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Resistance to Low Temperature Photoinhibition Is Not Associated with Isolated Thylakoid Membranes of Winter Rye[1]

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ABSTRACT

***In vivo* measurements of chlorophyll *a* fluorescence indicate that cold-hardened winter rye (*Secale cereale* L. cv Musketeer) develops a resistance to low temperature-induced photoinhibition compared with nonhardened rye. After 7.2 hours at 5°C and 1550 micromoles per square meter per second, the ratio of variable fluorescence/maximum fluorescence was depressed by only 23% in cold-hardened rye compared with 46% in nonhardened rye. We have tested the hypothesis that the principal site of this resistance to photoinhibition resides at the level of rye thylakoid membranes. Thylakoids were isolated from cold-hardened and nonhardened rye and exposed to high irradiance (1000–2600 micromoles per square meter per second) at either 5 or 20°C. The photoinhibitory response measured by room temperature fluorescence induction, photosystem II electron transport, photoacoustic spectroscopy, or [^{14}C]atrazine binding indicates that the differential resistance to low temperature-induced photoinhibition *in vivo* is not observed in isolated thylakoids. Similar results were obtained whether isolated rye thylakoids were photoinhibited or thylakoids were isolated from rye leaves preexposed to a photoinhibitory treatment. Thus, we conclude that increased resistance to low temperature-induced photoinhibition is not a property of thylakoid membranes but is associated with a higher level of cellular organization.**

The phenomenon of photoinhibition was described first by Kok and Businger (16) in green algae and subsequently has been observed in many oxygenic plant species when exposed to light conditions that are in excess of the photon requirement for photosynthesis (26). Susceptibility to photoinhibition is manifested as a reversible reduction in the quantum yield and light saturated rates of CO_2 uptake or CO_2-dependent O_2 evolution or as a decrease in the room temperature fluorescence ratio of F_v/F_m[3] (4).

The primary site of photoinhibitory damage has been shown to be localized to PSII *in vivo* and *in vitro* (2, 17, 26). However, the precise site within PSII that initially becomes photodamaged and the mechanism by which this damage occurs is still controversial (2, 17). For example, it has been proposed that the primary site of photoinhibitory dysfunction is the inactivation of the PSII reaction center itself or the donor side of PSII (2, 3, 6, 8, 9, 35, 39). This inactivation occurs with no apparent loss in herbicide binding capacity to the Q_B binding site of the 32 kD polypeptide of PSII. Most of these data have been obtained through *in vitro* photoinhibitory studies of isolated thylakoid membranes. In contrast, there is evidence that the primary site of damage during photoinhibition of PSII is the destruction and increased turnover of the 32 kD polypeptide of PSII (20, 27, 28). This leads to a decreased ability to bind herbicides at the Q_B binding site on the acceptor side of PSII. Much of this work is the result of photoinhibitory studies employing *Chlamydomonas reinhardtii*. Thus, whether the primary site of photoinhibitory damage is at the level of the PSII reaction center itself or at the level of the Q_B binding site cannot be ascertained unequivocally at this time.

The phenomenon of photoinhibition measured *in vivo* appears to be prevalent even under moderate light conditions when plants are subjected to environmental stresses such as chilling (5, 11, 23–26, 30, 32, 34, 37) and freezing (17, 24, 30, 33). This light-dependent reduction in photosynthetic efficiency *in vivo* is typically reversible and is not always associated with photodamage to PSII. Recently, photoinhibitory fluorescence quenching through the thermal deactivation of excited state pigments has been shown to be an important mechanism to prevent photodamage to PSII (10). Demmig and coworkers (10) have shown that photoinhibitory induced

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[3] Abbreviations: F_v, variable fluorescence; F_m, maximum fluorescence with all PSII traps closed; F_o, minimal fluorescence with all PSII traps open; RH, cold-hardened rye; RNH, nonhardened rye; LHCII, light-harvesting Chl *a*/*b* protein complex associated with PSII; DCPIP, 2,6-dichlorophenolindophenol; MV, methylviologen; PAS, photoacoustic spectroscopy; ϕ'_r, relative energy storage yield; Q_B, second electron-accepting plastoquinone of PSII.

increases in zeaxanthin content are correlated with greater protection of PSII through increased nonradiative decay of excitation energy. Schöner and Krause (30) concluded that changes in the xanthophyll levels of cold-hardened spinach thylakoids may be important in protecting cold-hardened spinach against photoinhibition. They also reported higher levels of scavengers of active O_2 species in cold-hardened than in nonhardened spinach plants, which could be implicated in the plant's resistance to photoinhibition at chilling temperatures.

