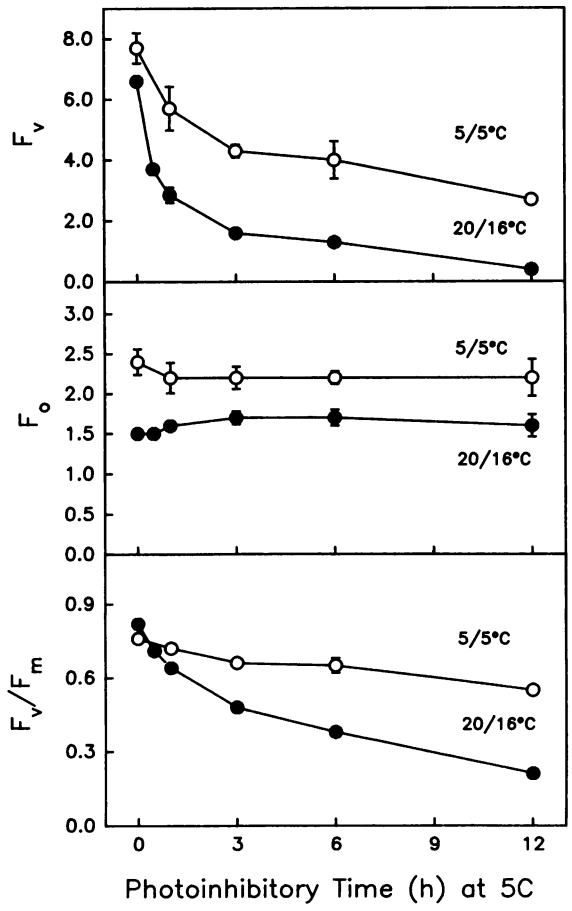


**Figure 1.** Changes in room temperature Chl a fluorescence characteristics of leaves of Monopol winter wheat. Plants grown at cold-hardening (5/5°C, O) and nonhardening (20/16°C, ●) temperatures were exposed to 1200 μmol m$^{-2}$ s$^{-1}$ PPFD at 5°C. Each point is the mean of four to six leaves. Values are relative fluorescence units. Bars represent SE. Where error bars are absent, the errors were smaller than the symbol size.

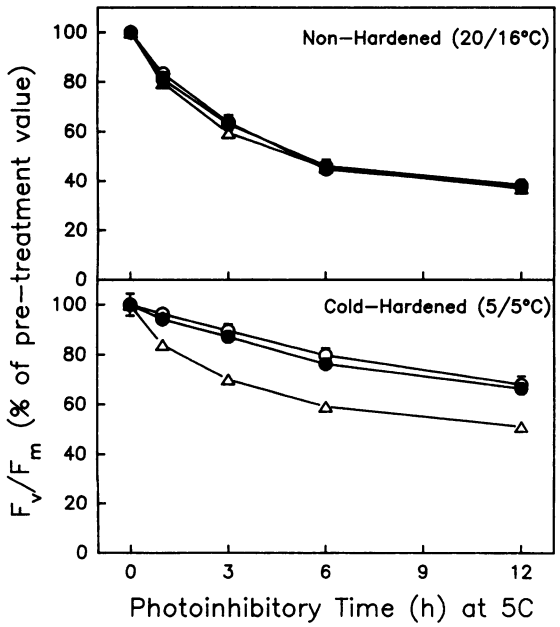


**Figure 2.** Sensitivity to photoinhibition of nonhardened (20/16°C) and cold-hardened (5/5°C) leaves of Kharkov (O) and Monopol (●) winter wheat and Glenlea (Δ) spring wheat exposed to 1200 μmol $m^{-2}$ $s^{-1}$ PPFD at 5°C. Each point is the mean of four to six leaves. Initial $F_V/F_M$ for cold-hardened Kharkov, Monopol, and Glenlea were 0.785 ± 0.035, 0.784 ± 0.002, and 0.774 ± 0.004, respectively. Initial $F_V/F_M$ for nonhardened Kharkov, Monopol, and Glenlea were 0.811 ± 0.002, 0.822 ± 0.002, and 0.818 ± 0.002, respectively. Bars represent SE. Where error bars are absent, the errors were smaller than the symbol size.

