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# Cold-Resistant and Cold-Sensitive Maize Lines Differ in the Phosphorylation of the Photosystem II Subunit, CP29[1]

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**The effects of low temperature on the relative contributions of the reaction center and the antenna activities to photosystem II (PSII) electron transport were estimated by chlorophyll fluorescence. The inhibition of PSII photochemistry resulted from photodamage to the reaction center and/or a reduced probability of excitation energy trapping by the reaction center. Although chill treatment did not modify the proportion of the dimeric to monomeric PSII, it destabilized its main light-harvesting complex. Full protection of the reaction center was achieved only in the presence of the phosphorylated PSII subunit, CP29. In a nonphosphorylating genotype the chill treatment led to photoinhibitory damage. The phosphorylation of CP29 modified neither its binding to the PSII core nor its pigment content. Phosphorylated CP29 was isolated by flat-bed isoelectric focusing. Its spectral characteristics indicated a depletion of the chlorophyll spectral forms with the highest excitation transfer efficiency to the reaction center. It is suggested that phosphorylated CP29 performs its regulatory function by an yet undescribed mechanism based on a shift of the equilibrium for the excitation energy toward the antenna.**

The photoconversion yields of both PSI and PSII are under the control of a regulatory network that tends to modulate their photoelectrochemical activity so as to match the rates of ATP and NADPH synthesis with the energetic requirements of the various energy-consuming pathways. The role of this regulatory network extends to the protection of the thylakoid membranes against photoinhibitory damage by preventing overreduction of the reaction centers even under light-saturating conditions (Genty et al., 1989; Harbinson et al., 1989; Foyer et al., 1990; Seaton and Walker, 1990).

Both photosystems are composed of a light-harvesting system that absorbs and transfers the excitation energy to a reaction center that catalyzes electron transfer. In contrast to PSI, in which the yield of electron transport is directly related to the degree of reduction of the P700 pool (Harbinson et al., 1989), PSII electron transport rates are essentially optimized by altering PSII light-harvesting properties. Indeed, the structure, composition, and function of the PSII antenna are very responsive to altered ATP/NADPH ratios resulting from changes in irradiance, light spectral composition, and C and N assimilation (Allen, 1992).

Under light-limiting conditions the $\Phi_{PSII}$ yield is kept constant at its maximum value. Adjustment of PSII electron transport rates is achieved by altering the PSII absorption cross-section. In contrast, with increasing light intensities, PSII is progressively geared to energy dissipation. Its photoconversion yield is lowered as a result of the production of additional excitation energy drains within the antenna system (Genty et al., 1989). It has been suggested that the thermal dissipation of excess excitation energy is associated with the synthesis of zeaxanthin (Demmig-Adams, 1990) under conditions of a high transmembrane pH gradient (Gilmore and Yamamoto, 1992) and is possibly coupled to changes in the aggregation state of the LHCII (Horton et al., 1994).

Several PSII proteins became phosphorylated upon illumination (Allen, 1992). Whereas the contribution of the phosphorylated LHCII to the optimization of the excitation energy distribution at low light intensities has been clearly demonstrated, the role of the phosphorylation of the inner antenna and reaction center polypeptides has not yet been clearly established. Recently, the CP29 complex has been shown to be phosphorylated under conditions of reduced photosynthetic capacity (Bergantino et al., 1995).

In this paper we present data indicating that the phosphorylation of CP29 represents a new regulatory mechanism that significantly contributes to the protection of PSII reaction centers against low-temperature-induced photoinhibition.

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Abbreviations: Deriphat, *N*-lauryl β-iminodipropionate; $F'_{max}$, maximum fluorescence produced by a saturating light pulse; $F'_o$, fluorescence level corresponding to the PSII electron acceptors being oxidized; $F_s$, steady-state fluorescence; LHCII, the major light-harvesting complex of PSII; $\Phi_{exc}$, efficiency of excitation energy capture; $\Phi_{PSII}$, PSII photoconversion yield; $q_p$, photochemical quenching.

## MATERIALS AND METHODS

Maize (*Zea mays* L.) lines 1873 and 1194, identified hereafter as R (cold-resistant) and S (cold-sensitive), respectively, were supplied by the Coop de Pau (Pau, France). The seedlings were grown in a controlled environment cabinet with a 16-h photoperiod (day/night temperatures, 25/17°C) until the third leaf was fully developed. Seedlings were illuminated with daylight fluorescent tubes to provide 250 $\mu$mol photons $m^{-2}$ $s^{-1}$ PAR at the plant level. For the chill treatment the seedlings were kept in the dark at 3°C for 3 h and then illuminated (250 $\mu$mol photons $m^{-2}$ $s^{-1}$ PAR) at 3°C for 6 h. The same treatment was given to control seedlings except that the temperature was kept at 25°C.

### Thylakoid Membrane Isolation and Fractionation

Leaves from seedlings exposed to 25 and 3°C were harvested at the end of the 6-h illumination period and ground without dark incubation. Mesophyll thylakoids were isolated as described by Bassi and Dainese (1992), with 10 mM NaF present in the various isolation buffers to prevent the dephosphorylation of the thylakoid membrane proteins, including the recently identified phosphorylated form of CP 29, which was demonstrated to have a half-time of approximately 2 h at 5°C in the absence of NaF (Covello et al., 1988). The thylakoids were immediately frozen in liquid N or subjected to further fractionation to yield grana membranes (Dunahay et al., 1984) or PSII core particles (Ghanotakis et al., 1987). The grana margins were isolated as described by Kettunen et al. (1991). The preparations were finally suspended in 0.33 M sorbitol, 10 mM Hepes, and 5 mM $MgCl_2$, pH 7.6, and stored at −80°C after freezing in liquid N.