Cold-resistant plant species such as winter rye (*Secale cereale* L. cv Musketeer) and spinach can be photoinhibited at low temperatures if given sufficiently high irradiance. However, it appears that growth and development at low temperature induces a unique resistance to the light-dependent reduction in F_v/F_m and in the apparent quantum yield for O_2 evolution when measured *in vivo* in winter rye (25) and spinach (5, 30, 32, 33, 34). Examination of the recovery kinetics from photoinhibition for RH and RNH plants indicates that resistance cannot be accounted for by faster recovery rates (25). Rye leaves must develop at the low temperature to exhibit this increased resistance to low temperature photoinhibition (25). Similar observations have been made in cold-hardened and nonhardened spinach (S.R. Boese and N.P.A. Huner, unpublished observation) and wheat (Hurry and N.P.A. Huner, unpublished observation).

Rye thylakoids have been shown to be modified during growth and development at low temperatures. Huner *et al.* (14, 15, 19) have reported that development at cold-hardening temperatures results in specific organizational changes in the LHCII. *In situ* electron microscopy and freeze fracture indicated that low temperature development results in smaller granal stacks and a decreased PSII-LHCII particle size (13.6 nm) compared with RNH thylakoids (16.0 nm) (12). *In vitro,* it has been shown that RH LHCII is stabilized primarily in its monomeric form, whereas RNH LHCII is stabilized in the oligomeric form (14, 15). These data have been supported by differential scanning calorimetry and 77 K fluorescence of isolated rye thylakoids (14). However, the development of rye leaves at cold-hardening temperatures did not induce any change in the ratio of carotenoid/Chl (12).

We have been interested in elucidating the mechanism of cold-hardening-induced resistance to photoinhibition. Because PSII of thylakoid membranes is the primary site of a photoinhibitory-induced decrease in photosynthetic efficiency as measured by *in vivo* fluorescence (F_v/F_m) or apparent quantum yield for O_2 evolution, it follows that a resistance to a photoinhibitory decrease in F_v/F_m may be a property of rye thylakoid membranes. If these modifications in PSII-LHCII units in rye thylakoid membrane organization observed during cold hardening impart resistance to photoinhibition, isolated RH thylakoids should exhibit more resistance to the photoinhibitory-induced decrease in PSII efficiency than RNH thylakoids. In this report, we test this hypothesis by an examination of the susceptibility of isolated thylakoids from cold-hardened and nonhardened rye to photoinhibition using Chl fluorescence, *in vitro* electron transport activity, photoacoustic spectroscopy, and [^{14}C]atrazine binding.

MATERIALS AND METHODS

Plant Material

Seeds of winter rye (*Secale cereale* L. cv Musketeer) were sown in vermiculite and germinated in a growth cabinet at 20/16°C (day/night) under a photoperiod of 16 h and a PPFD of 250 μmol m^{-2} s^{-1}. After 7 d, seedlings were either kept at 20/16°C (day/night) for an additional 2 weeks or transferred to 5/5°C (day/night) for 8 weeks with all other conditions held constant. According to Krol *et al.* (18), these cold- and warm-grown rye plants are of comparable physiological ages.

Thylakoid Isolation

Thylakoids were isolated from fully expanded rye leaves by grinding in a Waring blender (2 × 5 s bursts) in the following buffer: 0.4 M sorbitol, 50 mM Tricine (pH 7.8), 10 mM NaCl cooled to 0°C. The homogenate was filtered through two layers of Miracloth and centrifuged at 3000*g* for 2 min at 4°C. The pellet was washed once in buffer containing 0.1 M sorbitol, 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, and 1 mM NH_4Cl and kept on ice in the dark until used. Thylakoids were resuspended in the presence of uncoupler to eliminate the effect of nonphotochemical quenching caused by the buildup of a light-dependent *trans*-thylakoid pH gradient. However, preliminary study of photoinhibition of rye thylakoids in the absence of uncouplers showed the same trends as those described in this paper. Freshly prepared thylakoids were used in all cases.

Chl concentrations were determined according to Arnon (1). Thylakoid suspensions were diluted with resuspension buffer to a Chl concentration of 150 μg mL^{-1}.