grown at 20/16°C. However, following cold hardening at 5°C, the winter cv Monopol and cv Kharkov showed less susceptibility to photoinhibition than the freezing-sensitive spring cv Glenlea (Fig. 2). The two winter cultivars showed similar sensitivity to photoinhibition following cold hardening even though Kharkov showed greater freezing tolerance than Monopol. This suggests that, although the capacity to exhibit lower susceptibility to photoinhibition following cold hardening is more characteristic of winter wheat cultivars, the magnitude is not directly linked to freezing tolerance.

### Effect of CAP during Photoinhibition

In the steady state, the rate of photodamage may be matched by a continuous repair, involving de novo synthesis of D1 and possibly D2 protein (10, 11). The core PSII polypeptides D1 and D2 are encoded by the plastid genome and are synthesized by plastid ribosomes on the stromal lamellae (15). As such, synthesis of these polypeptides, and hence the repair process, can be inhibited by 70S inhibitors such as CAP. Application of CAP (1 mM final concentration) reduced incorporation of [$^{35}$S]methionine into protein associated with washed thylakoid preparations from cold-hardened cv Monopol winter wheat by 70% after 3 h at 20°C and 50 μmol $m^{-2}$ $s^{-1}$ PPFD. It also completely blocked incorporation of label into the 31- to 34-kD region of SDS-PAGE polypeptide gels from the same thylakoid preparations (data not shown). Thus, CAP treatment does, indeed, inhibit protein synthesis.

Treatment of cold-hardened and nonhardened leaves of Kharkov, Monopol, and Glenlea with CAP before exposure to 1200 μmol $m^{-2}$ $s^{-1}$ PPFD at 5°C led to increased sensitivity to photoinhibition, indicating that repair was active in both cold-hardened and nonhardened leaves at 5°C (Fig. 3). However, the difference between cold-hardened and nonhardened leaves in sensitivity to photoinhibition at 5°C when exposed to high light without CAP was maintained when leaves were exposed to high light plus CAP (Fig. 3). Thus,

