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Decreased Photosystem II Core Phosphorylation in a Yellow-Green Mutant of Wheat Showing Monophasic Fluorescence Induction Curve

Maria T. Giardi*, Tomas Kucera[1], Jean-Marie Briantais, and Michael Hodges

Institute of Biochemistry and Ecophysiology of Plants, Consiglio Nazionale delle Ricerche, Via Salaria km 29.3, 00016 Monterotondo Scalo, Italy (M.T.G., T.K.); Laboratoire d'Ecologie Végétale, URA1492, Bât 362, Université de Paris XI, F-91405 Orsay Cedex, France (J.-M.B.); and Laboratoire de Physiologié Végétale Moleculaire, URA1128, Bât 430, Université de Paris Sud, 91405 Orsay Cedex, France (M.H.)

In the present work we study the regulation of the distribution of the phosphorylated photosystem II (PSII) core populations present in grana regions of the thylakoids from several plant species. The heterogeneous nature of PSII core phosphorylation has previously been reported (M.T. Giardi, F. Rigoni, R. Barbato [1992] Plant Physiol 100: 1948–1954; M.T. Giardi [1993] Planta 190: 107–113). The pattern of four phosphorylated PSII core populations in the grana regions appears to be ubiquitous in higher plants. In the dark, at least two phosphorylated PSII core populations are always detected. A mutant of wheat (*Triticum durum*) that shows monophasic room-temperature photoreduction of the primary quinone electron acceptor of PSII as measured by chlorophyll fluorescence increase in the presence and absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea and by fluorescence upon flash illumination in intact leaves also lacks the usual distribution of phosphorylated PSII core populations. In this mutant, the whole PSII core population pattern is changed, probably due to altered threonine kinase activity, which leads to the absence of light-induced phosphorylation of CP43 and D_2 proteins. The results, correlated to previous experiments in vivo, support the idea that the functional heterogeneity observed by fluorescence is correlated in part to the PSII protein phosphorylation in the grana.

Many types of PSII heterogeneity have been observed at the level of the antenna and photochemistry, both at the donor and acceptor side (Black et al., 1986; Govindjee, 1990; Hansson and Wydrzynski, 1990; Krause and Weis, 1991; Melis, 1991). PSII heterogeneity has been described in different ways, many of them based on Chl fluorescence changes, but several of these observations are still controversial and no model exists that can explain all of the results.

The heterogeneity in the rate of reduction of Q_A in the presence of DCMU was attributed to the existence of two types of PSII centers. The fast sigmoidal phase of Q_A reduction was related to PSIIα and the subsequent slow phase to PSIIβ center. It has been suggested that the two phases correspond to two types of PSII reaction center that differ, among other properties, in their antenna size and location within the thylakoid membrane. PSIIα was thought to reside in grana partitions, whereas PSIIβ was located in the stroma-exposed membrane regions (Krause and Weis, 1991; Melis, 1991). However, this interpretation contrasts with other experimental data, such as the observation that the slow phase of fluorescence induction is eliminated by high concentrations of DCMU, indicating the existence of PSII units that vary in their sensitivity to DCMU (Lavergne, 1982; Black et al., 1986; Hodges and Barber, 1986), whereas it has been shown that PSII located in stroma-exposed regions and PSII present in grana regions are probably equally sensitive to DCMU (Henrynsson and Sundby, 1990). Moreover, fluorescence induction curves of DCMU-poisoned chloroplasts have been resolved by mathematical analysis, not only into two but also into three and even four phases, a major, rapid sigmoidal phase followed by two or three slower exponential phases (Sinclair and Spence, 1990; Hsu and Lee, 1991; Sinclair et al., 1991).

Another type of heterogeneity associated with PSII is the so-called reducing-side heterogeneity. Through several approaches it has been demonstrated that Q_B is not reduced by some PSII centers, which have thus been termed non-B-type centers (Black et al., 1986; Ort and Whitmarsh, 1990; Nedbal and Whitmarsh, 1992; Lavergne and Leci, 1993). The location of these centers in the membrane is still controversial (Black et al., 1986; Hodges and Barber, 1986; Hodges and Moya, 1986; Govindjee, 1990; Hsu and Lee, 1991; Krause and Weis, 1991; Melis, 1991). Moreover, biochemical evidence of the existence, in the grana regions, of numerous PSIIα, heterogeneous in antenna size, has been reported (Albertsson et al., 1990).

The photosynthetic membrane contains several phosphoproteins. The most conspicuous are components of the light-harvesting Chl *a*/*b* complex (Bennett, 1991; Horton

[1] Present address: Department of Biochemistry, Charles University, Albertov 2030, 12840 Praha 2, Czech Republic.

* Corresponding author; e-mail giardi@nserv.icmat.mlib.cnr.it; fax 39–6–9064492.