Photosynthetic Electron Transport Activity

PSII activity ($H_2O \rightarrow$ DCPIP) was measured in the resuspension buffer containing 12 μg Chl mL^{-1} and 60 μM DCPIP. The reduction of DCPIP was followed in a Unicam SP1800 spectrophotometer at 590 nm in a jacketed cuvette thermoregulated at 25°C. A light source, filtered through Cinemoid ruby No. 14 and an orange No. 5 (Strand Electric) filters, was used to excite the thylakoid sample. PSII activities were corrected for dark decay of thylakoids at room temperature as a function of time.

PSI activity (ascorbate/DCPIP $\rightarrow$ MV) was measured in the resuspension buffer containing 12 μg Chl mL^{-1} as well as 20 μM DCMU, 5 mM NH_4Cl, 200 μM MV, 60 μM DCPIP, 1 mM NaN_3, and 61 μg mL^{-1} of superoxide dismutase. The reduction of MV was followed polarigraphically at 20°C with a Hansatech aqueous phase O_2 electrode. The reaction was started by turning on the light. PPFD was attenuated with neutral density filters.

Room Temperature Fluorescence Induction

Room temperature fluorescence induction curves were obtained using a PAM 101 chlorophyll fluorometer (Walz) that has been described in detail (31). PPFD of the modulated beam at 650 nm was 0.12 m^{-2} s^{-1} and the frequencies used

were 1.6 kHz for F_o measurements and 100 kHz when used in conjunction with a pulse of saturating white light (2750 μmol m^{-2} s^{-1}). The duration of the flashes was 400 ms with 5 s between each pulse. The fluorescence signals were recorded on an X-Y recorder Omnigraphic 2000 (Houston Instruments). The samples were diluted to 20 μg Chl mL^{-1}. F_v and F_m were corrected for dark decay of thylakoids at room temperature as a function of time.

Photoacoustic Measurements

PAS measurements were performed as described in detail by Carpentier *et al.* (7). The fluence rate of the modulated measuring light beam of 680 nm was varied between 0.16 and 4.15 W m^{-2} at a frequency of 35 Hz. The nonmodulated saturating light beam had a fluence rate of 186 W m^{-2}. All measurements were made at room temperature. The thylakoids (240 μg Chl total) were aspirated through a nitrocellulose filter (Millipore Corp., AA type, 25 mm diameter, 0.8 μm pore size) that was then cut to a final diameter of 15 mm. A new sample was prepared for each photoacoustic measurement. The ϕ'_r was calculated from the difference between the intensity of the PAS signal in the presence and in the absence of the nonmodulated saturating beam. Correction factors were applied to ϕ'_r to take into account dark decay of thylakoids at room temperature as a function time.

Atrazine Binding

Atrazine binding was carried out according to Tischer and Strotmann (36). Resuspended thylakoids diluted to 48 μg Chl mL^{-1} were pipetted in an Eppendorf tube and 20 μL of [ethyl-1-^{14}C]atrazine (925 kBq μmol^{-1}) were added for a final concentration of 1 μM. The total reaction volume was 1 mL. Thylakoid membranes were incubated for 10 min at room temperature and low light (2 μmol m^{-2} s^{-1}), then centrifuged for 3 min at 16000*g* at 5°C. The supernatant (0.7 mL) was added to 10 mL of AQUASOL-2 (DuPont) and radioactivity determined in a Beckman scintillation counter (model LS6000IC). Control samples without thylakoids were processed in the same way. The amount of thylakoid-bound atrazine was calculated from the difference between controls and the corresponding thylakoid samples. An initial kinetic binding study was performed to determine the optimal concentration range required to saturate the high affinity binding sites. Double reciprocal plots of the binding data indicated that atrazine binding was biphasic as expected (36) and that 1 μM atrazine was sufficient to saturate the high affinity binding sites. The number of binding sites for atrazine in RNH and RH control thylakoids was not significantly different and was calculated to be 4.5 ± 0.3 and 6.3 ± 1.7 nmol of atrazine mg Chl^{-1}, respectively. Thus, for rye thylakoids, there is about 1 atrazine binding site for every 212 Chl molecules. These values represented control levels prior to photoinhibition and were used as an estimate of 100% binding capacity. The reduction in the number of atrazine binding sites after a photoinhibitory treatment was calculated relative to these control values.

Photoinhibitory Treatment of Thylakoids

Isolated rye thylakoids were exposed to white light from a Fiber-Lite light source (model 170-D) directed to the sample vial via an optic fiber bundle. The samples were stirred continuously during the treatment. The treatments were performed at either room temperature or in a cold room at a PPFD of 2600 μmol m^{-2} s^{-1} at the center of the vial. The temperature of the sample was measured at the beginning and at the end of each photoinhibitory treatment and varied from 5 to 7°C at the low temperature and from 20 to 25°C at the high temperature. Control samples were kept at either room temperature or 5°C in the dark. For Chl fluorescence measurements, all samples were dark adapted for 10 min after the photoinhibitory treatment prior to measurements of Chl fluorescence. For PSI, PSII, and PAS measurements, the samples were measured immediately after the photoinhibitory treatment.