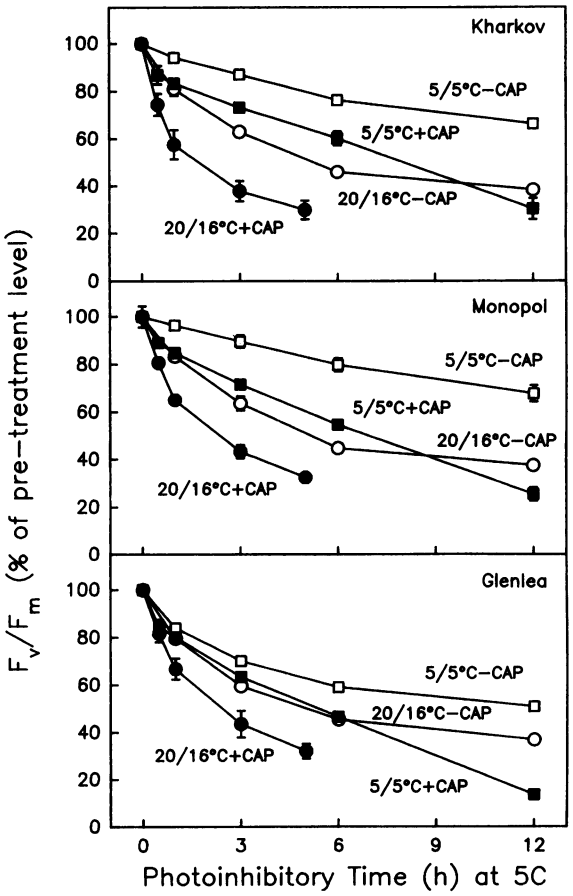


**Figure 3.** Effect of CAP on sensitivity to photoinhibition of nonhardened (20/16°C, O/●) and cold-hardened (5/5°C, □/■) leaves of Kharkov and Monopol winter wheat and Glenlea spring wheat. Leaves were exposed to 1200 μmol $m^{-2}$ $s^{-1}$ PPFD at 5°C. O, □, No CAP; ●, ■, 1 mM CAP. Initial $F_V/F_M$ for cold-hardened and nonhardened Monopol controls were 0.780 ± 0.010 and 0.802 ± 0.002, respectively, and for Monopol plus CAP initial values were 0.770 ± 0.010 and 0.746 ± 0.009, respectively. Initial $F_V/F_M$ for cold-hardened and nonhardened Kharkov controls were 0.791 ± 0.006 and 0.796 ± 0.003, respectively, and for Kharkov plus CAP initial values were 0.750 ± 0.010 and 0.749 ± 0.015, respectively. Initial $F_V/F_M$ for cold-hardened and nonhardened Glenlea controls were 0.775 ± 0.008 and 0.792 ± 0.007, respectively, and for Glenlea plus CAP initial values were 0.740 ± 0.010 and 0.798 ± 0.008, respectively. Each point is the mean of four to six leaves. Bars represent SE. Where error bars are absent, the errors were smaller than the symbol size.

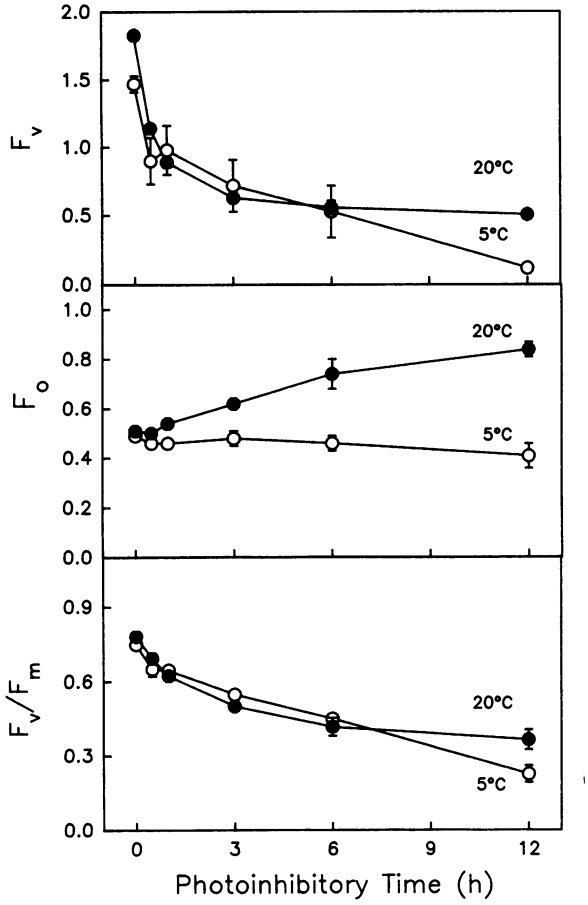


**Figure 4.** Changes in Chl *a* fluorescence characteristics at room temperature for cold-hardened (5/5°C) leaves of Monopol winter wheat. Leaf tissue was treated with 1 mM CAP during exposure to 1200 μmol $m^{-2}$ $s^{-1}$ PPFD at 20°C (●) or at 5°C (○). Each point is the mean of four to six leaves. Values are relative fluorescence units. Bars represent SE. Where error bars are absent, the errors were smaller than the symbol size.

the decrease in sensitivity to low-temperature-induced photoinhibition observed following cold hardening is not due to an increase in the capacity for chloroplastic protein synthesis but is an intrinsic property of the cold-hardened photosynthetic apparatus. However, from the results of these experiments, we cannot rule out completely the involvement of nuclear gene expression.

