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Recovery from Low-Temperature Photoinhibition is Related to Dephosphorylation of Phosphorylated CP29 Rather Than Zeaxanthin Epoxidation in Rice Leaves

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During recovery from chilling-induced photoinhibition in rice leaves, we compared the reactivation kinetics of PSII photochemical efficiency (Fv/Fm) with that of zeaxanthin (Z) epoxidation and the dephosphorylation of CP34 (i.e., the phosphorylated form of CP29). The latter two processes were kinetically similar to the slow increase in Fv/Fm measured in our control leaves. However, the rate of Z epoxidation was significantly retarded by an epoxidase inhibitor, 5 mM salicylaldoxime (SA), without any significant changes in the processes of PSII reactivation and CP34 dephosphorylation. When chilled leaves were incubated at 10°C in the dark, both reactivation and dephosphorylation were significantly blocked, but Z epoxidation was not. Finally, we observed that the kinetics of CP34 dephosphorylation matched very well with those of PSII recovery in two rice cultivars with different chilling sensitivities. These results suggest that PSII reactivation from low-temperature photoinhibition is more closely related to CP34 dephosphorylation than to Z epoxidation.

Keywords: CP29, dephosphorylation, low-temperature photoinhibition, PSII, rice

When rice leaves are chilled for 6 h at 4°C under a moderate PPFD, they experience severe photoinhibition, which is manifested in a sustained decrease in the photochemical efficiency of PSII (Kim et al., 1997). However, this decline is nearly fully reversible when plants are placed in the dark at room temperature. Recovery of PSII efficiency is independent of chloroplast protein synthesis but is sensitive to chloroplast phosphatase inhibitors. Therefore, we have suggested that this reversible decrease in efficiency represents a means for effective down-regulation of the photosynthetic apparatus through a reversible phosphorylation of some thylakoid proteins.

More than 10 thylakoid proteins are known to be phosphorylated in a light-dependent manner; most are associated with PSII and its light-harvesting antenna (Bennett, 1991). The reversible phosphorylation of major light-harvesting complexes in PSII (LHCII) serves as a mechanism for regulating the distribution of excitation energy between the two photosystems (Bennett, 1991), which then protects the photosynthetic apparatus from photoinhibitory damage (Horton and Lee, 1985). Phosphorylation of the PSII core proteins has been implicated in controlling the degradation of photodamaged D1 protein during its rapid turnover (Aro et al., 1992; Ebbert and Godde, 1994). More recently, Bergantino et al. (1995, 1998) have reported that an inner antenna complex of PSII, CP29, is phosphorylated under severe photoinhibitory conditions, such as a combination of chilling stress and light. Enhanced resistance to chilling-induced photoinhibition has been correlated with the higher capacity to phosphorylate this pigment-protein complex in maize (Bergantino et al., 1995; Mauro et al., 1997) as well as in rice, another chilling-sensitive plant (Kim et al., 1997). Bergantino et al. (1995) and Mauro et al. (1997) have also proposed that the reversible phosphorylation of CP29 represents a novel mechanism for protecting the photosynthetic apparatus from the potentially damaging effect of excess light.

Non-radiative dissipation of excitation energy, reflected by the non-photochemical quenching (NPQ) of chlorophyll fluorescence, is essential for photoprotection (Demmig-Adams and Adams, 1996; Horton et al., 1996; Gilmore, 1997). This process involves the xanthophyll cycle, while also having an obligatory requirement for a trans-thylakoid proton gradient (Horton et al.,

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Abbreviations: PPFD, photosynthetic photon flux density; PSII, Photosystem II; Z, zeaxanthin; SA, salicylaldoxime.

