Chilling Sensitivity in *Oryza sativa:* The Role of Protein Phosphorylation in Protection against Photoinhibition

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ABSTRACT

The effects of exposure to low temperature on photosynthesis and protein phosphorylation in chilling-sensitive and cold-tolerant plant species were compared. Chilling temperatures resulted in light-dependent loss of photosynthetic electron transport in chilling-sensitive rice (*Oryza sativa* L.) but not in cold-tolerant barley (*Hordeum vulgare* L.). Brief exposure to chilling temperatures (0–15°C, 10 min) did not cause a significant difference in photosynthetic O_2 evolution capacity *in vivo* between rice and barley. Analysis of *in vivo* chlorophyll fluorescence in chilling-sensitive rice suggests that low temperatures cause an increased reduction of the plastoquinone pool that could result in photoinhibitory damage to the photosystem II reaction centers. Analysis of ^{32}P incorporation into thylakoid proteins both *in vivo* and *in vitro* demonstrated that chilling temperature inhibited protein phosphorylation in rice, but not in barley. Low temperature (77 K) fluorescence analysis of isolated thylakoid membranes indicated that state I to state II transitions occurred in barley, but not in rice subjected to chilling temperatures. These observations suggest that protein phosphorylation may play an important role in protection against photoinhibition caused by exposure to chilling temperatures.

Stress conditions, such as high light intensity, chilling temperatures, or limiting CO_2 concentrations, result in light-dependent inactivation of photosynthetic electron transport. This phenomenon, termed photoinhibition, preferentially affects chloroplast membrane-bound PSII electron transport (24). Inactivation of the reaction center (10, 24), and turnover of the Q_B protein[1] (2, 14) have each been suggested to be a primary site of photoinhibitory damage under stress conditions. Photoinhibition at low temperatures (0–15°C) is well documented in a number of cold-intolerant crop species (22) and, in rice (13) and maize (3, 16), results in a loss of photosynthetic capacity that persists for several days.

Photoinhibitory damage to PSII under stress conditions has been related to excess light energy arriving at PSII (23). This can arise from either high light intensities *per se* or rate limitations at the reducing side of PSII which interfere with the efficient turnover of the PSII reaction center (23). Intolerance to chilling temperatures could therefore be explained either by rate limitations imposed at chilling temperatures which are unique to

chilling sensitive plants or by the inability of sensitive plants to effectively regulate the balance of light energy between the two photosystems at these temperatures. Investigations comparing chilling-tolerant pea, spinach, and barley with chilling-sensitive bean and mung bean (20, 21) did not find low temperature rate limitations to PSI, PSII, or pH gradient dissipation unique to chilling-sensitive plants, so it was of particular interest to study chilling effects on regulatory steps. A well documented mechanism for modulating the distribution of light energy between the two photosystems involves the reversible protein phosphorylation of LHCII (9). Phosphorylation is achieved through the light-stimulated activity of one or more thylakoid membrane-bound kinases (7) and can be reversed via a membrane-bound phosphoprotein phosphatase (8). Light stimulation of protein kinase activity is thought to be the result of kinase activation via reduction of the plastoquinone pool (1). The relative degree of LHCII phosphorylation is thought to physically mediate the interaction between PSII and LHCII (5) and thus regulate the balance of excitation energy arriving at the two reaction centers, providing a mechanism that controls the relative turnover of the two photosystems. In this study we have investigated the possibility that the effects of chilling on regulatory phenomena, and on protein phosphorylation in particular, are responsible for the sensitivity of rice to chilling temperatures.