### Biochemical Analysis

Chlorophyll determinations were carried out according to the method of Porra et al. (1989). SDS/6 M urea-PAGE, Coomassie blue, and silver-staining were carried out as described by Bassi and Dainese (1992). Proteins were transferred to nitrocellulose filters and immunodecorated using a polyclonal antibody raised against the CP29 apoprotein (Bassi and Dainese, 1992). For nondenaturing electrophoresis the method of Peter and Thornber (1991) as modified by Santini et al. (1994) was used. Nondenaturing IEF was carried out as described by Dainese et al. (1990).

### Spectroscopy

Chlorophyll fluorescence from the upper surface of attached leaves was measured with a fluorimeter (PAM 101, Walz, Effeltrich, Germany). The fluorescence signals were recorded at room temperature and quantitatively analyzed according to the method of Genty et al. (1989) and Havaux et al. (1991) using the nomenclature of Van Kooten and Snel (1990). Absorption and fluorescence spectra were obtained from the various sample preparations suspended in 0.06% dodecyl maltoside and 10 mM Hepes, pH 7.5. HPLC analysis of pigments was performed as previously described (Bassi et al., 1993).

## RESULTS

### Down-Regulation of PSII in Chilled Maize Leaves

To clarify the role of down-regulation of PSII activity in the protection of PSII reaction centers from photodamage, we have compared physiological and biochemical responses of R and S maize lines to short exposures of low temperature and light (Mauro et al., 1990). During the chill treatment care was taken to expose the seedlings to the same light intensity as given during the growth period.

The operation of the different mechanisms responsible for the optimization of the PSII electron transport was measured in planta using chlorophyll fluorescence (Seaton and Walker, 1990). For each light intensity, we measured the relative contribution of $q_p$ and $\Phi_{exc}$ to the $\Phi_{PSII}$. The measurements were performed at room temperature.

These results are shown in Figure 1. For the control seedlings of both R and S the down-regulation of PSII photochemistry was clearly demonstrated (Fig. 1A). At the highest light intensity used during our measurements (1200 $\mu$mol photons $m^{-2}$ $s^{-1}$), a significant portion of the reaction center was maintained in an oxidized state (Fig. 1B). This has been demonstrated (Genty et al., 1989) to result from a reduced $\Phi_{exc}$ as a consequence of an increase in the excitation energy dissipation within the PSII antenna system (Fig. 1C). Under control conditions R and S did not display any significant difference in their photochemistry.

As expected from previous results (Öquist et al., 1987; Kirilovsky et al., 1990; Mauro et al., 1990), a reduction of the $\Phi_{PSII}$ was observed for both R and S chilled seedlings (Fig. 1A). The extent of the cold-induced decrease in $\Phi_{PSII}$ was light-intensity dependent. The maximal reduction was observed at about 250 $\mu$mol photons $m^{-2}$ $s^{-1}$. The analysis of the PSII efficiency-light intensity curves obtained from the R and S chilled seedlings revealed a dual origin for the cold-induced decrease of PSII photochemistry. In R the PSII reaction center activity as measured by $q_p$ was essentially unaffected by the chill treatment (Fig. 1B). Reduced trapping efficiency alone accounted for the inhibition of PSII activity (Fig. 1C). In contrast, a significant inhibition of the reaction center activity was measured for S (Fig. 1B). $q_p$ for S was severely reduced and remained essentially constant with increasing light intensities (Fig. 1B). Compared with R, the $\Phi_{exc}$ of S was less affected by the chill treatment (Fig. 1C).

To further confirm the origin of the chill-induced reduction in $\Phi_{PSII}$, we measured the relative apparent rate constants for photochemical and nonphotochemical processes (fluorescence, heat emission, and energy transfer to PSI). The rate parameters are, respectively, the fluorescence ratios $(1/F'_o - 1/F'_{max})$ and $1/F'_{max}$ (Havaux et al., 1991). A direct comparison between control and chilled R and S was possible because we did not observe any difference in the chlorophyll content or in the chlorophyll *a/b* ratios. The data shown in Figure 1, D and E, confirm that there was no difference in the energy dissipation processes in PSII for