1996). The de-epoxidized xanthophyll cycle pigments are thought to act either as a direct quencher of excited chlorophyll pigments (Demmig-Adams and Adams, 1996; Gilmore, 1997) or as an amplifier of NPQ (Horton et al., 1996). Evidence also exists for the enrichment of xanthophyll cycle pigments in the minor antenna pigment-protein complexes of PSII, including CP29 (Bassi et al., 1993; Crimi et al., 2001). These proteins may exclusively contain quenching sites and, therefore, play a crucial role in xanthophyll cycle-associated NPQ (Crofts and Yerkes, 1994; Gilmore, 1997).

This current study is a continuation of our previous research on the possible involvement of reversible phosphorylation of thylakoid proteins in the recovery of PSII efficiency from chilling-induced photoinhibition in chilling-sensitive rice plants. Here, we monitored the rate of dephosphorylation of the phosphorylated form of CP29 (CP34) in relation to the kinetics of increased PSII photochemical efficiency during recovery. Because the recovery of PSII efficiency from photoinhibition is reportedly correlated with the epoxidation of zeaxanthin (Thiele et al., 1996, 1998; Verhoeven et al., 1996, 1998; Demmig-Adams et al., 1998), we also compared the kinetics of epoxidation with that of PSII recovery.

MATERIALS AND METHODS

Plant Materials

Two cultivars of rice (*Oryza sativa* L.) -- Dongjin-byeo and IR841 -- were reared in a growth chamber, where the PPFD at leaf level was 100 μmol m^{-2} s^{-1}. A 14-h photoperiod was used, and air temperatures ranged from 28°C (daytime) to 25°C (dark). For all experiments, fully expanded leaves were collected from three-week-old plants.

Photoinhibition and Recovery

Leaf segments (3 cm long) were floated with adaxial sides up on water at 4°C in Petri dishes, and were exposed to a PPFD of 500 μmol m^{-2} s^{-1} for 3 h. For the recovery period following photoinhibition, the leaves were incubated in the water in darkness at either 10°C or 25°C.

Inhibitor Treatments

To inhibit Z epoxidation, leaves were vacuum-infiltrated with 5 mM salicylaldoxime (SA), an inhibitor of epoxidase (Pfündel and Bilger, 1994), beginning at the end of the photoinhibition treatment and continuing in the same solution for the entire recovery period. Control leaves were treated with water only.

Measurement of Chlorophyll Fluorescence

The extent of photoinhibition and subsequent recovery was monitored by measuring fluorescence induction with a fluorometer (Plant Efficiency Analyzer, Hansatech Instrument, Norfolk, UK) as described by Kim et al. (1997). Leaf segments were dark-adapted for 5 min in the leaf clips of this portable instrument prior to recording the fluorescence. Maximum variable fluorescence (Fv) was obtained by subtracting the initial fluorescence (Fo) from the maximum fluorescence (Fm). The ratio of Fv/Fm was then used to quantify the maximum photochemical efficiency of PSII.

Pigment Analysis

Xanthophyll cycle components were determined according to the methods of Thayer and Björkman (1990), with some modifications. Leaves were frozen in liquid nitrogen and ground with a mortar and pestle in ice-cold 100% acetone. The pigment extracts were then passed through a 0.45-μm membrane filter. Pigment separation was performed in an HPLC system (HP 1100 series; Hewlett Packard, Waldbronn, Germany) on a Zorbax ODS-1 column protected by a guard column. A solvent mixture of acetonitrile:methanol (85:15, v/v) was used for 6 min, followed by a 10-min linear gradient to methanol:ethyl acetate (66:34, v/v). All pigments were recovered from the column within about 30 min, at a flow rate of 2 mL/min. The eluted pigments were monitored at 440 nm. Pigment concentrations were estimated by using factors for converting peak area to nanomoles, as determined for this solvent mixture by Thayer and Björkman (1990).