MATERIALS AND METHODS

Plant Material. Seeds of *Oryza sativa* L. cv IR 8 (a chilling-sensitive Indica strain) and cv M201 (a partially chilling-tolerant California rice variety) were germinated under water in ambient light for 2 weeks; seedlings were transplanted to 4-inch pots containing soil mix (Supersoil, Rod McLellan Co., South San Francisco, CA). Barley (*Hordeum vulgare* L. cv Himalaya) was germinated in soil mix. All plants were grown in a greenhouse (day/night temperatures 25/20 ± 4°C; day/night relative humidity 50/80 ± 10%; no supplemental lighting). For cold-grown barley, plants were transferred 1 week after germination to a Percival growth chamber and cold-adapted for at least 3 weeks (day/night temperature 10/10 ± 2°C; RH 80/80 ± 5%; 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ of PAR from cool white fluorescent bulbs; 16-h photoperiod).

Analysis of O_2 Evolution *In Vivo*. Photosynthetic capacity was analyzed in leaf segments using an O_2 electrode (model 53, Yellow Springs Instrument Co.) and a 1.5-ml water-jacketed glass chamber. The basal end of a leaf segment was submerged in 100 μl buffered solution containing 50 mM Tricine-NaOH (pH 7) and 1% (w/v) $NaHCO_3$. Samples were illuminated with a Kodak model 4000 slide projector equipped with a tungsten halogen FHS 300 W bulb. The light intensity in the sample chamber was adjusted to 2,000 $\mu E \cdot m^{-2} \cdot s^{-1}$. Prior to illumination the chamber was flushed with argon to reduce the O_2 concentration to 2% (v/v). O_2 evolution was recorded using an X/Y recorder (Soltec model VP 6414S).

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[1] Abbreviations: F_{max}, maximal fluorescence; F_o, initial fluorescence; F_v, (F_{max}–F_o); F_T, steady state fluorescence at temperature T; LHCII, light-harvesting Chl a/b-binding protein complex of PSII; Q_A, the primary electron accepting plastoquinone of PSII; Q_B, the second electron accepting plastoquinone of PSII; Q_B protein, the 32-kD herbicide binding protein of PSII that binds Q_B.

Isolation of Thylakoid Membranes. Leaf tissue (0.2–0.5 g) was homogenized in a 50-ml Waring Blendor at high speed for 7 s in 20 ml 20 mM Tricine-NaOH (pH 7.8) buffer containing 5 mM MgCl$_2$, 0.4 M sorbitol, and 0.01% (w/v) DTT. The resulting homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 2000g for 5 min. The pellet was resuspended in 20 mM Tricine-NaOH buffer (pH 7.8), containing 5 mM MgCl$_2$, and 0.01% (w/v) DTT, and centrifuged at 2000g for 5 min. The pellet, containing stroma-free thylakoids, was resuspended in the resuspension buffer including 0.1 M sorbitol. Chl concentration was determined by the method of MacKinney (17).

Fluorescence Measurements. The effects of illumination conditions on the state of the pigment bed *in vivo* were analyzed by floating 5-cm long leaf segments on water in a crystalizing dish modified with a temperature-controlled water jacket. A Kodak 4000 projector was used as a light source with 650 and 710 nm bandpass intereference filters. Thylakoids were prepared as described previously, with the addition of 20 mM NaF and 1 μM diuron in the isolation medium and 20 mM NaF in subsequent solutions. Thylakoid samples for low temperature (77 K) fluorescence measurements were resuspended in 20 mM Tricine-NaOH (pH 7.8) buffer containing 5 mM MgCl$_2$, 20 mM NaF, and 40% (v/v) glycerol to 10 μg Chl/ml and frozen in liquid N$_2$ in a transparent Dewar. Emission spectra were taken from 650 to 800 nm using a Perkin-Elmer model 650-40 fluorometer and model R-100 chart recorder and the peak heights at 735 and 685 nm compared.

Steady-state fluorescence *in vivo* was measured at 685 nm using the fluorometer and recorder previously described. Leaf segments were attached to a brass plate equipped with water jacket for temperature control and were placed at 45°C to the excitation beam. After fluorescence reached a steady-state level at 25°C, the temperature was decreased and fluorescence permitted to reach a new steady-state level, and then the temperature was returned to 25°C.