Abbreviations: D_1, 32-kD reaction center II protein; D_2, 34-kD reaction center II protein; D_1/D_2 heterodimer, 64-kD reaction center heterodimer of D_1 and D_2 proteins; F_v/F_m, the ratio of the yield of variable fluorescence to the yield of maximal fluorescence when all reaction centers are closed; LHCII, light-harvesting protein of PSII; MAIM, *N*-ethylmaleimide; Q_A and Q_B, primary and secondary quinone electron acceptor of PSII.

and Ruban, 1992). Several other PSII proteins have been reported to be phosphorylated by light-dependent kinases in a process reversed by endogenous phosphatases; among these are CP43, D_1, D_2, and PsbH, that together with CP47 and Cyt b_{559} form the PSII core (Bennett, 1991; Allen, 1992; Satoh, 1992).

Four heterogeneous PSII core populations have been recently isolated by IEF of spinach grana particles solubilized with nonionic detergent (Giardi et al., 1990, 1991). The four complexes, referred to as a, b, c, and d in increasing order of phosphorylation, showed either a different level of phosphorylation on PSII core polypeptides or a different content of the *psb*H gene product. The most phosphorylated PSII core population, d, was about 40 times more phosphorylated on D_1 protein than the core complex a and was inactive in electron transfer from diphenylcarbazide to dichlorophenolindophenol (Giardi et al., 1991, 1992; Giardi, 1993a). These differently phosphorylated PSII core populations have been shown to have different affinities for the binding of photosynthetic herbicides (Giardi et al., 1990, 1992; Giardi, 1993b) and different sensitivities to photoinhibitory conditions (Giardi, 1993a). It has been shown that phosphorylation of thylakoid membrane results in a partial inhibition of the light-saturated rate of oxygen evolution, which may reflect a decreased affinity of plastoquinone for the Q_B site (Horton and Lee, 1984; Hodges et al., 1987).

In the present work we analyze the distribution of PSII core phosphorylation heterogeneity in different plant species. We report a modified PSII core phosphorylation heterogeneity in a Chl-deficient mutant of wheat (*Triticum durum*) that does not show a part of functional PSII heterogeneity pointed out by Q_A measurement using (a) Chl fluorescence induction in the presence of DCMU, and (b) Chl fluorescence increase upon flash illumination.

MATERIALS AND METHODS

Plant Material

Seedlings of various plant species were tested: spinach, basil, and parsley were purchased from the local market; mallow and mint were harvested in the field; oat, maize, senecio, and a *Senecio vulgaris* atrazine-resistant mutant were grown in a growth chamber at 25°C, during a 16-h photoperiod at 400 μmol m^{-2} s^{-1} and 80% RH. Leaves of 2- to 3-week-old plants were used in the experiments. *Triticum durum* (wheat) and a yellow-green mutant of *T. durum*, described by Triolo et al. (1982), were grown under the same conditions reported above, but at 18°C during the day and at 10°C during the night. Wild-type barley (Triumph) and the yellow-green mutant chlorina f2 were grown in similar conditions. According to Peter and Thornber (1991), the chlorina f2 mutant lacks LHCII complexes.

Isolation of Membranes and Phosphorylation

Plant thylakoids were extracted at 5°C in an isolation buffer containing 50 mM Tricine (pH 7.8), 15 mM NaCl, 5 mM $MgCl_2$ with additional 0.3 M Suc. After centrifugation, pelleted membranes were washed with the same buffer containing 70 mM Suc. Thylakoids were obtained under different conditions according to the following numbered procedures. 1. Leaves were kept in the dark for 12 h and thylakoids were isolated strictly in the dark. To allow for visibility during extraction, a very low, indirect green light (less than 5 μmol m^{-2} s^{-1}) was used. Isolated thylakoids were incubated in the dark for an additional 2 h. 2. Thylakoids were obtained as reported in procedure 1 except for the additional dark incubation time of only 30 min. 3. Thylakoids were extracted from dark-adapted plants in the normal light of the laboratory (approximately 20 μmol m^{-2} s^{-1}). 4. Light control was obtained extracting light-adapted plants (12 h at 400 μmol m^{-2} s^{-1}) in the light of the laboratory. 5. Extraction was performed using buffers in which, instead of $MgCl_2$, 150 mM KCl was added to keep the membranes stacked. 6. Extraction was performed adding 10 μM DCMU. 7. Ten millimolar MAIM was added in the isolation buffers.

In procedures 5 through 7 the isolations were carried out using dark-adapted plants in the presence of room light. Phosphorylation profiles of polypeptides were produced by incubating the cut petiole of the plants in the presence of ^{32}Pi (0.2 mCi/seedling, 9000 Ci/mmol) with additional 0.01% Tween for 8 h in the dark under a current of air and 2 h in the light (1000 μmol m^{-2} s^{-1}). Isolation of thylakoids was performed with the isolation buffer with or without additional 5 mM NaF, a phosphatase inhibitor. PSII-enriched particles were immediately isolated from thylakoids according to Berthold et al. (1981), obtained under the conditions described in procedures 1 through 7. The PSII particles were further solubilized with 1% *n*-dodecyl-β-D-maltoside (0.5 mL mg^{-1} Chl) and applied to the cathode region of a flat bed of granulated gel as previously described (Giardi et al., 1990, 1992). PSII core fractions were eluted from the gel with a buffer containing 50 mM Mes (pH 6.5), 15 mM NaCl, 5 mM $MgCl_2$, and 0.1% dodecyl-β-D-maltoside. Four isolated PSII bands were applied to a linear gradient from 0 to 1 M Suc in the above extraction buffer and centrifuged at 29,000 rpm (SW41 Beckman rotor) for 12 h to obtain complete separation of four differently phosphorylated PSII core populations (Giardi et al., 1991). Separation into grana and stroma-exposed thylakoids was obtained by solubilization of thylakoids with digitonin and followed by differential centrifugation (Giardi, 1993a). Preparation of the PSII-enriched particles from the wheat mutant and its wild type was performed according to Berthold et al. (1981) using the Triton/Chl ratio of 15.