In Vivo Photoinhibitory Treatments

Rye segments were placed on moist filter paper and exposed to a PPFD of 1500 to 1550 μmol m^{-2} s^{-1} using high pressure sodium vapor lamps. The samples were shielded from the light by a 10 cm heat filter that consisted of continuously flowing water. The air above the samples was circulated using two small fans. Sample temperatures remained between 5 and 7°C under these conditions. Leaf sections were dark adapted at room temperature for 30 min before Chl *a* fluorescence was measured and then thylakoids were isolated from the treated leaf segments. Fluorescence was measured with a Plant Stress Meter Mark II (Bio Monitor, Sweden) on leaf segments as well as on thylakoids isolated from the leaf segments.

All PPFD were measured with a Li-Cor light meter (model LI-185A) from the end of the optic fiber bundles. Each experiment presented here was repeated at least three times for electron transport and PAS measurements and four times for the Chl fluorescence measurements and atrazine binding studies. All data are presented as the average ± SE. Where error bars are not obvious in the figures, the SE was the same size as the symbol.

RESULTS

The *in vivo* Chl *a* fluorescence results for rye leaf segments illustrated in Figure 1 indicate that rye leaves developed under cold-hardening conditions develop a resistance to low temperature-induced photoinhibition compared with rye leaves developed under nonhardening conditions. After 7.2 h at 5°C and an irradiance of 1550 μmol m^{-2} s^{-1}, the F_v/F_m was depressed by 46% in RNH leaves in contrast to only 23% in RH leaves. In addition, the F_v/F_m of RH leaves prior to the photoinhibitory treatment was slightly lower (0.74 ± 0.03) than that observed at time zero for RNH leaves (0.79 ± 0.01). These results are consistent with those recently published for attached RH and RNH leaves (25).

To test the hypothesis that the site for the development of resistance to low-temperature photoinhibition was PSII in rye thylakoids, we isolated thylakoids from RH and RNH leaves and exposed them to photoinhibitory conditions. F_v/F_m ratios of control samples taken from different experiments varied

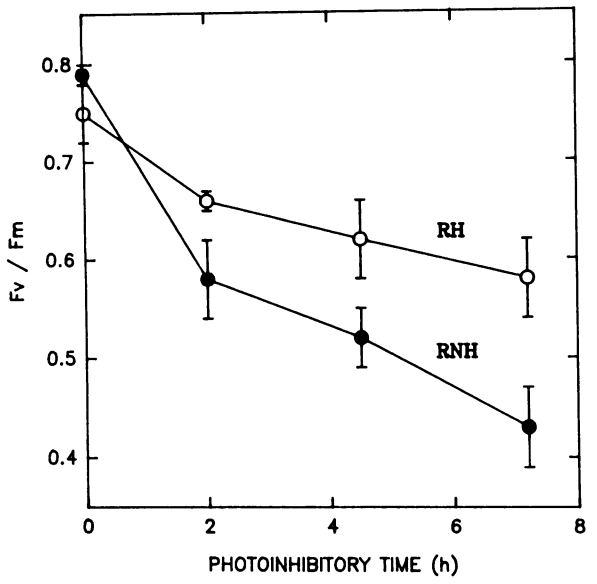

Figure 1. The effect of low temperature photoinhibition on F_v/F_m measured *in vivo* for RH and RNH leaves. (●) RNH leaf segments. (○) RH leaf segments. All data represent the average of five different leaf segments taken from the middle portion of the third leaf of RH and RNH plants. Photoinhibitory condition consisted of 1550 μmol m^{-2} s^{-1} and a temperature of 5 to 7°C.