The $F_O$ of cold-hardened Monopol leaves exposed to 1200 μmol $m^{-2}$ $s^{-1}$ PPFD at 20°C for 12 h in the presence of CAP increased by 65%, with most of the increase occurring after 3 h. The $F_O$ of leaves exposed to high light for 12 h at 5°C in the presence of CAP, however, decreased by 16% (Fig. 4). Similar responses were exhibited by Kharkov and Glenlea wheat (data not shown). Thus, the high light-induced decline in $F_V/F_M$ during the first 3 h at 20°C plus CAP was due primarily to quenching of $F_V$. The slow decline in $F_V/F_M$ after 3 h at 20°C plus CAP, however, was due largely to increases in $F_O$ (Fig. 4), which is indicative of a decline in trapping efficiency rather than a loss in the yield of electron transport for PSII. In contrast, at 5°C the decline in $F_V/F_M$ was due almost entirely to quenching of $F_V$ (Fig. 4), which is indicative of strong nonphotochemical quenching of $F_V$, perhaps by PSII quenching centers (17).

### Atrazine Binding

Photoinhibition of both cold-hardened and nonhardened wheat leaves was associated with a loss in atrazine binding in isolated thylakoids (Table II). However, loss in atrazine binding, and hence damage to the secondary quinone electron acceptor for PSII- or herbicide-binding site of the D1 protein, accounted for only 47 to 68% of the total decrease in $F_V/F_M$ due to photoinhibition in wheat leaves. Furthermore, loss in atrazine binding accounted for only 54% of the reduction in $F_V/F_M$ of cold-hardened leaves showing photoinhibition relative to 65% in nonhardened wheat leaves. Leaves of *Zea mays*, however, show loss in atrazine-binding capacity, accounting for the total reduction in $F_V/F_M$ (Table II), consistent with the strong correlation between reductions in atrazine binding and $F_V/F_M$ reported for this chilling-sensitive species (26). Thus, damage to PSII as measured by atrazine binding cannot account for the entire decrease in $F_V/F_M$ observed upon low-temperature-induced photoinhibition in wheat.

### Effect of Cold Hardening on Recovery from Photoinhibition

Leaves of cold-hardened and nonhardened Kharkov and Monopol winter wheat and Glenlea spring wheat were ex-

**Table II.** *Effect of Photoinhibition on [$^{14}$C]Atrazine Binding to Isolated Thylakoids of Cold-Hardened and Nonhardened Spring and Winter Wheat and Zea mays*

Nonhardened and cold-hardened leaves of spring and winter wheat, and leaves of the chilling-sensitive plant *Zea mays* were exposed to 1200 μmol $m^{-2}$ $s^{-1}$ PPFD for varying periods at 5°C. Photoinhibition was assayed by measuring the $F_V/V_M$ of thylakoids isolated from these leaves. That portion of photoinhibition resulting from damage to the herbicide-binding site on the 32-kD PSII reaction center polypeptide was assessed by loss of [$^{14}$C]atrazine binding. All data represent the averages of two experiments in which a variation of 5% or less was exhibited.

| Growth Temperature | Cultivar | $F_V/F_M$ | [$^{14}$C]Atrazine Binding |
|---|---|---|---|
| | | % reduction following photoinhibition for: | |
| °C | | 6 h at 5°C and 1200 μmol $m^{-2}$ $s^{-1}$ PPFD | |
| 20 | Glenlea | 40 | 27 |
| | Monopol | 40 | 27 |
| | Kharkov | 40 | 23 |
| | | 12 h at 5°C and 1200 μmol $m^{-2}$ $s^{-1}$ PPFD | |
| 5 | Glenlea | 29 | 16 |
| | Monopol | 23 | 14 |
| | Kharkov | 17 | 8 |
| | | 3 h at 5°C and 1200 μmol $m^{-2}$ $s^{-1}$ PPFD | |
| 24 | *Zea mays* | 40 | 44 |