SDS-PAGE, Autoradiography, and Immunoblotting

SDS-PAGE was performed in the presence of 6 M urea using a 12 to 18% linear acrylamide gradient (Barbato et al., 1991). To obtain radiolabeled profiles of the polypeptides, gels were stained with Coomassie brilliant blue R, treated with Amplify (Amersham) for 30 min, and exposed to x-ray film (Hyperfilm TM MP, Amersham). The gels and the autoradiograms were scanned in a Shimadzu (Kyoto, Japan) 9000 densitometer. Polyclonal antibodies against PSII proteins were a kind gift from Dr. Barbato of Padua University (Italy). Monoclonal antibodies against phosphothreonine were purchased from Sigma. The 64-kD polypep-

tide attributed to the LHCII kinase was isolated according to the method reported by Coughlan and Hind (1986) with a few minor modifications. Mega-9 was used instead of octyl-glucoside, and after the histone-affinity column a further affinity step using ATP-agarose was applied. The final kinase activity was eluted at 200 mM NaCl, dialyzed, and concentrated. Antibodies were raised against the purified pea LHCII kinase. The rabbit serum was precipitated with 33% ammonium sulfate and the IgGs were purified on a protein-A affinity column. Western blot experiments in various wild-type and mutant thylakoids and PSII particles were carried out on samples whose proteins were separated on 12 to 17% acrylamide gels and transferred to nitrocellulose filters. The immunocomplexes were detected using anti-rabbit or anti-mouse secondary antibodies, coupled to alkaline phosphatase (Barbato et al., 1991), except the 64-kD antibodies, which were coupled to peroxidase (Hodges et al., 1990).

Chl Fluorescence Measurements

Chl fluorescence induction in continuous light was performed on 1-h dark-adapted leaves and on cut leaves incubated in the presence of 10 μM DCMU before measurement. Chl fluorescence induction curves were measured using a PAM fluorometer (Walz, Effeltrich, Germany) and the kinetics were recorded with a digital storage oscilloscope (Thurlby 524, London, UK). The change in the area above the fluorescence rise was analyzed by computer using one to three exponential components and a Marquardt search algorithm program (Hodges et al., 1989). Fluorescence changes were followed using a PAM 101 fluorometer, and single-turnover flashes were obtained from a XST xenon lamp (Walz). The intensity of the flash was varied with neutral density filters.

RESULTS

Regulation of Distribution of Phosphorylated PSII Core Populations in Higher Plants

With the aim of obtaining more information on the regulation of the distribution of the phosphorylated PSII core populations, the isolation procedure described by Giardi et al. (1990, 1992) was applied to grana particles from various species (reported in "Materials and Methods"). In light-extracted plants at least four PSII core bands were always detected with a similar distribution of phosphorylated PSII core populations (data not shown). Thus, we concluded that the pattern of four phosphorylated PSII cores is ubiquitous in higher plants. The relative distribution of the PSII core populations was studied in spinach plants adapted to different conditions of light prior to and during extraction of thylakoids. The light present in the laboratory during extraction of dark-adapted plants was observed to be high enough to induce phosphorylation of PSII core polypeptides (Table I, procedures 1–4). We found that the distribution of PSII core populations responds to conditions that are known to inhibit light-induced kinase activity (Bennett, 1991; Allen, 1992), such as the depletion of Mg^{2+}, the addition of herbicides, and the presence of MAIM during the extraction of dark-adapted thylakoids (Table I, procedures 5–7). Incubation in the dark of light-adapted plants for 12 h prior to extraction and for 2 h after extraction did not give rise to complete reconversion of the most-phosphorylated populations into the least-phosphorylated PSII core a (Table I, procedure 1). Moreover, the radioactivity incorporated in vivo by treatment of the leaves with ^{32}Pi was still present in the PSII core proteins after such long, dark incubation times, particularly in the 8-kD protein attributed to the *psb*H gene product (data not shown; Giardi et al., 1994). We concluded that a phosphorylation component that is not completely reversed by dark incubation under physiological conditions is always present in the PSII core in accordance with recent observations (Elich et al., 1992, 1993; Giardi, 1993b; Silverstein et al., 1993; Giardi et al., 1994).