between 0.65 and 0.74 for RNH thylakoids and from 0.61 to 0.64 for RH thylakoids prior to photoinhibitory treatment. Although the absolute values of F_v/F_m observed for isolated thylakoids were about 15% lower than those observed *in vivo*, the lower values for RH thylakoids than for RNH thylakoids are consistent with that observed *in vivo* (Fig. 1) (25). The lower F_v/F_m ratios in RH thylakoids prior to photoinhibitory treatment were essentially due to higher F_o values, and their F_v values were similar to those of RNH thylakoids (Fig. 2B and C). Regardless, the kinetics for the decrease in F_v/F_m upon exposure to 2600 μmol m^{-2} s^{-1} at 20°C was similar for RH and RNH thylakoids during 1 h of this photoinhibitory treatment (Fig. 2A). After 60 min, F_v could no longer be detected and, as a consequence, F_v/F_m had decayed to zero. The observed decrease in F_v/F_m was light dependent because, in the dark at 20°C, F_v/F_m of RH and RNH thylakoids decreased by only 15% after 1 h (data not shown). A more detailed examination of the fluorescence parameters during the photoinhibitory treatment indicated that the decrease in F_v/F_m was due to a decrease in F_v over the 60 min treatment period coupled with a substantial increase in F_o during the first 30 min (Fig. 2B and C).

For comparative purposes, the fluorescence data for RH and RNH thylakoids were normalized (Fig. 2D and E). The results showed that RH thylakoids exhibited a 25% increase, whereas RNH thylakoids exhibited a 40% increase in F_o after 30 min of photoinhibitory treatment (Fig. 2E). However, the F_v of RH thylakoids tended to be lower than that of RNH thylakoids during the photoinhibitory period (Fig. 2E). When the effects on F_v and F_o were combined to calculate F_v/F_m (Fig. 2D), RH thylakoids tended to be more sensitive to the photoinhibitory treatment than RNH thylakoids. Similar trends were observed when lower PPFD (1000 μmol m^{-2} s^{-1}) were used for the photoinhibitory treatment at 20°C or when RH and RNH thylakoids were isolated and resuspended in high osmoticum (0.8 M sorbitol) to stabilize the rye membranes (13) during the photoinhibitory treatment (data not shown).

In vivo exposure of attached rye leaves or rye leaf segments to moderate irradiance and low temperature markedly increased susceptibility to photoinhibition compared with the same light treatment at 20°C (25). In contrast to these *in vivo* observations, isolated RH and RNH thylakoids appeared to be less sensitive to photoinhibition at 5°C than at 20°C, as indicated by a slower rate of change in F_o, F_v, and F_v/F_m during photoinhibition at 5°C (Fig. 3A and B). Similar observations have been reported previously (22, 29). The relative trends between RH and RNH thylakoids with respect to F_o, F_v, and F_v/F_m observed during photoinhibition at 5°C were consistent with those observed at 20°C. Again the F_v/F_m of RH thylakoids tended to be more sensitive to the photoinhibitory treatment than that of RNH thylakoids (Fig. 3A).

The photoinhibitory decrease in F_v/F_m in RH and RNH thylakoids implies a decrease in the photosynthetic efficiency of PSII. This was corroborated by independent measurements of PSII electron transport under light-saturating (Fig. 4A) or light-limiting conditions (Fig. 4B). Both RH and RNH thylakoids exhibited similar kinetics for the photoinhibitory decrease in PSII activity, with RH thylakoids tending to be more sensitive to the photoinhibitory treatment than RNH thyla-

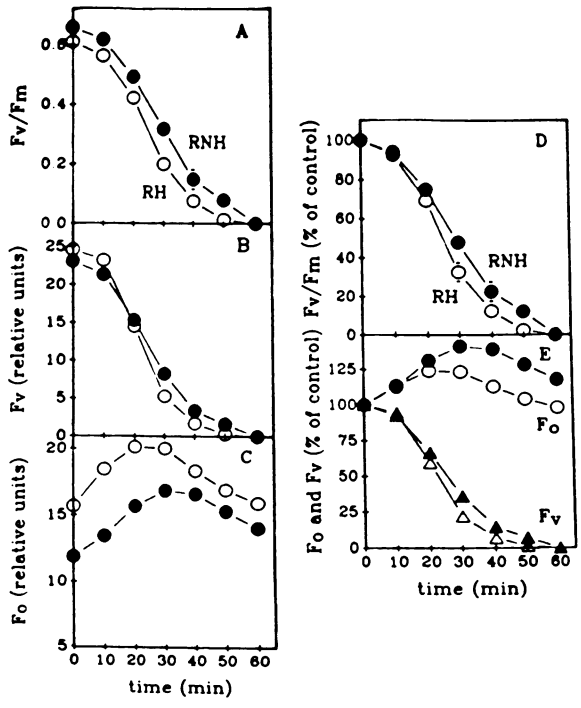

Figure 2. The effect of *in vitro* photoinhibitory treatment at 20°C on the fluorescence characteristics of RH and RNH thylakoids. A through C, Relative values of F_v/F_m, F_v, and F_o. D, E, Normalized data. Thylakoids were exposed to 2600 μmol m^{-2} s^{-1}. SE were equal to or smaller than the symbol size.