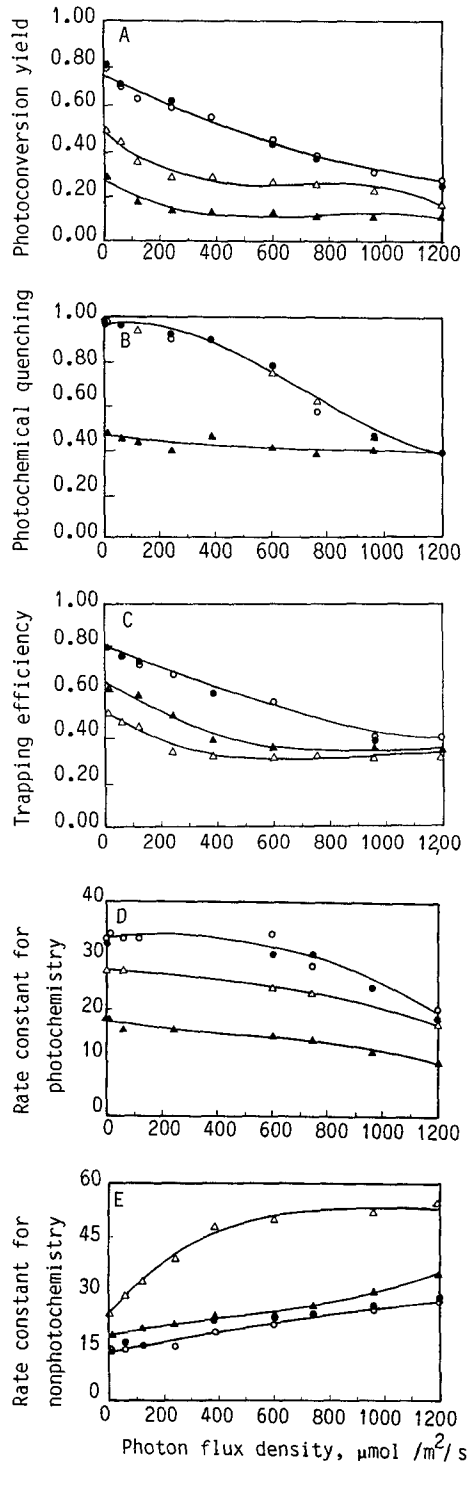


**Figure 1.** Down-regulation of PSII efficiency with light intensity. For each light intensity $F_s$, $F'_{max}$, and $F'_o$ were measured; $\Phi_{PII}$ is given by the ratio$(F'_{max} - F_s)/F'_{max}$; $q_p$ is given by $(F'_{max} - F_s)/(F'_{max} - F'_o)$; and $\Phi_{exc}$ is the ratio $(F'_{max} - F'_o)/F'_{max}$. The relative apparent rate constants for $q_p$ and nonphotochemical quenching are given by $(1/F'_o - 1/F'_{max})$ and $1/F'_{max}$, respectively. ○, Control R seedlings; △, chilled R seedlings; ●, control S seedlings; and ▲, chilled S seedlings. SES were calculated from four to six replicates and were less than 10%.

control R and S. However, a significant decrease in $1/F'_o - 1/F'_{max}$ was found for chilled S, whereas it was less affected in chilled R. In contrast, the chill treatment clearly increased $1/F'_{max}$ in R, indicating a cold-stimulated, nonphotochemical energy dissipation.

These results clearly indicate that the R and S lines differed in their responses to chilling in the light. In contrast to S, in which the PSII activity reflected an altered reaction center function, R achieved a low conversion yield as a result of an increased rate of nonradiative energy dissipation and maintained a maximal reaction center activity. Thus, the reduced photochemical activity measured for chilled R is equivalent to a down-regulation.

## Photoprotection and Zeaxanthin

The reason for the differences in differential sensitivity of R and S to photoinhibition could be reasonably hypothesized to be the activation of the major photoprotection mechanism previously described, the zeaxanthin-dependent nonphotochemical quenching. To verify this we analyzed the pigment composition of both R and S leaves following treatment at 250 µmol photons $m^{-2}$ $s^{-1}$ and either 25 or 4°C. These results are shown in Table I. Although this light intensity was not effective in inducing the conversion of violaxanthin to zeaxanthin at 25°C, a limited amount of zeaxanthin was found at 4°C, yielding a de-epoxydation state of 0.58. There was no difference between the R and S maize lines, indicating that the zeaxanthin-dependent nonphotochemical quenching could not account for the contrasted sensitivity of our maize lines to the chill treatment. We therefore proceeded to a further biochemical characterization of R and S thylakoids to identify the origin of the differential sensitivity of R and S to low temperatures.

## Differential Production and Location of the Phosphorylated CP29

Following the 6-h light treatment given at either 4 or 25°C, the thylakoid membranes were rapidly isolated and analyzed by SDS-PAGE (Fig. 2). Under control conditions the polypeptide pattern of the thylakoid membranes iso-

**Table I.** *De-epoxidation state of leaves from control and chilled R and S maize lines*

Results are expressed as molar ratios of zeaxanthin to zeaxantin (Z + V) and violanxanthin as determined by HPLC. Small amounts of antheraxanthin were also detected but did not significantly affect the measured pigment ratios. The data are typical of results seen in three different experiments. The relative proportion of the phosphorylated and nonphosphorylated CP29 was determined by densitometric scans performed in Coomassie blue-stained gels. Both the pigment and protein ratios were determined after a 5-h light treatment (250 µmol photons $m^{-2}$ $s^{-1}$) given at either 5 or 25°C.

| Treatment | CP29 | Phosphorylated CP29 | Z/Z + V |
|---|---|---|---|
| Control S | 100 | 0 | 0.093 |
| Chilled S | 93 | 7 | 0.582 |
| Control R | 100 | 0 | 0.089 |
| Chilled R | 57 | 43 | 0.562 |

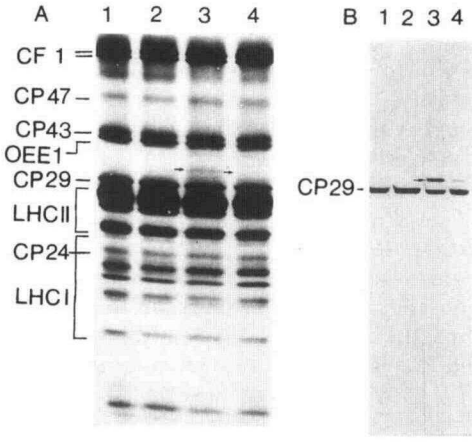


**Figure 2.** Analysis of the chill-induced changes in the polypeptide pattern of the thylakoid membranes. A, Coomassie blue-stained gels. Major bands are identified at the left. B, Immunoblots treated with the CP29 antibody. The arrows indicate the position of the cold-induced 34-kD protein. Lanes 1 and 2, Thylakoid membranes from control R and S seedlings, respectively; lanes 3 and 4, thylakoid membranes from chilled R and S seedlings, respectively. LHCI, Major light-harvesting complex of PSI.