Table I. *Relative distribution of Chl into phosphorylated PSII core populations from thylakoids isolated from plants under different conditions*

PSII cores were isolated from PSII particles by IEF and further Suc gradient ultracentrifugation of the isolated PSII fractions according to Giardi et al. (1992). PSII particles were isolated according to Berthold et al. (1981) from spinach thylakoids isolated under different conditions (see "Materials and Methods" for details of procedures 1–7). nr, Not revealed. The values represent typical results of an average of three measurements in a preparation, SE approximately 10%.

Conditions	PSII Core Populations			
	a	b	c	d
1. Dark 12 h + 2 h dark	45	42	13	nr
2. Dark 12 h + 30 min dark	41	40	12	7
3. Dim light	28	39	8	25
4. Light	24	37	6	33
5. $-MgCl_2$	65	25	6	4
6. +10 μM DCMU	38	39	14	9
7. +10 mM MAIM	52	41	4	3

PSII Protein Characterization and Structural Studies of the Yellow-Green Wheat Mutant

A yellow-green mutant of wheat, whose photosynthetic apparatus has previously been studied (Di Marco et al., 1989), has also been observed to lack a part of functional PSII heterogeneity, since it gives rise to a monophasic Chl fluorescence induction curve with DCMU-poisoned leaves. In this study we have thoroughly examined the mutant.

The wheat mutant was obtained by x-ray treatment and was shown to have a single dominant mutation inherited in a Mendelian manner; the mutation is thought to affect regulation of gene expression and not the structure of coding regions, since, if the plants are grown at a temperature higher than 30°C, they show Chl content and biochemical characteristics similar to the wild type (Dr. B. Giorgi, personal communication, and M.T. Giardi, unpublished results). These observations suggest that under these conditions the mutation is silent and all Chl complexes can be synthesized.

It has previously been observed in the mutant that PSII polypeptides appear to be quite depleted compared to the wild type per leaf area (see Di Marco et al., 1989) (Fig. 1).

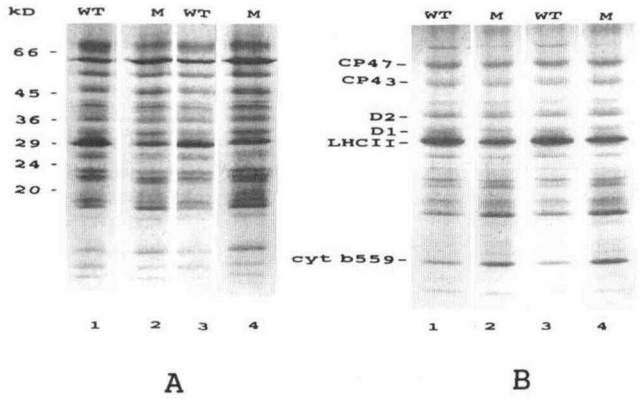

Figure 1. SDS-PAGE analysis of thylakoids (A) and PSII-enriched particles (B) of the wheat mutant (M) and its wild type (WT). The gels were loaded on the basis of a similar amount of core (lanes 1 and 2) calculated according to Table II, and of a similar amount of Chl (lanes 3 and 4).

We analyzed the polypeptide composition of the mutant. Thylakoids and PSII-enriched particles were isolated from the wheat wild type and mutant using the procedure of Berthold et al. (1981) with a Triton X-100:Chl ratio of 15 (see "Materials and Methods"). SDS-PAGE analysis of the membranes showed that the PSII polypeptide composition of the mutant was apparently qualitatively the same as that observed in the wild type, although the relative stoichiometry of the polypeptides was quite different (Fig. 1). Using polyclonal antibodies raised against the PSII components, it was observed that all of the main PSII polypeptides are present in the mutant (Fig. 2). A greater reaction in a polypeptide of about 16 kD, cross-reacting with CP47 antibodies, and in the 20-kD region, cross-reacting with CP43 antibodies, was observed, perhaps representing some breakdown products (Fig. 2). Fractionation of the PSII particles by IEF showed that when the gel was loaded with the same amount of PSII particle Chl as the mutant and wild type, the LHCII Chl content in the mutant was reduced more than 2-fold, the content of Chl associated to PSII core was increased, and the content of the minor antennae was similar (Table II). Immunoblot analysis with polyclonal antibodies raised against a 64-kD protein, attributed to the LHCII kinase (Coughlan and Hind, 1987; M. Hodges, unpublished data), indicated that the wheat mutant was considerably depleted of LHCII compared with the wild type (Fig. 3).

Micrograph analyses showed that a clear distinction between the appressed and nonappressed regions was present in the mutant (data not shown). The mutant was depleted in the content of thylakoids per leaf area, but despite the partial depletion of the PSI previously observed, it was still possible to separate PSII-enriched grana particles and stroma lamellae (Table II) with the digitonin method (Giardi, 1993a), confirming a distinction between appressed and nonappressed regions.

Phosphorylation State of the Wheat Mutant

We were unable to phosphorylate the mutant thylakoids with added ATP under plastoquinone-reducing conditions induced by light, probably due to a rapid inhibition of electron transfer observed in the thylakoids of the wheat seedlings (data not shown). However, phosphorylation of PSII proteins was observed in vivo by incubating the cut petiole of the seedlings in the presence of ^{32}Pi in the light (Fig. 4). Since the efficiency of this radiolabeled marking was low, a high specific radioactive activity (9000 Ci/mmol) was used.