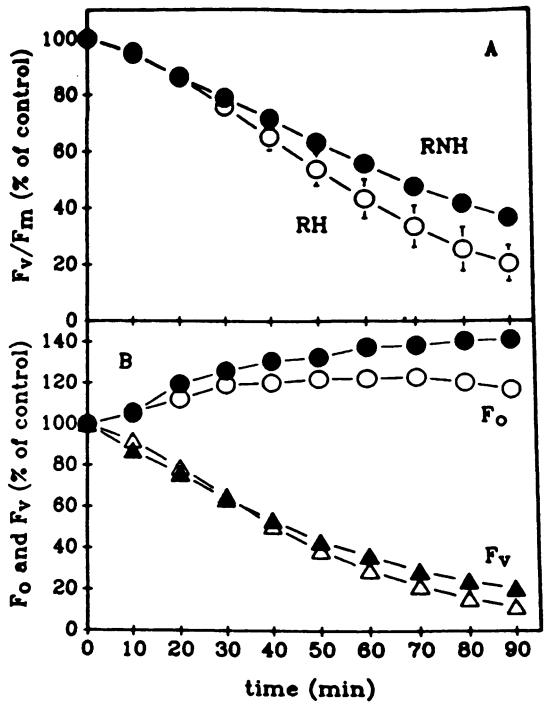

Figure 3. The effect of photoinhibitory treatment at 5°C on the fluorescence characteristics of RH and RNH thylakoids. All data have been normalized for convenience. Thylakoids were exposed to 2600 μmol m^{-2} s^{-1}. Initial F_v/F_m were 0.64 ± 0.01 (RH) and 0.74 ± (RNH). Most SE were smaller than the symbol size.

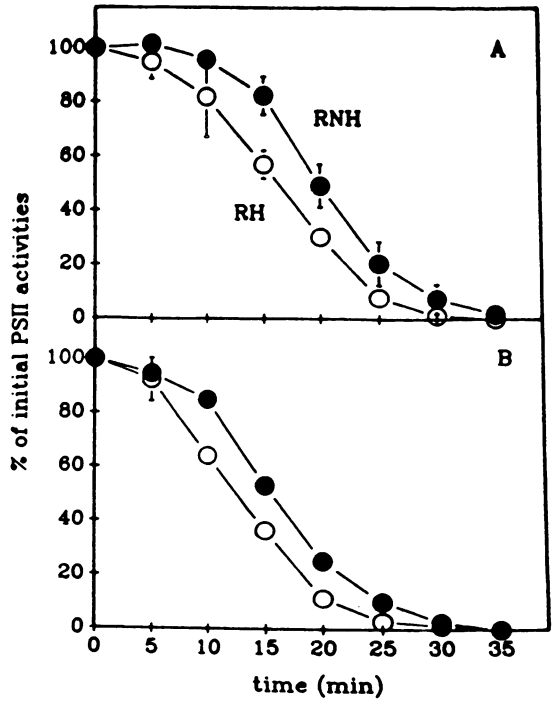

Figure 4. Changes in the light-saturated (3000 μmol m^{-2} s^{-1}) (A) and light-limited (250 μmol m^{-2} s^{-1}) (B) rates of PSII electron transport in rye thylakoids as a function of exposure time to 2600 μmol m^{-2} s^{-1} at 20°C. Initial rates of PSII electron transport as measured at 25°C under light-saturating conditions were 473 ± 108 (RH) and 399 ± 53 (RNH) μmol DCPIP reduced mg Chl^{-1} h^{-1} and 89 ± 8 (RH) and 84 ± 11 (RNH) μmol DCPIP reduced mg Chl^{-1} h^{-1} under light-limiting conditions. Most SE were smaller than the symbol size.

koids. In addition, after 15 min at 2600 μmol m^{-2} s^{-1} at 20°C, the initial slopes of the light response curves for PSII activity for RH thylakoids exhibited a 2.5-fold decrease, whereas RNH thylakoids exhibited a 1.4-fold decrease in initial slope (Table I).

Concomitantly, light response curves for PSI electron transport were measured in RH and RNH thylakoids before and after photoinhibitory treatment at 2600 μmol m^{-2} s^{-1} and 20°C. The results in Table I indicate that both RH and RNH thylakoids exhibited a similar 2.7-fold decrease in the initial slopes for PSI electron transport as a consequence of the photoinhibitory treatment.