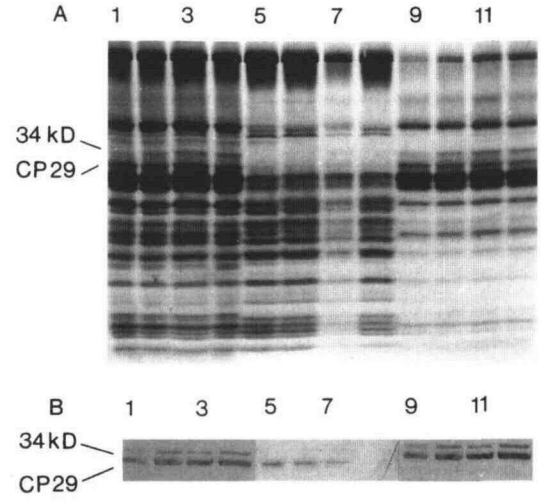


**Figure 3.** Kinetics and distribution of the 34-kD protein during the light-chill treatment. A, Coomassie blue-stained gels. B, Immunoblots treated with the CP29 antibody. Lanes 1 to 4, Thylakoids; lanes 5 to 8, stromal lamellae; lanes 9 to 12, PSII membranes isolated from 0.5-, 1-, 2-, and 6-h light-chilled seedlings, respectively.

lated from R and S was identical (Fig. 2A). However, following the cold treatment, a band with an apparent molecular mass of 34 kD was present in R. This 34-kD protein was present only to a small extent in S thylakoids from chilled seedlings. Immunoblot analysis using a set of antibodies directed to various antenna proteins confirmed that the newly appearing band was immunologically related to the chlorophyll *a*/*b*-binding protein, CP29 (Fig. 2B). Its reduced mobility has been shown to result from a conformational change associated with its phosphorylation (Bergantino et al., 1995).

The 34-kD band was detectable by Coomassie blue staining after 30 min and reached its maximal value after 5 to 6 h of cold treatment (Fig. 3A). The appearance of the 34-kD protein was not restricted to our R maize line and the LG11 maize hybrid in which it was first detected (Hayden and Baker, 1990). Indeed, the cold-tolerant lines B37, Oh 7N, and VA 36 (Bertolini et al., 1982) and the commercial maize hybrids LG 22.57, Cuzco, and F2xF7 were all found to accumulate similar amounts of the 34-kD, cold-induced protein. The maximum amount of the 34-kD protein was never found to be higher than 0.5 times the amount of its immunologically related 31-kD apoprotein of CP29 (not shown).

The existence of a positive correlation between the cold tolerance of maize and its ability to accumulate the cold-induced protein was confirmed by a failure to observe its accumulation in significant amounts in the cold-sensitive maize line A619 (not shown). We then proceeded to locate the 34-kD protein within the thylakoid membrane domains. With this aim in view, thylakoids isolated from seedlings exposed to various durations of cold treatment were fractionated into their grana and stroma domains. SDS-PAGE and immunoblots (Fig. 3) showed that the cold-induced protein was at all times associated with the grana membranes and excluded from the stromal lamellae. Fur-

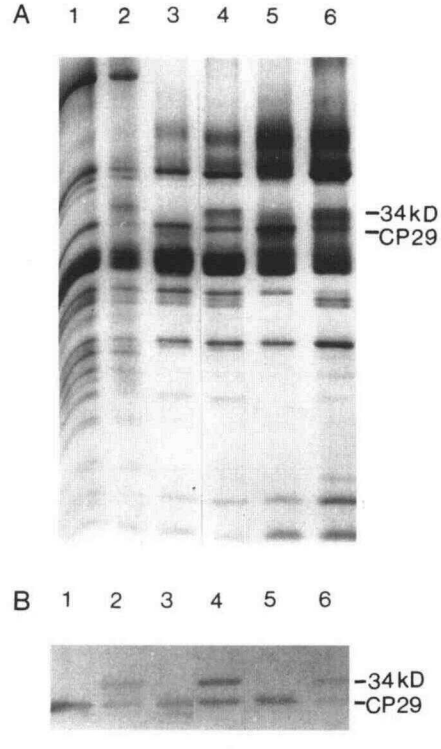


**Figure 4.** Analysis of various thylakoid membrane preparations with increasing content of the PSII reaction center complex. For reasons independent of the experiments presented here, the PSII core preparation from control membranes has been Tris-washed, which resulted in the removal of the 16-, 23-, and 33-kD proteins of the $O_2$-evolving complex. A, Coomassie blue-stained gels. B, Immunoblots treated with the CP29 antibody. Lanes 1 and 2, Thylakoid membranes from control and chilled R seedlings, respectively; lanes 3 and 4, PSII membranes from control and chilled R seedlings, respectively; lanes 5 and 6, PSII core particles from control and chilled R seedlings, respectively.

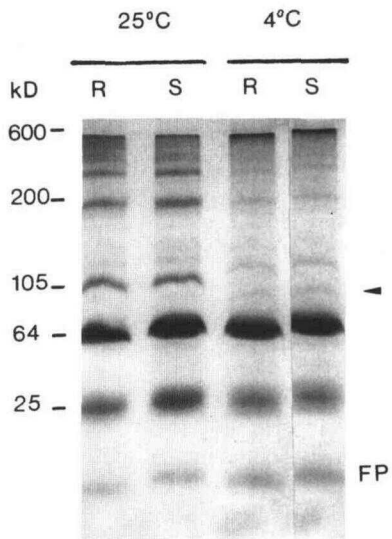


**Figure 5.** Nondenaturing Deriphat-PAGE separation of chlorophyll-protein complexes of R and S PSII membranes after treatment at 25 or 4°C. PSII membranes obtained from treated and untreated leaves were solubilized with 1% dodecyl maltoside and then fractionated overnight by Deriphat-PAGE. The gels were not stained and therefore the green bands represent chlorophyll-protein complexes or their association. Arrowhead, Position of the CP43-less PSII core; FP, position of the free pigments. See text for further description.

thermore, the margins of the grana discs failed to accumulate the 34-kD protein, as was observed for the phosphorylated PSII reaction centers of the D1 protein (not shown). This indicates that, unlike the phosphorylation of D1, the phosphorylation of CP29 does not constitute a signal for the correct positioning of damaged reaction centers before entering the stroma lamellae (Kettunen et al., 1991).