Electrophoresis and Immunoblotting

Thylakoid membranes were prepared from rice leaves as described by Aro et al. (1993), with some modification. For immunoblotting, the thylakoid was solubilized with gel-loading buffer, 50 mM Tris (pH 6.8), 100 mM dithiotheitol, 2% sodium dodecyl sulfate, 0.1 % bromophenol blue, and 10% glycerol. The thylakoid polypeptides were then separated by 12% SDS-PAGE according to Laemmli (1970), with 5 μg Chl loaded into each well. After the polypeptides were transferred to a Hybond ECL membrane (Amersham Phamarcia

Biotech, UK), antibody against CP29 was used to identify that protein and its phosphorylated form, CP34 (Bergantino et al., 1995). The bound antibody was detected with an Enhanced ChemiLuminescence kit (Amersham Phamarcia Biotech, UK), and the immunoblot bound with the antibody was exposed to X-ray film (Fuji, Japan). To quantify CP29 and CP34, the film was scanned with a laser densitometer (Molecular Dynamics, USA).

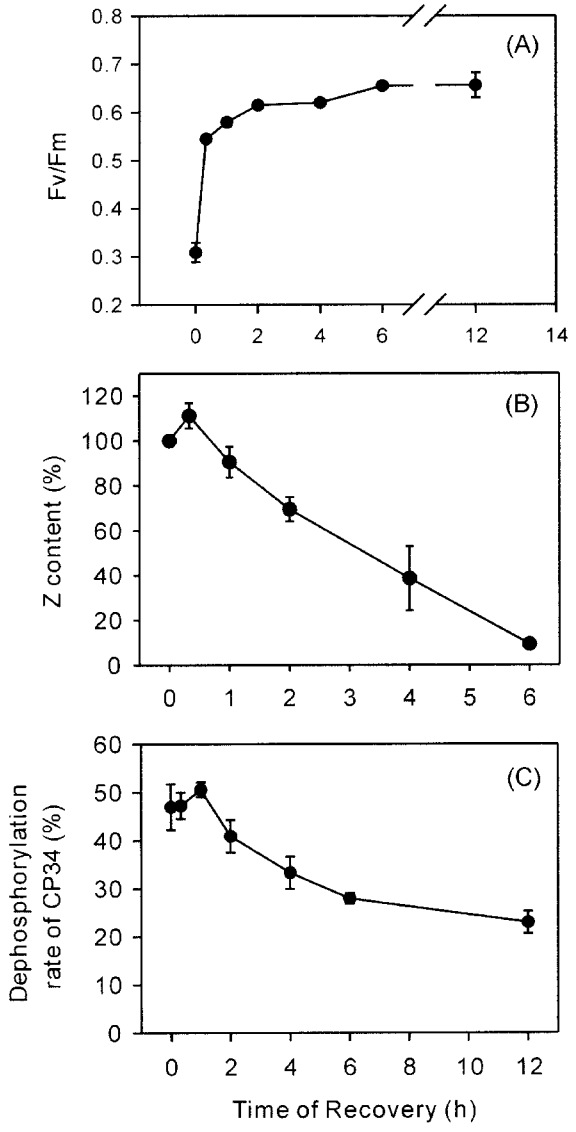

Figure 1. Changes in PSII photochemical efficiency (Fv/Fm) (A), content of zeaxanthin (B), and dephosphorylation rate of CP34 (C) in leaves of rice cv. Dongjin-byeo during recovery in the dark at 25°C after chilling in the light for 3 h. Fv/Fm at time 0 was measured at 4°C after dark-adaptation for 5 min. Error bars represent standard errors (n = 3 - 5).

RESULTS

Reactivation of PSII, Z Epoxidation, and CP34 Dephosphorylation at 25°C

During dark-incubation at 25°C after light-chilling, the kinetics of the increase in PSII photochemical efficiency (Fv/Fm) consisted of two phases. An initial fast-recovery phase of about 20 min was followed by a