posed to 1200 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD for 12 h at 5°C and then allowed to recover at room temperature at 50 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD. Following cold hardening, all three cultivars showed a strong biphasic recovery pattern, with an initial fast-recovery phase that was complete within 30 min to 1 h, followed by a second slower phase (Fig. 5). Nonhardened leaves also exhibited biphasic recovery kinetics. However, the initial $F_V/F_M$ was lower, and the rate of the initial fast phase was reduced (22% recovery $h^{-1}$) relative to cold-hardened leaves (42% recovery $h^{-1}$). The relative freezing tolerance of the wheat cultivar had no effect on recovery kinetics; rather, recovery was a function of the cold-hardened state of the leaf tissue. The fast-recovery phase accounted for up to 45% of the photon yield lost during photoinhibition. Similar results were found for the chilling-tolerant dicotyledon *S. oleracea* L. (29).

Recovery of $F_O$ (data not shown) was similar to that during photoinhibition (Fig. 1) in which $F_O$ was stable regardless of the state of cold hardening. Recovery of $F_V/F_M$ from photoinhibition, therefore, reflects recovery of $F_V$ and, hence, the yield of PSII photochemistry.

### Effect of Temperature and CAP on Recovery from Photoinhibition

The fast phase of recovery of $F_V/F_M$ at 20°C and 50 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD in cold-hardened Monopol winter wheat, which represented about half of the total recovered $F_V/F_M$,

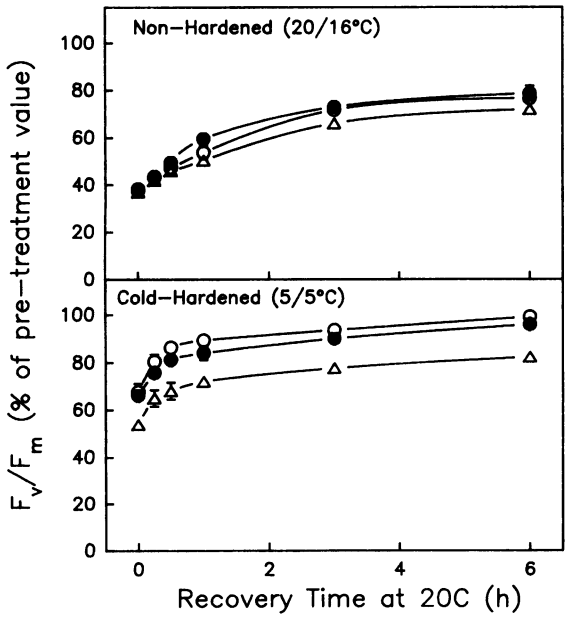


**Figure 5.** Recovery of $F_V/F_M$ at 20°C and 50 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD in nonhardened (20/16°C) and cold-hardened (5/5°C) leaves of Kharkov (○) and Monopol (●) winter wheat and Glenlea (△) spring wheat following exposure to 1200 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD at 5°C for 12 h. See Figure 2 for initial $F_V/F_M$ for cold-hardened and nonhardened samples. Each point is the mean of four to six leaves. Bars represent SE. Where error bars are absent, the errors were smaller than the symbol size.