Further evidence for the close association of the 34-kD protein with PSII was obtained by solubilizing the grana membrane preparations with octylglucoside in high-salt-concentration conditions (Ghanotakis et al., 1987). This procedure selectively extracts the peripheral LHCII antenna proteins and yields a PSII core complex with its pericentral antenna (CP24, CP26, and CP29) still connected. By this procedure, the cold-induced protein band was seen to partition with the PSII core rather than with the outer LHCII antenna (Fig. 4).

The organization of the chlorophyll proteins of PSII membranes was then studied by Deriphat-PAGE (Peter and Thornber, 1991; Santini et al., 1994). This procedure yields several green bands representing either individual chlorophyll-binding proteins or their association into supramolecular complexes. Therefore, a Deriphat-PAGE pattern represents a fingerprint image of the protein-protein interaction within the grana membranes. Figure 5 shows the patterns of PSII membranes isolated from control and chilled R and S leaves. The polypeptide composition of the green bands seen in the Deriphat-PAGE system was then analyzed by a second-dimension SDS-urea PAGE (Fig. 6).

In the control R and S PSII membranes, the green bands could be classified into three groups according to their polypeptide composition (Fig. 6A). The first group, with apparent molecular masses between 25 and 105 kD, mainly contained the light-harvesting complexes. The lower band (25 kD) consisted of CP24, CP26, CP29, and monomeric LHCII migrating close to each other and forming a composite band. At the higher molecular mass range (60 kD), a CP29-CP24 complex migrated with the major trimeric LHCII complex. The last band in this group, at 105 kD, corresponded to a previously characterized supramolecular complex containing CP29, CP24, and LHCII (Bassi and

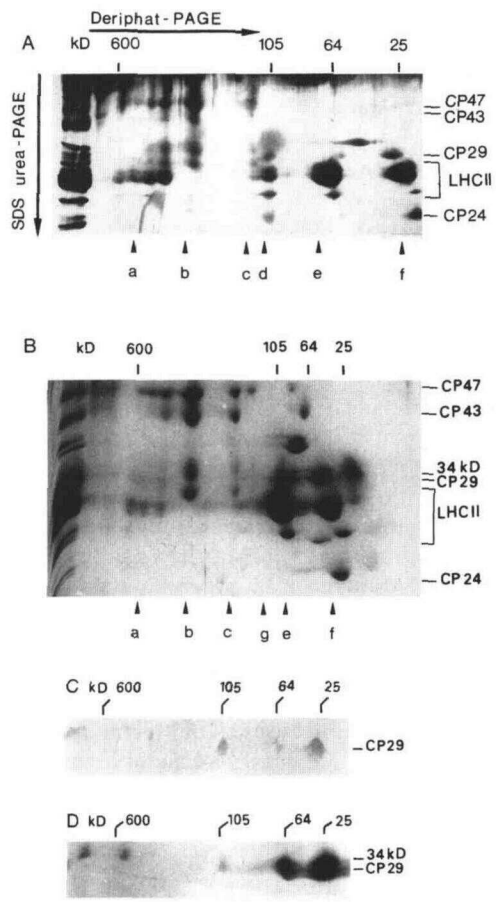


**Figure 6.** Analysis of the Deriphat-SDS R-PSII membranes by two-dimensional SDS-PAGE. Deriphat-PAGE separation gel lanes of Figure 5 were excised from the gel and further analyzed by perpendicular fractionation by SDS-PAGE to detect polypeptides contained in each green band. Polypeptides disposed on vertical rows are considered to belong to a common complex as isolated in the first dimension. The various polypeptides were identified using a set of polyclonal antibodies as described by Santini et al. (1994). For simplicity, only gels of the R line are shown; gels obtained from the S line were identical except for the absence of the 34-kD protein band. A, Control PSII membranes. B, Chilled PSII membranes. C, Immunoblot of control PSII membranes treated with CP29 antibody. D, Immunoblot of chilled PSII membranes treated with CP29 antibody. Lanes a, PSII complexes containing the PSII core and antenna components such as LHCII and CP29; lanes b, dimeric PSII core complexes; lanes c, monomeric PSII core complexes; lanes d, supramolecular complex containing CP29, CP24, and LHCII; lanes e, trimeric LHCII migrating with a CP29-CP24 complex; lanes f, monomeric LHCII migrating with CP29, CP26, and CP24; and lanes g, CP43-less PSII core.

Dainese, 1992). In the second group, CP47, CP43, D1, D2, and Cyt $b_{559}$ were organized into the monomeric and dimeric forms of the PSII core complex (Santini et al., 1994). The third group contained high-molecular-mass complexes, including the PSII core associated with various chlorophyll *a/b* complexes, which yielded green bands with apparent molecular masses between 300 and 600 kD.

PSII membranes from cold-treated R and S samples analyzed as described above showed as the major difference the absence of the 105-kD green band, indicating a change in the organization of the antenna system. Minor changes were also detected. A faint PSII band lacking CP43 was detected at the position corresponding approximately to the 105-kD green band (Fig. 5). Its CP43 complement migrated between monomeric and trimeric LHCII proteins (Fig. 6B), indicating that chilling induced a limited PSII core dissociation (Aro et al., 1990).