Figure 4 shows the distribution of radioactivity due to phosphate incorporation into the PSII polypeptides of the mutant and its wild type. Due to the high breakdown, for a better illustration two exposition times of the film are presented. Moreover, the thylakoids for this autoradiography analysis were obtained by extracting the leaves without additional phosphatase inhibitors. This procedure of allowing reduction of the radioactivity incorporated into LHCII highlights the radioactivity in the PSII core polypeptides. In fact, LHCII dephosphorylation by endogenous phosphatases is more rapid than dephosphorylation of the core proteins (half-time of minutes compared to half-time of hours; Elich et al., 1992, 1993). However, similar results were obtained using the phosphatase inhibitor NaF during the membrane isolation (data not shown). The radioactivity

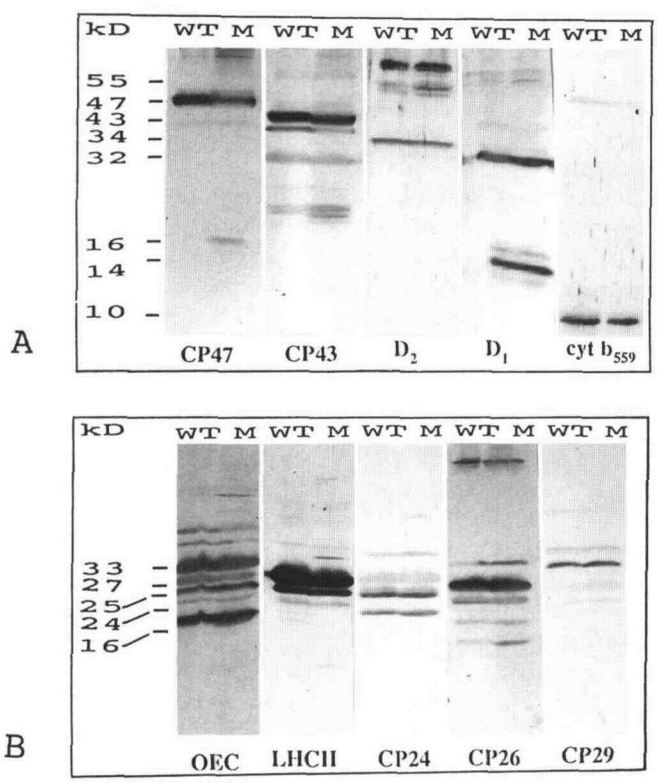

Figure 2. Immunoblot analyses of the mutant (M) and wild type (WT) with the main PSII polypeptide polyclonal antibodies. The gels for the immunoblottings were loaded with similar amounts of core (A) or with similar amounts of Chl (B) for the mutant and wild type thylakoids. Cyt b_{559} polyclonal antibodies recognized only the 10-kD subunit. The analyses were performed as reported by Barbato et al. (1991). The bands were attributed as follows: CP47, 47 kD; CP43, 43 kD; D_2, 34 kD; D_1, 32 kD; Cyt b_{559}, 10 kD; OEC, 33, 24, and 16 kD; LHCII, 27 and 25 kD; CP24, 24 kD; CP26, 26 kD; CP29, 29 kD.

Table II. *Thylakoid fractionation of the yellow-green mutant of wheat and its wild-type and relative distribution of Chl in PSII components*

Stroma lamellae and PSII particles of the mutant were obtained from thylakoids extracted in dim light. PSII particles were further fractionated by IEF according to Giardi et al. (1992). Yield in stroma lamellae and grana particles from thylakoids is relative to the recovery from the wild type. The values are typical results obtained as an average of three independent measurements in a preparation, SE approximately 10%. WT, Wild type; M, mutant.

Type	Grana Fraction					
	Minor antennae	LHCII	Core	LHCP/core	Grana	Stroma Lamellae
	% Chl				Yield	
M	13	30	57	0.75	35	40
WT	14	63	23	3.30	100	100

incorporated into the mutant was considerably different compared to the wild type (Fig. 4). For the wild type the phosphorylation pattern was typical of that observed in higher plants: radioactivity was incorporated into CP43, D_2, D_1, LHCII, 22-kD, 9-kD, 8-kD, and 5-kD proteins. The latter proteins were attributed, on the basis of previous studies, perhaps to a D_1 breakdown product (22 kD; Mattoo et al., 1989), a peripheral component of OEC (9 kD, Ikeuchi et al., 1987), and the *psb*H gene product (8 kD, Bennett, 1991). The attribution of the 5-kD protein is problematic, being in the molecular weight region of both the *psb*I gene product and the β subunit of Cyt b_{559} (Satoh, 1992).