Variable fluorescence was measured from leaf discs incubated for 2 h in a solution of 0.01% (v/v) Tween 80 and 50 μM diuron on ice in the dark. Leaf discs were placed in the sample chamber of the fluorometer (modified for kinetic measurements) and permitted to dark-adapt for 5 min. Samples were illuminated with the excitation beam at 440 nm controlled by a Uniblitz model 100 shutter. The induction transient was recorded on a Nicolet 4040 digital oscilloscope.

Labeling of Thylakoid Proteins. Thylakoid phosphoproteins were labeled *in vivo* by incubation of leaf segments with basal ends placed in water containing ^{32}Pi (100 μCi/leaf). Following 3 h incubation under constant illumination (floodlamp, Sylvania 150 W, 1000 μE·m^{-2}·s^{-1}), the leaves were cut into segments and floated on water in Petri dishes under dim far red light for 30 min to ensure dephosphorylation of LHCII polypeptides. Segments were then subjected to light and temperature regimes as described in the text and thylakoids isolated as previously described.

In vitro labeling of thylakoid proteins was carried out by incubation of isolated thylakoids (0.1 mg Chl/ml) in resuspension medium supplemented with [γ-^{32}P]ATP (15 μCi/ml) and 0.5 mM ATP for 20 min in the light (250 μE·m^{-2}·s^{-1}).

SDS-PAGE and Autoradiography. SDS-PAGE of thylakoid proteins was carried out as previously described (26) using 10 to 17% (w/v) polyacrylamide gradient gels and the buffer system of Laemmli (15). Gels were stained and destained as described (26), dried, and exposed to Kodak XAR-5 x-ray film with one intensifying screen at −80°C.

RESULTS AND DISCUSSION

Cold-sensitive rice varieties undergo chilling photoinhibition at temperatures below 15°C. IR 8, a chilling-sensitive rice culti-

var, exposed to a nonpermissive temperature of 10°C and light (PAR = 500 μE·m^{-2}·s^{-1}) for 16 h, showed a 60% reduction in O$_2$ evolution capacity *in vivo*. Under the same conditions a cold-tolerant barley plant showed no loss of O$_2$ evolution (data not shown).

It is possible that one or more rate limitations are differentially imposed on the overall photosynthetic capacity of rice at chilling temperatures thus setting the stage for chilling photoinhibition. Analysis of the temperature-dependence of O$_2$ evolution capacity was measured in plants grown in a greenhouse. Temperature treatments were carried out as described in "Materials and Methods." Under the experimental conditions used, no photoinhibitory damage was observed; the rate of O$_2$ evolution in leaf material treated at low temperature, then returned to 25°C was comparable to the original control rates for all samples (data not shown). The results with rice and barley are shown in Figure 1. In the range of 25 to 5°C no significant difference in relative O$_2$ evolution capacity was observed between rice and barley. The lack of different short term temperature effects on O$_2$ evolution between the two plants suggests that temperature-dependent limitations on overall photosynthetic capacity at low temperatures do not account for the difference in chilling sensitivity between these plants.