Photoacoustic measurements represent another independent means of assessing the photosynthetic capacity of PSII (21). The results presented in Figure 5 indicate that RH and RNH thylakoids exhibited similar decreases in ϕ'_r, that is, a similar decrease in their capacity for PSII electron transport, upon exposure to a photoinhibitory treatment at 20°C. Furthermore, RH and RNH thylakoids exhibited similar kinetics for the inhibition of atrazine binding during a photoinhibitory treatment at 20°C and 2600 μmol m^{-2} s^{-1}. After 1 h of photoinhibitory treatment, the number of atrazine binding sites was reduced by 70 ± 10% in both RH and RNH thylakoids (data not shown). Because atrazine is a competitive inhibitor of Q_B binding to the D_1 polypeptide of PSII (38), this indicates that the photoinhibitory treatment did not cause differential damage to the Q_B binding site of the D1 polypeptide of PSII in RH and RNH thylakoids. These results are consistent with the fluorescence and electron transport data which indicate that, *in vitro,* RH thylakoids are not more resistant to photoinhibition than RNH thylakoids, in contrast to the *in vivo* data (Fig. 1).

One may argue that the discrepancy between the *in vivo* data (Fig. 1) and the *in vitro* data (Figs. 2–5) is due to the fact that the microenvironment of rye thylakoids during photoinhibition *in vivo* is quite different compared with that during photoinhibition *in vitro.* Thus, isolated RH thylakoids would no longer exhibit the same resistance to photoinhibition ob-

Table I. *Initial Slopes of the Light Response Curves for PSII and PSI Activities of Control and Photoinhibited RH and RHN Thylakoids*

Rye thylakoids were exposed to an irradiance of 2600 μmol m^{-2} s^{-1} for 15 min (PSII) or for 30 min (PSI). The initial slopes were estimated by linear regression from the initial, linear portion of the light response curve, which represented six data points per sample. All data represent the average of three replicate experiments ± SE.

Sample	PSII[a]		PSI[b]	
	RH	RNH	RH	RNH
Control	0.35 ± 0.03	0.37 ± 0.02	0.12 ± 0.01	0.11 ± 0.01
Treated	0.14 ± 0.01	0.26 ± 0.04	0.04 ± 0.01	0.04 ± 0.01

[a] μmol DCPIP reduced mg Chl^{-1} h^{-1}/μmol photons m^{-2} s^{-1}. [b] μmol O_2 consumed mg Chl^{-1} h^{-1}/μmol photons m^{-2} s^{-1}.

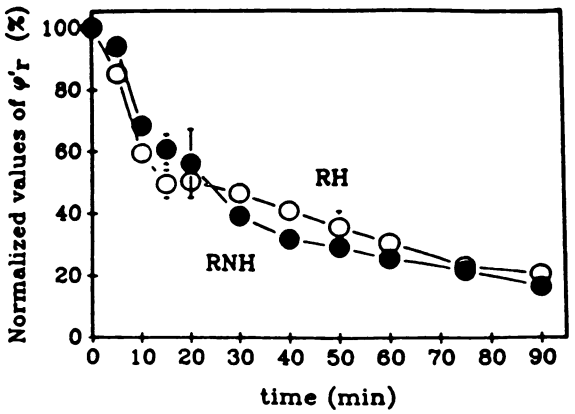

Figure 5. Changes in the initial ϕ'_f of rye thylakoids as a function of exposure time to 2600 μmol m^{-2} s^{-1} at 20°C. Initial values of ϕ'_f were 16.5 ± 1.5% (RH) and 15.9 ± 0.6% (RNH). The modulated measuring light beam had a fluence rate of 0.49 W m^{-2} at a frequency of 35 Hz. The nonmodulated saturating light beam had a fluence rate of 186 W m^{-2}. Most SE were smaller than the symbol size.

served *in vivo*. Thus, we examined RH and RNH thylakoids that had been isolated from rye leaves exposed to a photoinhibitory treatment at 5°C and 1200 μmol m^{-2} s^{-1}. The F_v/F_m of RNH and RH leaves was 0.77 ± 0.01 and 0.73 ± 0.02, respectively, prior to the photoinhibitory treatment. The F_v/F_m of isolated thylakoids from the control leaves was 0.73 ± 0.01 and 0.66 ± 0.01 for RNH and RH, respectively. As expected, after 5.5 h at 5°C and 1200 μmol m^{-2} s^{-1}, the F_v/F_m of RNH leaves was reduced by 61 ± 5% of the initial control values, whereas the F_v/F_m of RH leaves was reduced by only 37 ± 4%. In contrast, thylakoids isolated from these same leaves exhibited similar reductions in F_v/F_m of about 30% compared to control thylakoids. Thus, although RH and RNH leaves exhibited a differential sensitivity to photoinhibition, thylakoids isolated from the same leaves did not.