In the present study, no efforts were made to clearly identify the phosphorylated proteins in the low molecular weight region. The mutant showed an altered phosphorylation pattern; there was almost no radioactivity incorporated into the CP43, D_2, and 9-kD proteins, whereas a high level of radioactivity was incorporated into the D_1, 22-kD, 8-kD, and 5-kD proteins (Fig. 4). Despite the depletion of a 64-kD kinase, the mutant LHCII showed some degree of phosphorylation (Figs. 4 and 5). The phosphorylation state

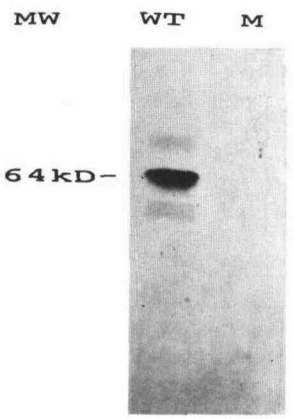

Figure 3. Immunoblot analysis of the mutant (M) and wild-type (WT) thylakoids with 64-kD LHCII kinase antibodies. The gel for the blotting was loaded with a similar amount of wild-type and mutant thylakoid Chl. The analysis was performed according to Hodges et al. (1990). MW, Molecular weight.

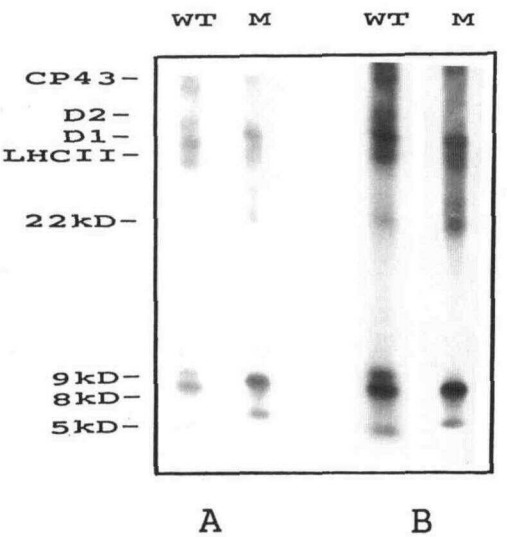

Figure 4. Autoradiography of an SDS-PAGE analysis of the mutant (M) and wild-type (WT) thylakoids isolated from ^{32}Pi-treated plants. The two genotypes were compared on the basis of similar amounts of thylakoid Chl. Two exposition times of the film are reported: A, 5 d; B, 15 d.

of the mutant was also tested using monoclonal antibodies against phosphorylated Thr (Fig. 5). These antibodies were observed to react with the proteins attributed to CP47, CP43, D_2, and LHCP, and to react very slightly with D_1 and two proteins of low molecular mass (9 to 8 kD). Moreover, they reacted with some proteins in the molecular mass region of 25 to 20 kD, in part attributed to minor antennae components (Melis, 1991; Horton and Ruban, 1992). The main differences between the mutant and the wild type were observed in the reaction of CP43 and D_2 proteins, which was almost absent in the mutant (confirming what was seen by the incorporation of radioactive Pi above). It is noteworthy that the reaction of a serial dilution of the mutant and wild-type thylakoids with antibodies shown in Figure 5 refers to gels in which the lanes were loaded with a similar amount of Chl from the two genotypes; this means, as shown in the SDS-PAGE analysis of Figure 1 and fractionation of PSII components in Table II, a greater amount of core polypeptide for the mutant. Thus, the phosphorylation differences per unit of protein are probably more accentuated than can be seen in Figure 5.

Applying the IEF method followed by Suc gradient centrifugation (Giardi et al., 1991, 1992), it was found that the mutant lacked the usual pattern of four phosphorylated PSII core populations. The five core polypeptides CP43, D_2, D_1, 9 kD, and 8 kD were phosphorylated in the wild type, whereas only the two proteins D_1 and 8 kD were phosphorylated in the mutant (Table III; Figs. 4 and 5). In Table III the pI values, the Suc gradient positions of the PSII core populations, and the phosphate incorporation into the core proteins of the mutant and wild type are reported. In the mutant, a preponderant PSII core population e (about 80%) and small quantities of core populations f and g were obtained, instead of two main populations a and b and small quantities of c and d (Fig. 6; Table III); in particular,

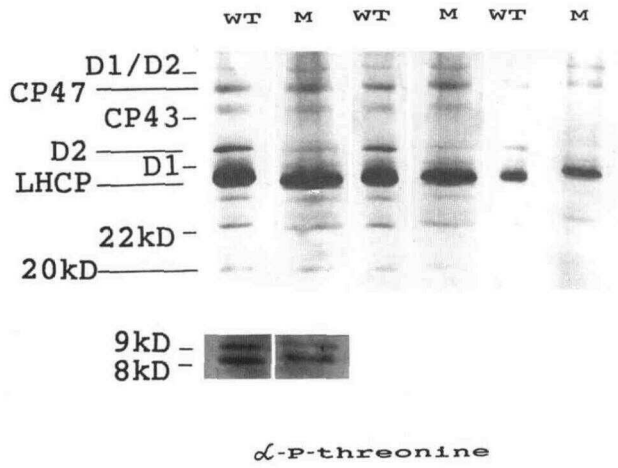

Figure 5. Immunoblot analysis with monoclonal phosphothreonine antibodies of a serial dilution of the mutant (M) and wild-type (WT) thylakoids. The gels for immunoblot analyses were loaded with similar amounts of Chl for the mutant and wild-type samples.