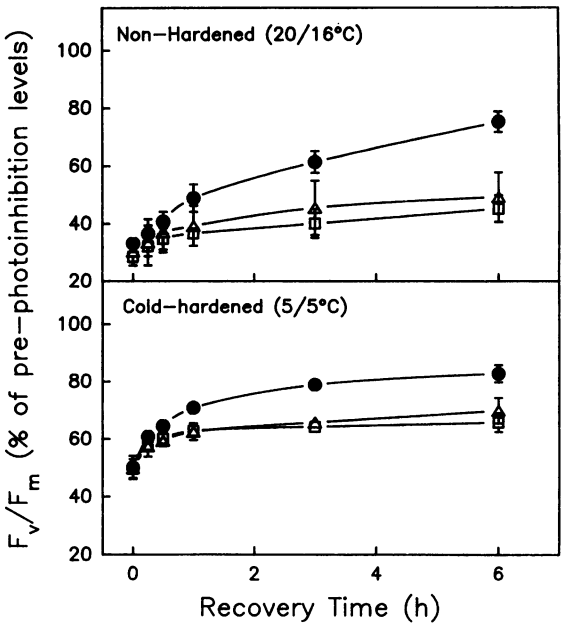


**Figure 6.** Temperature dependence and the effect of CAP on recovery of $F_V/F_M$ at 50 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD in nonhardened (20/16°C) and cold-hardened (5/5°C) leaves of Monopol winter wheat following exposure to 1200 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD at 5°C for 12 h. ●, Recovery at 20°C; □, recovery at 20°C + CAP; △, recovery at 5°C. Initial $F_V/F_M$ for cold-hardened and nonhardened samples: exposed to recovery at 20°C in the presence of CAP, 0.736 ± 0.014 and 0.779 ± 0.004, respectively; exposed to recovery at 20°C, 0.792 ± 0.005 and 0.808 ± 0.004, respectively; and exposed to recovery at 5°C, 0.779 ± 0.011 and 0.805 ± 0.009, respectively. Each point is the mean of four to six leaves. Bars represent SE. Where error bars are absent, the errors were smaller than the symbol size.

was also present during recovery at 5°C and at 20°C plus CAP (Fig. 6). Similar responses were exhibited by Kharkov winter wheat and Glenlea spring wheat (data not shown). The fast-recovery phase of $F_V/F_M$ after exposure to 1200 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD at 5°C for 12 h was also exhibited by cold-hardened Kharkov leaves at −3°C, but it was blocked at this temperature in the less cold-tolerant cultivars and in all nonhardened leaves (data not shown). In contrast, the slow phase present during 20°C recovery was inhibited at 5°C and by CAP at 20°C (Fig. 6).

The existence of a fast-recovery phase at temperatures as low as −3°C and in the absence of de novo chloroplast protein synthesis indicates the presence of a nonphotochemical quenching process in these leaves that relaxes within the first 30 min, even at low temperatures. Relaxation is, however, light dependent, because no significant recovery occurred when leaves were kept in darkness and room temperature for 3 h following photoinhibition (data not shown).

## DISCUSSION

Cold hardening at 5°C and 250 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD reduces the sensitivity of wheat cultivars to low-temperature-induced photoinhibition. Cold-hardened winter wheat exhibits lower susceptibility to low-temperature-induced photoinhibition

relative to cold-hardened spring wheat. Because Kharkov and Monopol exhibit different tolerances to freezing, we conclude that the decreased susceptibility to photoinhibition is associated with winter phenotype rather than freezing tolerance per se. Similar reductions in sensitivity to photoinhibition following cold hardening have been shown in leaves of winter rye (20, 23) and spinach (5, 29). However, this is the first time differences in sensitivity to photoinhibition have been reported among closely related cold-hardened cultivars.

The increased sensitivity of the photosynthetic apparatus to photoinhibition at low, compared with high, temperatures is thought to be due to reduced rates of synthesis of damaged PSII reaction center proteins. In addition, low temperature is thought to reduce rates of diffusion, removal, and degradation of damaged reaction PSII centers (10, 11, 19). Diffusion limitation of this repair cycle by low temperatures is supported by data showing that migration of the apoprotein of the oligomeric form of the light-harvesting complex associated with PSII from the stroma to the grana is blocked at 10°C in barley thylakoids (32). Although the application of CAP inhibited in vivo protein synthesis, CAP-treated leaves from cold-hardened plants were still less susceptible to low-temperature photoinhibition than nonhardened plants. Furthermore, the winter wheat cultivars were consistently less susceptible than the spring cultivar. Thus, we conclude that the differential susceptibility between cold-hardened and nonhardened leaves and between winter and spring cultivars cannot be accounted for by a differential capacity to repair PSII by de novo chloroplastic protein synthesis.