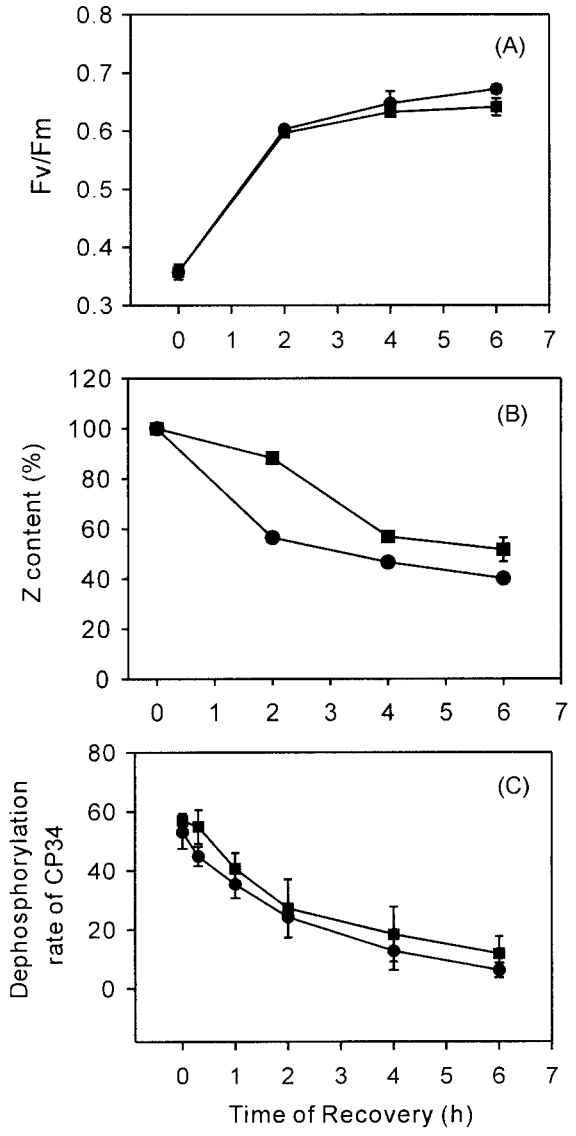

Figure 2. Changes in PSII photochemical efficiency (Fv/Fm) (A), content of zeaxanthin (B), and dephosphorylation rate of CP34 (C) in control (●) and SA-treated (■) leaves of rice cv. Dongjin-byeo during recovery in the dark at 25°C after chilling in the light for 3 h. Fv/Fm at time 0 was measured at 4°C after dark-adaptation for 5 min. Error bars represent standard errors (n = 3).

slow phase of several hours, leading to almost complete PSII reactivation in leaves (Kim et al., 1997). We have previously shown that the first phase is due to the relaxation of the proton gradient across the thylakoid membranes. The second phase, however, is independent of that proton gradient (Xu et al., 1999) and is insensitive to lincomycin, a chloroplast synthesis inhibitor (Kim et al., 1997).

To investigate the mechanistic basis for the second slow phase, we monitored the changes in PSII efficiency and the levels of zeaxanthin and CP34 during recovery at 25°C (i.e., room temperature, or RT) in the dark. Chilling in the light for 3 h resulted in severely reduced Fv/Fm, heavy phosphorylation of CP29, and substantial accumulation of Z (Fig. 1). During the subsequent 12-h recovery period, the increase in Fv/Fm mirrored the process of Z epoxidation as well as the decrease in the CP34 level.

Effects of SA on Reactivation of PSII, Z Epoxidation, and CP34 Dephosphorylation

To investigate the role of Z during dark recovery, leaves were vacuum-infiltrated with an inhibitor of epoxidase (SA) just after termination of the photoinhibitory treatment. As expected, the infiltration markedly inhibited epoxidation of Z (Fig. 2). Interestingly, SA did not affect either the reactivation kinetics of PSII or the kinetics of CP34 dephosphorylation.

Reactivation of PSII, Z Epoxidation, and CP34 Dephosphorylation at 10°C

When chilled leaves were dark-incubated at 10°C, PSII efficiency increased slowly and reached a plateau after 2 h (Fig. 3). Epoxidation of Z was also considerably slowed at 10°C, but the content of zeaxnathin continued to decrease even after PSII efficiency reached its maximum. Notably, the dephosphorylation of CP34 was significantly inhibited during recovery at 10°C.