It has been suggested that the dissociation of the 105-kD band was correlated with the dissociation of the dimeric to the monomeric PSII core complex (Peter and Thornber, 1991). However, in our study densitometric analysis of Coomassie blue-stained gels showed that the dimeric to monomeric ratio was not significantly affected by the chill treatment.

The two-dimensional separation was used to further elucidate the relationship of the 34-kD protein with the other thylakoid polypeptides. This was done by immunoblotting with a CP29 antibody (Fig. 6, C and D). In the cold-treated samples, the antibody recognized the 34-kD protein and the 31-kD apoprotein of CP29 in the position corresponding in the first dimension to green bands with apparent molecular masses of approximately 30 and 60 kD, respectively. Additional locations were at very high apparent molecular mass bands corresponding to chlorophyll *a/b* complexes (including the PSII core). The two-dimensional SDS-PAGE fractionation of thylakoid membranes (Fig. 6, A and B) also allowed detection in membranes from cold-treated plants of monomeric CP43 and CP47 PSII subunits, which appear as spots with apparent molecular masses of 65 to 70 kD. The presence of these spots correlates with that of the above-mentioned 105-kD PSII complex lacking CP43. Since monomeric CP43 and CP47 were absent in the control sample (Fig. 6A) and their amount increased in cold-treated S membranes (not shown), we suggest that they represent a product of the stress-induced dissociation of the PSII core complex. Although the chill treatment significantly modified the composition of the PSII antenna, the phosphorylation of the CP29 subunit altered its interactions neither with the PSII core complex nor with the outer antenna proteins.

### Isolation and Spectral Characterization of the Phosphorylated CP29

In an attempt to clarify the consequences of the chill-induced modification to the CP29 protein, we fractionated PSII membranes from cold-treated R seedlings to isolate the protein in its native form. This was obtained by solubilization with 1% dodecyl maltoside and separation by flat-bed IEF. The fractionation pattern was very similar to that which we previously described for control membranes (Dainese et al., 1990) except for the presence of an additional green band at pH 4.65 (Fig. 7). This pH was slightly lower than that previously determined for CP29 (pH 4.72) (Dainese et al., 1990). The green bands from the gradients were characterized by SDS-PAGE, immunoblotting, and absorption and fluorescence spectroscopy. The lack of cross-contamination between the two CP29 fractions was confirmed by immunoblotting (Fig. 7).

Phosphorylated CP29 had a chlorophyll *a/b* ratio of 2.7, which is identical to that of CP29. It displayed (Fig. 8) the characteristic long-wavelength absorption peak at 678 nm and the shoulder at 641 nm detected for CP29 (Bassi and Dainese, 1992). Although the fourth derivative of the absorption spectra confirmed that the peak positions of various spectral components were identical in both chlorophyll *a/b*-binding complexes (not shown), the absorption spectra difference between phosphorylated and nonphosphorylated CP29 emphasized reproducible differences (Fig. 8). Two negative contributions peaking at 648 and 676 nm were detected. Two positive contributions were also detected, with the broad one peaking at approximately 685 nm and the small one at 660 nm. We attribute these differences to a redistribution of the chlorophyll absorption spectral forms in the two complexes. Thus, phosphorylated CP29 was enriched in chlorophyll absorption spectral form components, peaking at 647 and 677, whereas the contribution of the spectral forms at 665 and 685 was decreased.

Room temperature fluorescence spectra were also very similar, with a maximum at 681 nm (Fig. 9). The fluorescence spectra difference confirmed the altered relative contribution in phosphorylated CP29 of the various spectral forms suggested by the absorbance data. The peak positions of the negative and positive contributions revealed by the fluorescence emission spectra difference corresponded well with those observed in the absorption spectra difference when allowing for the appropriate wavelength shifts (Zuchelli et al., 1992). However, the overall emission yield of both complexes was identical, as demonstrated by the

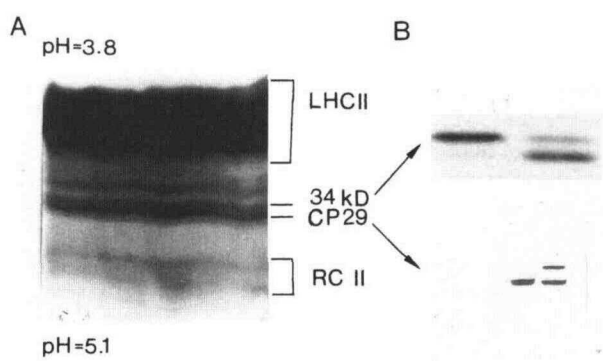


**Figure 7.** Isolation of CP29 and its cold-induced phosphorylated form by nondenaturing. A, Photograph of separation pattern of 1% dodecyl-maltoside-solubilized PSII membranes. The two bands were eluted from the gel with 0.06% dodecyl maltoside in 10 mM Hepes, pH 7.5, and ultracentrifuged through a Suc gradient to eliminate possible contaminants and carrier ampholytes. B, Immunoblot of the two CP29 fractions with CP29 antibody. RC II, Reaction center II.