Structural or functional changes which do not result in short term changes in observed O$_2$ evolution at saturating light intensities could directly or indirectly mediate the photoinhibitory process. Any change that results in an alteration in the balance of light energy utilization by PSI and PSII will result in a change in the proportion of the plastoquinone pool that is reduced and hence in the number of closed (Q$_A$ reduced) reaction centers. Factors that can affect this balance include pH gradients within the chloroplast (4, 25) and phosphorylation of PSII proteins (12). Closed reaction centers are highly fluorescent compared to open (Q$_A$ oxidized) centers. Thus, an increase in the proportion of closed centers results in increased steady state fluorescence. The influence of temperature on the regulation of the balance of PSI and PSII activity was investigated by measuring *in vivo* steady state fluorescence before and after temperature shifts (Fig. 2). Steady state fluorescence increased as temperature decreased. The greatest temperature-dependent fluorescence increase was
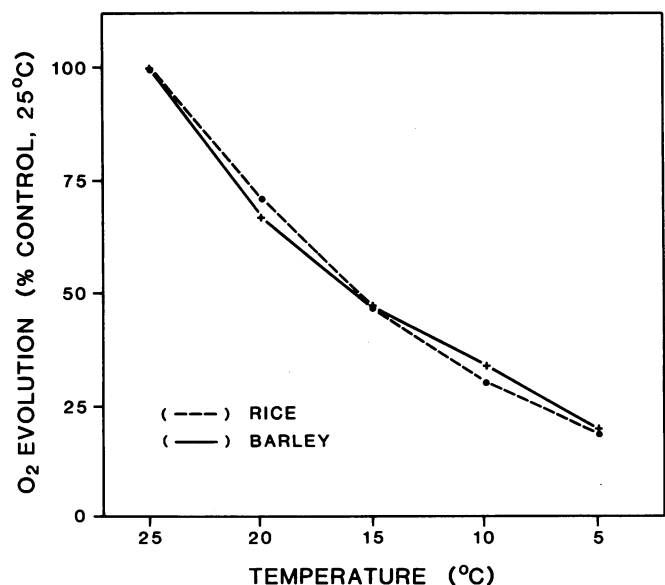

FIG. 1. O$_2$ evolution by leaf segments of rice and barley at various temperatures. Rates of O$_2$ evolution were determined 10 min after initial illumination or shift to a new temperature. The standard deviation for all measurements was less than 5% of the mean.
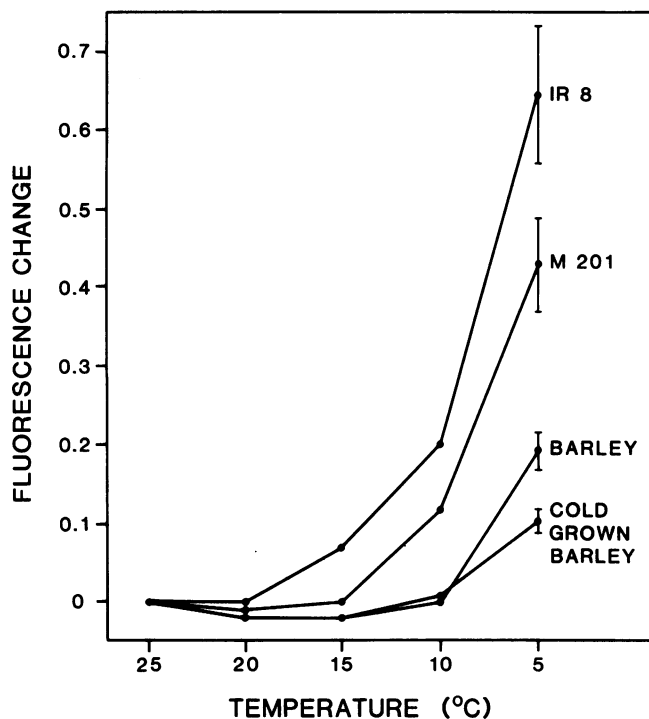
FIG. 2. Steady state *in vivo* fluorescence measured as a function of temperature in rice cultivars IR 8 and M201, barley, and cold-grown barley. Data are presented for individual leaves as $(F_{TC} - F_{25°C})/F_{25°C}$. The standard deviation for all measurements was less than 15% of the mean. Vertical bars represent the SE at 5°C.

shown by rice cv IR 8, followed by rice cv M201, barley, and cold-grown barley.