Reactivation of PSII and CP34 Dephosphorylation in Two Cultivars with Different Chilling Sensitivities

The possible involvement of CP34 dephosphorylation in the recovery of PSII efficiency was further investigated by comparing two rice cultivars with different chilling sensitivities. We previously demonstrated that the Japonica rice cultivar, Dongjin-byeo, is more resistant than the Indica, IR841, to chilling stress, mainly because of its capacity for quicker recovery (Kim et al., 1997). This observation was confirmed in the present study

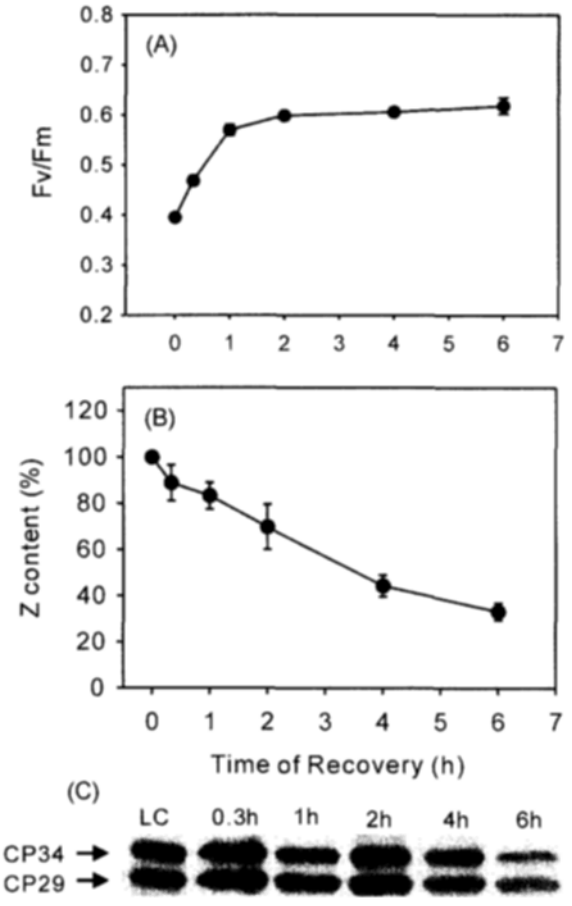

Figure 3. Changes in PSII photochemical efficiency (Fv/Fm) (A), content of zeaxanthin (B), and dephosphorylation of CP34 (C) in leaves of rice cv. Dongjin-byeo during recovery in the dark at 10°C after chilling in the light for 3 h. Fv/Fm at time 0 was measured at 4°C after dark-adaptation for 5 min, and others during dark-recovery were measured at 10°C. Error bars represent standard errors (n = 3). LC, light chilling.

(Fig. 4), in which, remarkably, Dongjin-byeo also exhibited a faster rate of CP34 dephosphorylation. Half times for dephosphorylation were about 3 h and 6 h in Dongjin-byeo and IR841, respectively. Likewise, fitting the sum of two exponentials to the reactivation curves (Fig. 4A) revealed that the half times of the second slow components were 2.0 h and 5.4 h in Dongjin-byeo and IR841, respectively.

DISCUSSION

Photoinhibition occurs in at least two phases (Krause, 1994). The first is thought to be associated with the down-regulation of photosynthesis through an increase in non-radiative energy dissipation within the photo-