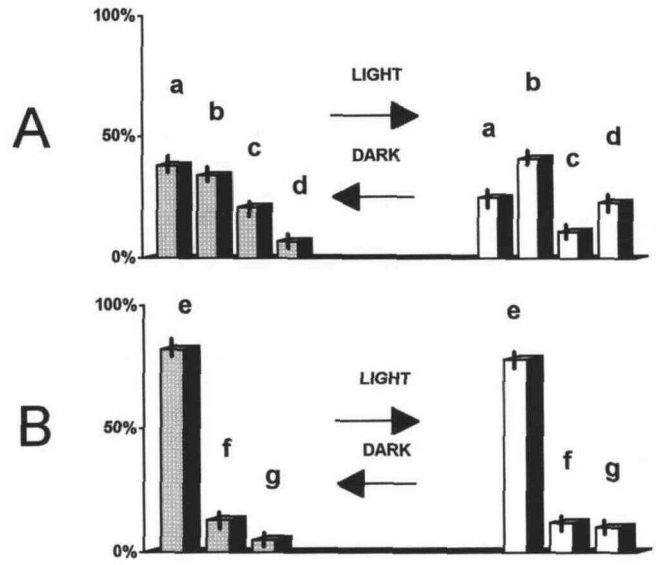

Figure 6. Distribution of the PSII core populations, measured as percentage of Chl of the populations, in light-dark conditions for the wild type (A) and the mutant (B). The values are the means ± SE from at least four Chl measurements in a preparation. The plots show typical results using conditions of light and of extraction as reported in "Materials and Methods."

a PSII core population similar to population b, with Suc gradient position at 0.57 M of Suc, was absent (Table III). The least-phosphorylated population e of the mutant, compared to the least-phosphorylated population of the wild type, shifted toward more negative pI values, as expected by the lack of phosphorylation, whereas the shift was not observed in the minor populations f and g (Table III). This occurrence can be explained by the observation that the 8-kD protein has a differential association in the PSII core of the mutant. In previous studies (Giardi, 1993b) it was shown that in a core population the pI is determined mainly by the relative content of the 8-kD protein, as well as the extent of phosphorylation of the other core proteins. Figure 6 shows the distribution of the Chl relative to the phosphorylated PSII cores for the mutant and the wild type under light and dark conditions.

Chl Fluorescence Induction upon Continuous Illumination and Fluorescence upon Flash Illumination

The room-temperature Chl fluorescence characteristics of the yellow-green mutant and of the wild type were compared. Since the thylakoids from wheat seedlings are easily damaged and give very poor electron transfer and F_v/F_m (data not shown), fluorescence measurements were carried out in vivo. After 1 h of dark adaptation, leaves of the mutant exhibited a significantly higher F_v/F_m ratio than the wild type (Table IV). We observed that this dif-

Table III. *Distribution of radiolabeled phosphate incorporated into polypeptides of the phosphorylated heterogeneous cores in the mutant of wheat and its wild type*

PSII particles were isolated from wheat thylakoids radiolabeled with ^{32}Pi (9000 Ci/mmol) in the light, and then PSII cores were isolated from these particles as described in "Materials and Methods." The values, in arbitrary units, were obtained by dividing each individual polypeptide area of the autoradiography densitogram by the total area of polypeptides obtained from the corresponding densitogram of a Coomassie blue-stained SDS-polyacrylamide gel. SG, Position in Suc gradient expressed as molarity of Suc. nr, Not revealed. Experiments were repeated twice and reproducible results were obtained. Mean values are given for three measurements of one typical preparation, SE approximately 12%.

pI, SG, or Phosphate Incorporation	PSII Core Populations						
	Wild type				Mutant		
	a	b	c	d	e	f	g
pI	5.3	4.9	4.8	4.7	5.8	4.8	4.6
SG	0.35	0.57	0.78	0.50	0.30	0.75	0.58
Polypeptide phosphate incorporation							
D_1/D_2 (heterodimer)	1	1	0	0	1	nr	nr
CP43	3	3	5	2	nr	nr	nr
D_2	2	10	15	25	nr	nr	nr
D_1	1	13	26	40	5	12	33
9 to 8 kD	10	2	0	0	7	0	0

ference persists all along the leaf area (data not shown). This difference can be explained by a lower amount of PSI, which preferentially affects F_o level. As judged by the so-called O-I rise in Chl fluorescence induced by low light intensities, both the wild-type and mutant plants contained similar amounts of non-B-type PSII reaction centers (Table IV). However, the mutant plants differed from the wild-type plants in that the area above the fluorescence induction curve in the presence of DCMU could be fitted by a single component. The mutant did not show the slow β phase, and the fast α phase was slower than in the wild type, as would be expected if certain LHCII polypeptides were missing in the mutant (Fig. 7; Table IV). Since the leaves of the two types of plant were not identical in either color or thickness, the possibility that the observed differences were artifactual was controlled for by carrying out similar measurements on another yellow-green mutant of barley as described by Peter and Thornber (1991). This time the wild-type and mutant plants both required two components to fit the overall induction curve (data not shown). It was not possible to compare the fluorescence induction heterogeneity on chloroplasts or thylakoids, since these isolated membranes from wheat wild-type seedlings and both wheat and barley mutants do not show fluorescence heterogeneity (data not shown; Ghirardi et al., 1986).