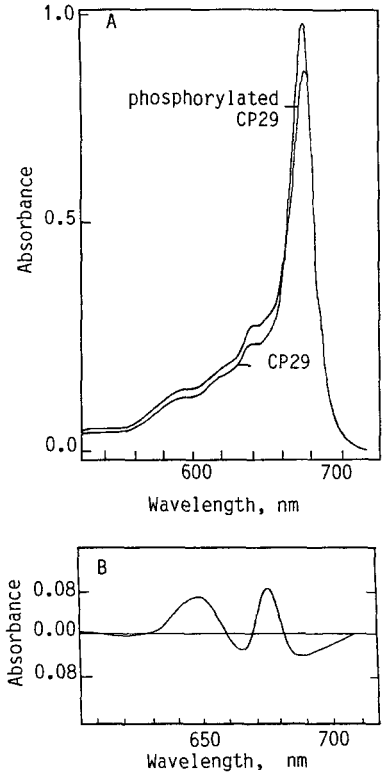


**Figure 8.** A, Absorption spectra of isolated CP29 and its cold induced phosphorylated form. B, Absorption spectra difference (phosphorylated CP29 − nonphosphorylated CP29) after normalization at 600 nm.

equality of the area under the fluorescence spectra measured under equal $A_{439}$. HPLC pigment analysis of the phosphorylated and nonphosphorylated CP29 complexes showed that they have a very similar if not identical xanthophyll composition, which is in agreement with previous results (Croce et al., 1996).

## DISCUSSION

In this work we have identified a particular mechanism responsible for the photoprotection of the PSII reaction centers under conditions in which optimal utilization and processing of the absorbed light energy were altered by low temperature. At low temperatures, it is the imbalance between the light absorption and utilization capabilities that promotes the photodestruction of the PSII reaction centers (Öquist et al., 1987; Barber and Andersson, 1992). The stress effect is further exacerbated by the slowing down of the major photoprotection mechanisms acting at room temperature (Moll and Steinback, 1986; Demmig-Adams et al., 1989; Bingsmark et al., 1992).

As previously described, we observed a decrease in $\Phi_{PSII}$ after a mild chill treatment; however, the analysis of this effect showed that it was not due to an increase in $F_o$ (Kirilovsky et al., 1990), which would reflect the formation of inactive PSII reaction centers (Vass et al., 1992). The constancy of the $F_o$ intensity was observed using both the pulse amplitude modulation technique and DCMU-treated samples (Mauro et al., 1990).

In the S maize line, damage to the PSII reaction center was inferred from the low value of $q_p$, as would be expected when a significant fraction of the reaction centers is brought to a low fluorescence state by irreversible photoinhibition (Ohad et al., 1990). This interpretation is strengthened by the cold-induced decrease in $1/F'_o - 1/F'_{max}$, which was proposed to unequivocally monitor the reaction center photochemistry (Havaux et al., 1991). It was significant that the Deriphat-PAGE and the two-dimensional SDS-PAGE analysis emphasized the presence of a green band containing a CP43-depleted PSII, which probably had damaged reaction centers.

In contrast, the inhibition of PSII photochemistry of the cold-resistant R could be exclusively accounted for by a reduced probability of trapping efficiency. If $1/F'_{max}$ really monitors the rate constant (although apparent) for nonphotochemical energy dissipation, its increase in chilled R is puzzling because all regulatory mechanisms (Moll and Steinback, 1986; Demmig-Adams et al., 1989; Bingsmark et al., 1992), including active $O_2$-scavenging enzymes (Janhke et al., 1991), are inhibited at low temperature. However, the chloroplastic Cu/Zn superoxide dismutase activity has been found to increase with decreasing temperature (Burke and Oliver, 1992). If this applies to chilled R, it would mean that some form of activated $O_2$ is involved in the regulation of PSII at low temperature as well (Neubauer and Yamamoto, 1992).

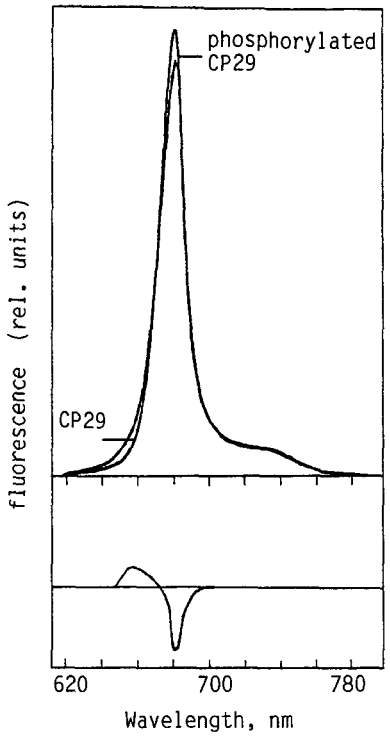


**Figure 9.** Room temperature fluorescence emission spectra of isolated CP29 and its cold-induced phosphorylated form (A) and the fluorescence emission spectra difference (B). The fluorescence of both complexes was recorded under equal absorption at 439 nm.

The conclusions drawn from the fluorescence analysis of the responses of our maize lines to a mild chill treatment are reminiscent of those obtained from simultaneous measurements of chlorophyll fluorescence and $O_2$ evolution performed on maize hybrids with contrasted sensitivity to low temperature (Massaci et al., 1995). The resistant maize genotype has been reported to efficiently coregulate C assimilation and PSII photochemistry, whereas in the cold-sensitive genotype the PSII photochemistry is not tightly down-regulated, leading to oxidative damages (Massaci et al., 1995).

Our fluorescence analysis provided physiological evidence for the existence of a regulatory mechanism that down-regulates PSII photochemistry by acting on its antenna components and consequently preserves the reaction centers from photoinhibition.

The difference between R and S maize lines for nonphotochemical energy dissipation capacities can be explained either by a common mechanism with a higher activity in R than in S or by the activation of a particular mechanism operating in the R maize line under the chilling conditions. The biochemical analysis indicates that at least two of the modifications brought to the antenna system are common to the R and S maize lines. In fact, the extent of the conversion of violaxanthin to zeaxanthin upon chilling is the same, as is the dissociation of the CP29-CP24 LHCII complex.