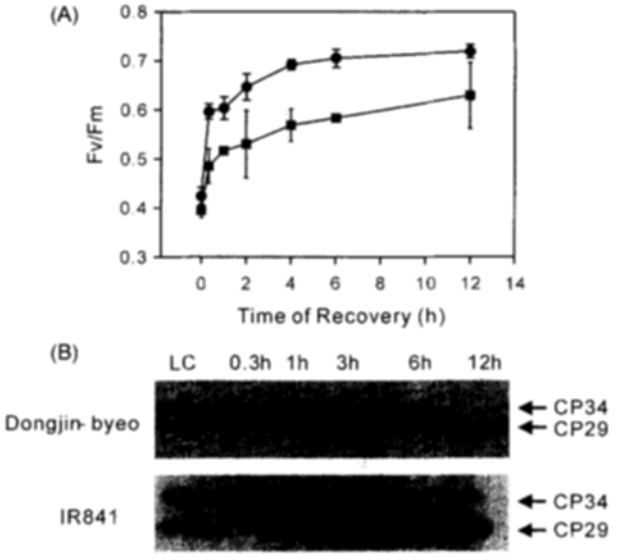

Figure 4. Changes in PSII photochemical efficiency (Fv/Fm) (A) and dephosphorylation of CP34 (B) in leaves of rice cv. Dongjin-byeo (●) and IR841 (■) during recovery in the dark at 25°C after chilling in the light for 3 h. Fv/Fm at time 0 was measured at 4°C after dark-adaptation for 5 min. Error bars represent standard errors (n = 3). LC, light chilling.

synthetic apparatus. In contrast, the second stage is characterized by its dependence on de novo protein synthesis. Our focus in the current study was the mechanistic basis for recovery of PSII efficiency from the first, protein synthesis-independent, stage of photoinhibition in chilling-sensitive rice leaves. Therefore, leaf samples were only exposed to chilling in moderate light for 3 h. The photoinhibition that resulted was mostly reversible in the dark at RT (Fig. 1A). As expected, recovery of PSII efficiency from this reversible photoinhibition was independent of chloroplast protein synthesis, as we had demonstrated previously (Kim et al., 1997).

It has been suggested that recovery from the first stage of photoinhibition is related either to direct reactivation via a reversible conformational change in the D1 protein (Hurry and Huner, 1992), or to the relaxation of energy-dependent fluorescence quenching (Fryer et al., 1995). The recovery of PSII efficiency has been attributed to the epoxidation of zeaxanthin via the xanthophyll cycle, which assumes that the de-epoxidized xanthophyll cycle pigments still act as fluorescence quenchers competing with both fluorescence emission and photochemistry for excitation energy during recovery at RT (Demmig-Adams and Adams, 1996; Gilmore, 1997). Indeed, that recovery process is kinetically very similar to the epoxidation of zeaxanthin (Thiele et al., 1996, 1998; Verhoeven et al., 1996, 1998), as was also shown in the present study (Fig. 1B). However, the presence of those pigments alone is not sufficient to induce fluorescence quenching (Bilger and Björkman, 1991, 1994; Thiele and Krause, 1994; Tardy and Havaux, 1996; Hurry et al., 1997), and the trans-thylakoid proton gradient is an obligatory requirement for xanthophyll cycle-associated quenching to occur (Noctor et al., 1991; Goss et al., 1995; Horton et al., 1996). In fact, the proton gradients that build up during photoinhibitory treatments seem to relax within minutes of either treatment termination or the transfer of samples to RT (Ruban and Horton, 1995; Gilmore, 1997; Verhoeven et al., 1998). Complete relaxation of the gradient after the chilled rice leaves are returned to RT takes less than 20 min (Xu et al., 1999). Therefore, because the sustained fluorescence quenching is totally proton gradient-independent, it is rather unlikely to be associated with the accumulation of zeaxanthin. Under field conditions, photoprotection is not strictly dependent on levels of antheraxanthin and zeaxanthin (Sun et al., 2001). This speculation is strongly supported by the fact that the epoxidase inhibitor blocked Z epoxidation (Fig. 2B) while exerting little effect on PSII recovery (Fig. 2A).