The CAP data presented here indicate that at 20°C, in the presumed absence of the repair cycle (plus CAP), loss of $F_V/F_M$ is initially due to nonphotochemical quenching of $F_V$. However after 3 h, reduction in $F_V/F_M$ is due to increasing $F_O$ and, hence, is probably due to reduced trapping efficiency in the antennae. This is consistent with the hypothesis that damaged PSII reaction centers have disengaged from the antennae (11) but have not been repaired and replaced, leaving the antennae unable to transfer energy to a reaction center, thus increasing the $F_O$. At 5°C, the decline in $F_V/F_M$ during high-light exposure is due to strong nonphotochemical quenching of $F_V$, and associated with this is minimal quenching of $F_O$. This is consistent with in situ conversion of photochemically active PSII reaction centers to inactive quenching centers, dissipating excitation energy as heat (16, 30). Thus, we suggest that, at least in the absence of chloroplastic protein synthesis, the manifestation of photoinhibition at 20°C is quite distinct from that observed at 5°C.

Up to one half of the lost photochemical efficiency of photoinhibited cold-hardened spring and winter wheat leaves was regained within 1 h of exposure to low light at temperatures as low as −3°C or at 20°C plus CAP, both of which should significantly reduce the repair of PSII. Thus, the repair process alone cannot explain the total photoinhibition response seen in wheat leaves at 5°C. Although the CAP data presented show the presence of repair, even at 5°C, we conclude that the fast, reversible component of photoinhibition is not a diffusion-limited phenomenon associated with the repair process of PSII. Krause and Weis (17) speculated that active PSII$\alpha$ centers can be converted rapidly and reversibly into PSII$\alpha$-quenching centers. Our data represent strong evidence in support of a fast, reversible component of recovery that is independent of chloroplastic protein synthesis.

The mechanistic basis of such reversible PSII regulation remains obscure. A model proposing a reversible light-induced quenching mechanism was proposed by Ohad et al. (22). This model suggests a reversible, light-induced change in the conformation of D1, which leaves the reaction center capable of primary photochemistry but destabilizes $Q_B^-$ and inhibits electron transport to plastoquinone. Alternatively, the quenching of $F_V$ observed in wheat leaves may involve an increase in thermal dissipation in the antenna Chl of PSII. Demmig-Adams and Adams (8, 9) proposed that the xanthophyll, zeaxanthin, is a primary mechanism for dissipation of excess excitation energy in the light-harvesting antennae. However, the fast phase of recovery is observed at 5°C, and the epoxidation of zeaxanthin back to violaxanthin is probably too slow at this temperature to account for the relaxation of $F_V$ quenching (3). Furthermore, the fast-recovery phase of $F_V/F_M$ is light dependent, whereas the epoxidation of zeaxanthin is not (33). Thus, we conclude that the fast-recovery phase observed in wheat is likely not due to the rapid relaxation of antenna fluorescence quenching by conversion of zeaxanthin back to violaxanthin at low photon fluxes but could be explained by a reversible change in conformation of PSII reaction centers.

In conclusion, cold hardening at 5°C decreases susceptibility to photoinhibition of all three wheat cultivars tested. However, the winter phenotype confers less susceptibility to photoinhibition following cold hardening than the spring phenotype. Increased photoinhibition at 5°C in the presence of CAP indicates an active repair process even at low temperatures. However, not all of the reduced activity of PSII can be accounted for by irreversible damage to D1. The presence of a significant, rapid recovery, even at low temperatures or at 20°C plus CAP, points to a reversible alteration of PSII that produces reaction centers capable of quenching variable fluorescence. Thus, the decreased susceptibility observed in cold-hardened leaves does not appear to be associated with an increase in the capacity of the PSII repair cycle in cold-hardened plants. Rather, it is associated with an increased capacity to develop a fast-relaxing form of photoinhibition not associated with permanent protein damage and possibly associated with reversible light-dependent changes in conformation of D1.

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