Although in agreement with the measured reduced trapping efficiency, the physiological significance of its dissociation and its fate are not straightforward. It is tempting to speculate that its dissociation represents a short-term physiological adaptation to low temperature. The 105-kD complex has been proposed to contribute to the regulation of the excitation energy distribution between the two photosystems (Bassi and Dainese, 1992). Diversion from PSII to PSI of a significant portion of the absorbed light energy would render the PSII and the whole thylakoid membrane less prone to photodestruction, since, in vivo, PSI has been demonstrated to be intrinsically more resistant to photoinhibition (Öquist et al., 1987; Harbinson et al., 1989). Alternatively, it could represent an early stage of a long-term adaptation leading to smaller photosynthetic units. In the latter case, dissociation of the complex could be an early step in the process of degradation and proteolysis of the various antenna components (Öquist et al., 1987; Maxwell et al., 1995).

What, then, causes the difference between R and S? Many mechanisms can be invoked to explain the different behaviors of R and S, including the synthesis of early light-inducible proteins. However, the appearance of these proteins in green, mature tissue was observed in conditions of intense degradation of PSII reaction centers induced by a very high-intensity light treatment (Adamska et al., 1992) or during growth at low temperature under high light intensity (Montané et al., 1997). Accordingly, close examination by SDS-PAGE of thylakoids isolated from the R line at various times during the chill treatment (Fig. 3) does not indicate the appearance of new protein bands in the 13- to 17-kD range, whereas it is very clear that the major modification of the thylakoid peptidic pattern is the appearance of the 34-kD protein band representing the phosphorylated apoprotein of CP29. Although we do not have direct evidence that the cold tolerance of the R line is controlled by the phosphorylation of CP29, we cannot exclude this possibility. In fact, consistent with a photoprotective function, localization studies indicate that phosphorylated CP29 partitions with the PSII core.

The PSII photosynthetic units have been proposed to behave as a shallow funnel for the trapping of excitation energy. The probability for excitation trapping depends on the intrinsic properties of individual chlorophyll spectral forms. All of the chlorophyll-binding proteins so far isolated have been shown to contain, in various proportions, these chlorophyll spectral forms with unchanged spectral properties (Jennings et al., 1993). The main topological constraint to the migration of the excitation energy toward the reaction center is constituted by the pigment protein complexes CP29, CP26, and CP24, which connect the outer antenna LHCII to the inner antenna CP43 and CP47. Furthermore, the identical location of phosphorylated and nonphosphorylated CP29, together with their similar spectral properties, suggests that they perform alternative functions in different physiological conditions.

A clue to the molecular mode of action of phosphorylated CP29 has been provided by the comparison of the absorption and fluorescence emission spectra of both forms of CP29. The constancy of the fluorescence quantum yield indicates that when phosphorylated the complex does not perform its regulatory function by directly increasing the thermal degradation of excitation energy. However, the absorption difference spectrum established that it was depleted in the most efficient excitation-energy-transferring chlorophyll spectral form, which absorbs at 684 nm, whereas it was enriched in the least efficient excitation-energy-transferring chlorophyll form, which absorbs at 645 nm. The changes of the relative contribution of these spectral forms led to the negative and positive contributions of the absorption difference spectrum, peaking at 684 and 648 nm, respectively.

The redistribution of the various chlorophyll *a* spectral forms within phosphorylated CP29 was confirmed by the fluorescence emission spectrum difference. Although the differences may appear rather small, simulation of the fluorescence spectra using the available characteristics of chlorophyll spectral forms (Jennings et al., 1993) indicates that these differences could account for a change by a factor of 2 in the relative contribution of the high energy-transfer efficiency spectral forms.

Because under steady-state illumination the distribution of excited states among the various complexes is governed by the concentration of the different spectral forms, the relatively low equilibrium constant for sharing the excitation between the antenna and the PSII core would be further reduced by the depletion of the spectral forms with high energy-transfer efficiency. Thus, photoprotection of the reaction center from excessive excitation is achieved by altering the properties of a small number of strategic sites through which the excitation energy has to pass on its way to the reaction center. The fact that this regulatory site is itself a chlorophyll-binding protein provides an easy means to control the fate of the excitation energy absorbed

by the antenna. Its chlorophylls do not significantly contribute to the overall light absorption but readily participate in the excitation energy transfer to the reaction center.

The cold-induced CP29 form is characterized by an internal redistribution of the various chlorophyll spectral forms with the important result that the excitation energy is redistributed toward the antenna. Such a shift is very rapidly turned on and off, as is demonstrated by the rapid kinetics of its appearance and disappearance on return to normal temperature (Hayden and Baker, 1990).

The 34-kD protein was first detected as a 31-kD protein band in a different gel system and was proposed to represent an unprocessed form of the CP29 apoprotein. However, the changes in the spectral properties of the chilled thylakoid membranes were loosely analyzed and were explained at least partly by the insertion of the unprocessed 31-kD protein in the PSII complex. We have extended these initial experiments and coupled chlorophyll fluorescence measurements to investigate the particulars of PSII regulation with a careful biochemical analysis of PSII antenna organization in thylakoid membranes isolated from chilled maize leaves. We suggest that the cold-induced phosphorylation of the CP29 apoprotein may contribute to the photoprotection of PSII. In fact, the characterization of purified phosphorylated and nonphosphorylated CP29 indicates that differences in their spectral properties exist and may lead to a different partition of the excitation energy between the PSII reaction center and the PSII antenna, thus protecting PSII from photoinhibition.

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