One diagnostic assay for the light-dependent damage in chilling-sensitive plants is the loss of F_v as a function of time. The rate of decrease in F_v depends on both the light intensity (2, 14) and temperature. At light intensity sufficient to bring about rapid photoinhibitory damage in rice, there is a decrease of F_v even at permissive temperatures. The decrease in F_v due to high light intensity alone is reversed almost completely within 2 h at 25°C in both rice and barley, but in rice the loss of F_v at nonpermissive temperatures is reversed much more slowly (data not shown). Chilling sensitivity was determined by measurement of F_v before and after treatment of attached leaves with 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR at 5°C for 2 h, followed by 2 h recovery in room light at 25°C. Following this treatment all plants showed decreases in F_v: IR 8 (72%), M201 (58%) barley (31%), and cold-grown barley (20%). The relative tendencies of these plants to undergo chilling photoinhibition correlated with the relative degree of change seen in steady state fluorescence (Fig. 2) in the temperature shift experiments.

The effect of chilling temperatures on the phosphorylation of LHCII proteins was investigated by *in vivo* labeling with ³²Pi, followed by SDS-PAGE and autoradiography. The effects of different light treatments at 25 and 5°C are shown in Figure 3A. LHCII phosphorylation occurs at 25°C with 650 nm light, which preferentially excites PSII, but not with 710 nm light, which primarily stimulates PSI. However, there is almost no LHCII phosphorylation at 5°C in IR 8 treated with 650 nm light. For comparison, the same experiment was done with cold grown barley (Fig. 3B). In this case, there is little difference in ³²Pi labeling of LHCII following 650 nm light treatment at 25°C and 5°C.

State I–state II transitions (the alteration of the relative absorption cross section of PSI and PSII, or a change in excitation
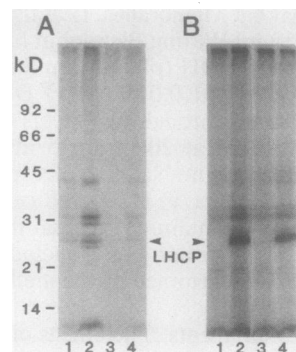

FIG. 3. Protein phosphorylation in rice and barley. After 3 h of ³²Pi uptake, leaves of rice cv IR 8 (A) and cold grown barley (B) were treated in dim far red light for 30 min and then subjected to (a) illumination with 710 nm light at 25°C; (b) 650 nm, 25°C; (c) 710 nm, 5°C; (d) 650 nm; 5°C. Thylakoids were isolated and proteins separated by SDS-PAGE; the gel was autoradiographed as described in "Materials and Methods."

spillover from PSII to PSI (11, 19) occur as a consequence of LHCII protein phosphorylation (27). The relative state of the pigment bed was measured by analyzing 77 K fluorescence emission spectra. At 77 K, there is no electron transport beyond Q_A, so fluorescence can be influenced only by changes in energy coupling within the photosystems. The ratio of the peak heights at 735 nm, due largely to PSI, and at 685 nm, due to PSII, indicates the relative state of the pigment beds. In parallel with the ³²Pi labeling experiments, 77 K fluorescence emission spectra were analyzed in thylakoids prepared following the various light and temperature treatments. The results are shown in Table I. For both rice (IR 8) and barley, illumination with 650 nm light at 25°C results in 735/685 nm ratios higher than those for treatments with 710 nm light. This held true for cold-grown barley treated at 5°C. However for rice treated at 5°C, the 735/685 nm ratio from the 650 nm treated leaves was essentially the same as for the 710 nm treatment. Thus the state I–state II transition data obtained by 77 K fluorescence analysis agree with those from the ³²Pi experiments on LHCII phosphorylation; both show inhibition by 5°C in rice cv IR 8 but not in cold-grown barley.