In contrast, the parallel changes in PSII efficiency and CP34 dephosphorylation (Fig. 1, A and C) point to the possible involvement of reversible phosphorylation of this minor chlorophyll a/b protein in the down-regulation of efficiency in our chilled rice leaves. Phosphorylation of CP29 can induce a long-range conformational change that modulates the spectral properties of this PSII subunit (Croce et al., 1996). Recently, the chilling-induced CP34 has been characterized by an internal redistribution of the various chlorophyll spectral forms, in which the excitation energy tends toward the antenna, thus protecting PSII from photodamage in maize chilled in the light (Mauro et al., 1997). CP29 phosphorylation presumably isolates a major portion (30%) of PSII antenna, which, being connected to the PSII core via CP29, results in diminished energy transfer to the core (Bergantino et al., 1995). As pointed in Crimi et al. (2001), the source of quenching is the conformational changes in the CP29 protein structure, rather than the direct chlorophyll to carotenoid energy transfer. Moreover, Dongjin-byeo also exhibited a faster rate of CP34 dephosphorylation than IR841 (Fig. 4B). Kim et al. (1997) have reported that the chilling of rice leaves in moderate light causes Fo to increase. Therefore, based on the properties stated for this minor complex, as well as its topological location between the major LHCII complex and PSII core (Jansson, 1994), we might speculate that the recovery of PSII efficiency

from chilling-induced down-regulation is related to the dephosphorylation of CP34 in rice leaves.

We also hypothesize that CP34 dephosphorylation, rather than Z epoxidation, is related to the recovery of PSII efficiency in rice leaves. This is based on the following results: 1) the considerably slow but continuously declining Z content at 10°C after PSII efficiency reached its maximum (Fig. 3B); and 2) rather complete inhibition of CP34 dephosphorylation during recovery at 10°C (Fig. 3C).

Low light has been shown to inhibit both the recovery of PSII after light-chilling (Kim et al., 1997) and the dephosphorylation of CP34 (Bergantino et al., 1995). Park et al. (1995) have explained this phenomenon in terms of the low-light requirement for D1 synthesis. However, in our study, D1 presumably was not damaged because full recovery of efficiency was achieved in the dark (Fig. 1A), and was insensitive to the chloroplast synthesis inhibitor (Kim et al., 1997). The mechanistic basis for the effect of light level on this recovery is still unclear. Chow (1994) has suggested that the low-light requirement is due to an optimal stromal pH for the synthesis of proteins, including D1, which is already achieved in low light. From a study of pumpkin leaves subjected to photoinhibition at a chilling temperature, Salonen et al. (1998) have reported that D1 kinase is maximally active even at low PSII excitation pressures (i.e., measured as 1-qP (photochemical quenching)), and that D1 dephosphorylation is inhibited in low-light conditions during recovery due to light-activation of D1 kinase. Because D1 dephosphorylation is a prerequisite for the degradation and de novo synthesis of this protein (Aro et al., 1992; Ebbert and Godde, 1994; Rintamäki et al., 1996, 1997), the low-light requirement for optimal recovery of PSII function may reflect the need for low light for D1dephosphorylation as well. Dephosphorylation of CP34 also seemed to be inhibited by low light, possibly because of light-activation with CP34 kinase. Similar to D1 kinase, CP34 kinase is controlled by the redox state of the plastoquinone pool (Bergantino et al., 1995). During recovery after chilling in low light, kinases in the thylakoid might be activated by that light level. Therefore, even low light during recovery may result in an excitation pressure high enough to activate kinases for both D1 and CP29 phosphorylations, thereby inhibiting PSII recovery by blocking D1 turnover or CP34 dephosphorylation.

In conclusion, we have shown here that recovery of PSII efficiency from chilling-induced photoinhibition is kinetically correlated with CP34 dephosphorylation in rice leaves. Ruban and Horton (1995) have suggested that sustained, protein synthesis-independent photoinhibition may result when protonation modifies the LHCII organization. However, it is unclear whether such a protonation-induced conformational change can be prolonged for extended periods after the complete relaxation of the proton gradient. Moreover, the reversible stage of photoinhibition may be caused by a conformational change in the D1 protein (Hurry and Huner, 1992). Further investigations are necessary to discriminate between these possibilities.

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