DISCUSSION

A combination of low temperature and moderate to high irradiance can induce photoinhibition in intact leaves as indicated by a decrease in F_v/F_m and in the quantum yield for O_2 evolution (11, 23, 24, 26, 32, 37). However, recently it has been reported that cold hardening of spinach (5, 30, 32) and winter rye (25) (Fig. 1) and winter wheat (Hurry, NPA Huner unpublished observation) results in the unique capacity to exhibit increased resistance to low temperature-induced photoinhibition. Thus, under conditions of low temperature and moderate to high light (400–1500 μmol m^{-2} s^{-1}), nonhardened leaves exhibit a twofold greater suppression of F_v/F_m than cold-hardened leaves exposed to the same photoinhibitory regimen. This differential change in F_v/F_m implicates PSII as the principal site of resistance. Hence, it seems reasonable to assume that the cold hardening-induced resistance to low temperature photoinhibition would be associated with thylakoid membranes. It has been suggested that the resistance induced by cold hardening is a function of reported changes in carotenoid composition of thylakoid membranes and the level of scavengers of active O_2 species in spinach chloroplasts (30). Furthermore, it has been suggested by Huner and coworkers (15) that the organizational changes observed in RH thylakoids may be related to an increased resistance to low temperature-induced photoinhibition. On the basis of *in vitro* Chl *a* fluorescence, PSII electron transport, atrazine binding, and PAS, the observed *in vivo* differential resistance to photoinhibition is not apparent at the level of isolated thylakoid membranes. Similar conclusions were reached whether isolated thylakoids were photoinhibited or whether thylakoids were isolated from photoinhibited leaves. Thus, we conclude that the mechanism of resistance to photoinhibition must require a higher level of cellular organization. Recently, we have shown that isolated, intact mesophyll cells from RH leaves are indeed more resistant to low temperature photoinhibition than intact mesophyll cells from RNH leaves based on fluorescence analyses (L Lapointe, NPA Huner, manuscript in preparation). This indicates that increased resistance to photoinhibition probably involves complex regulation of PSII such that higher PSII efficiency is maintained in RH dark-adapted samples after exposure to a photoinhibitory event. This will be the subject of a forthcoming paper.

The *in vitro* results presented here indicate that thylakoids exposed to photoinhibitory conditions at either 5 or 20°C exhibited a significant increase in F_o (Figs. 2 and 3). Furthermore, RNH thylakoids exhibited a 40% increase in contrast to the 25% increase in RH thylakoids. An increase in F_o may reflect a decrease in the efficiency of energy transfer from LHCII to PSII reaction centers as a result of physical dissociation of LHCII from the PSII core (3). The differential effects of photoinhibition on F_o may reflect the organizational differences reported between RH and RNH LHCII (14, 19). A closer association between LHCII and PSII in RNH than RH thylakoids could explain the lower F_o and higher F_v/F_m in RNH than RH leaves and thylakoids. However, it is clear from the experiments presented here that these organizational differences in RH and RNH thylakoids cannot account for the differential resistance to photoinhibition observed *in vivo*.

It is interesting to note that photoinhibition appears to have a greater effect on the F_v/F_m of intact leaves than on the F_v/F_m of thylakoids isolated from the same leaves. This may be due to the fact that only the uppermost layer of mesophyll cells is detected by *in vivo* fluorescence, whereas thylakoids isolated from the same leaves reflect the total population of mesophyll cells within the leaf. Those thylakoids obtained from deeper within the leaf would be less photoinhibited than those closer to the exposed leaf surface.

Although PSI is stable to photoinhibition *in vivo* (17), the results in Table I indicate that the initial slopes for the light response curves for RH and RNH thylakoids decreased 2.7-fold after exposure to high light at 20°C for 30 min. Thus, *in vitro* the apparent quantum efficiency of both PSII and PSI are reduced by photoinhibition. The susceptibility of PSI to photoinhibition *in vitro* has been reported previously (3, 29). Clearly, photoinhibition *in vitro* is quite distinct from photoinhibition *in vivo*.

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