It is possible that a number of *in vivo* conditions such as ATP concentration, Mg^{2+} concentration, or stromal pH are changed by low temperature. Changes in these factors could in turn affect kinase activation. Thylakoid protein phosphorylation with [γ-³²P]ATP was carried out at several temperatures to determine if temperature influences thylakoid protein phosphorylation under *in vitro* conditions of constant ATP supply, pH, and Mg^{2+} concentration. Figure 4 shows an SDS gel autoradiograph of thylakoid proteins labeled *in vitro* in rice (cv IR 8) and cold-grown barley. There is considerably less LHCII phosphorylation in rice incubated at 0, 5, and 10°C than at 20°C. However, in barley there is almost no effect of temperature. The inhibition of LHCII phosphorylation at 5°C in rice can be attributed at least in part to a temperature effect on the action of the protein kinase(s).

The experiments described in this paper show that in rice chilling temperatures result in inhibition of thylakoid protein phosphorylation. Since this is true *in vitro* as well as *in vivo*, this inhibition must be due at least in part to an effect on the activity of the LHCII protein kinase. This could be the result of lowered kinase activity, or it could be that the substrate, shown to be threonine residues on a surface exposed peptide segment of the LHCII proteins (6, 18), is not accessible to the kinase at chilling temperatures. Further experiments will be required to distinguish between these possibilities.

Our observations lead to an explanation of chilling sensitivity

Table I. *Effect of Chilling Temperatures in Vivo on 77 K in Vitro Fluorescence Emission Spectra*

Leaves of rice (IR 8) and cold grown barley (CGB) were pretreated with 650 nm (state II) or 710 nm (state I) light for 20 min at 5 or 25°C. Chloroplast thylakoid membranes were isolated and 77 K fluorescence emission spectra analyzed as described in "Materials and Methods." The ratios of peak fluorescence emission at 735 nm to that at 685 nm are listed, and the numerical difference between fluorescence ratios from 5 and 25° treatments are shown.

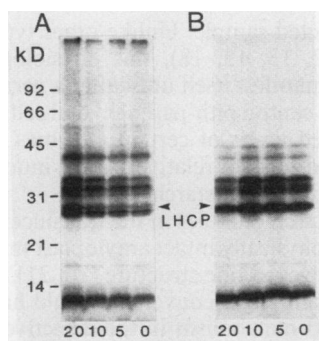| Conditions		IR 8 Ratio F_{735}/F_{685}	Δ	CBG Ratio F_{735}/F_{685}	Δ
°C	λ(nm)				
5	650	0.73		1.44	
	710	0.71	0.02	1.05	0.39
25	650	0.90		1.73	
	710	0.70	0.20	1.26	0.47

Fig. 4. *In vitro* protein phosphorylation in thylakoids. Thylakoids isolated from (A) rice cv IR 8 and (B) cold grown barley were resuspended in buffer and incubated with [γ-^{32}P]ATP in the light for 20 min at 20, 10, 5, and 0°C. Reactions were stopped by addition of 20 mM NaF and 1 M diuron. Thylakoid proteins were separated by SDS-PAGE and autoradiographed. Equal amounts of Chl were loaded in each lane.

in rice, although other influences on the regulation of electron transport, such as pH gradient formation or Mg^{2+} ion movement, may also contribute to this sensitivity. If LHCII phosphorylation is inhibited by chilling temperatures, the required regulation of excitation energy distribution between PSI and PSII will not occur. There will be capacity for electron transport through PSII in excess of the rate of electron transport, and consequently there will be increased reduction of the plastoquinone pool; the resulting unavailability of electron carriers on the reducing side of PSII may result in an increase in the rate of light-induced damage to PSII via effects on the 32 kD Q_B protein as suggested by Kyle et al. (14) or damage to the reaction center itself (10, 24). The precise nature of the damage sight(s) in cold-sensitive rice is not understood. It is clear that the kinetics of recovery from photoinhibition in cold-stressed rice plants is long compared to plants inhibited at high light (10, 24), suggesting either a different site or multiple sites for photoinhibitory damage. Alternatively chilling temperatures may have direct effects on either protein turnover or protein synthesis. These aspects of chilling sensitivity in rice are currently being investigated.

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