Since DCMU penetration and translocation into the leaf could differ in the two genotypes and this could result in artifactual information on PSII heterogeneity, Q_A photoreduction in 1-h dark-adapted leaves was also analyzed as previously described by Genty et al. (1990), using an approach introduced by Falkowsky et al. (1986). This approach analyzes the light dosage-response curve of the Chl fluorescence yield increase if induced by a single turnover flash of intensity I. The plot of ΔF normalized to the maximal value, ΔFmax, obtained with the saturating flash intensity Imax versus I/Imax, is described by the exponential function $\Delta F/\Delta F\text{max} = 1 - e^{\sigma(I/I\text{max})}$. At a given intensity σ is determined both by the antenna size and by photochemical efficiency of PSII in order to perform a stabilized charge separation. A semilog plot of the complement of $\Delta F/\Delta F$max versus I/Imax gives similar information to that obtained by the analysis of the fluorescence induction in the presence of saturating concentrations of DCMU. However, in this one-turnover method, the product of light intensity and time of illumination is increased by enhancement of the flash intensity. Kinetics of Chl decays after a saturating one-turnover flash has also been measured.

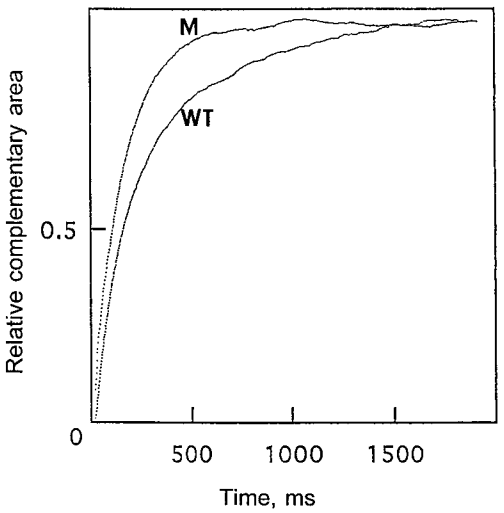

Figure 7. Fluorescence induction kinetics of wild-type (WT) and mutant (M) leaves in the presence of DCMU. Intact leaves were infiltrated with DCMU and dark adapted before carrying out the fluorescence induction measurements. The complementary area above the induction curve indicative of Q_A reduction was calculated and analyzed using a single or double exponential model. The quantitative analysis and reproducibility are reported in Table IV.

Figure 8 shows the increases of PSII fluorescence upon a single turnover flash (A) and the corresponding semilog plot analysis (B) in the wild-type and the wheat mutant leaves. Half increase of fluorescence ($\Delta F = 1/2\ \Delta F$max) requires a higher flash intensity in the mutant than in the wild type (Fig. 8A). As F_v/F_m are only slightly different in the two leaves (Table IV), this increase probably reflects essentially the lower content in LHCII of the mutant (Table II). Figure 8B and Table IV show that two phases of Q_A photoreduction are observed in the wild-type leaves, but that only a single component is present in the mutant, confirming what was previously seen in the case of fluorescence induction in continuous light.

Table IV. *Comparison of different Chl fluorescence parameters of wild-type and mutant wheat leaves*

IΔFmax/2 is the relative flash intensity induced by a saturating flash. The analyses were repeated at least three times and reproducible results were obtained.

Fluorescence Parameter	Wild Type	Mutant
F_v/F_m	0.79 ± 0.02	0.86 ± 0.01
$F_{o\text{-}i}/F_v$	0.038 ± 0.009	0.031 ± 0.008
Complementary area of fluorescence induction in the presence of DCMU		
α	108 ms (0.42)	140 ms (1.00)
β	430 ms (0.58)	–
IΔFmax/2 (amplitude)	0.05 (0.65)	0.13 (1.00)
	0.23 (0.35)	

DISCUSSION

The reported in vivo functional heterogeneity of PSII derives, for the main part, from the following observations. (a) Mathematical analysis of fluorescence induction curves of DCMU-treated thylakoids and leaves show two-three-four phases (Hodges and Barber, 1986; Hsu and Lee, 1991, 1995; Sinclair et al., 1991). (b) Certain phases are saturated by different concentrations of herbicides (Black and Horton, 1986; Hodges and Barber, 1986). (c) The relative distribution of the four phases is regulated by light conditions (Sinclair et al., 1991) apparently in a manner similar to that observed for the phosphorylated PSII core populations (for comparison, see Sinclair et al., 1991). (d) The phases have different sensitivities to strong photoinhibitory light able to

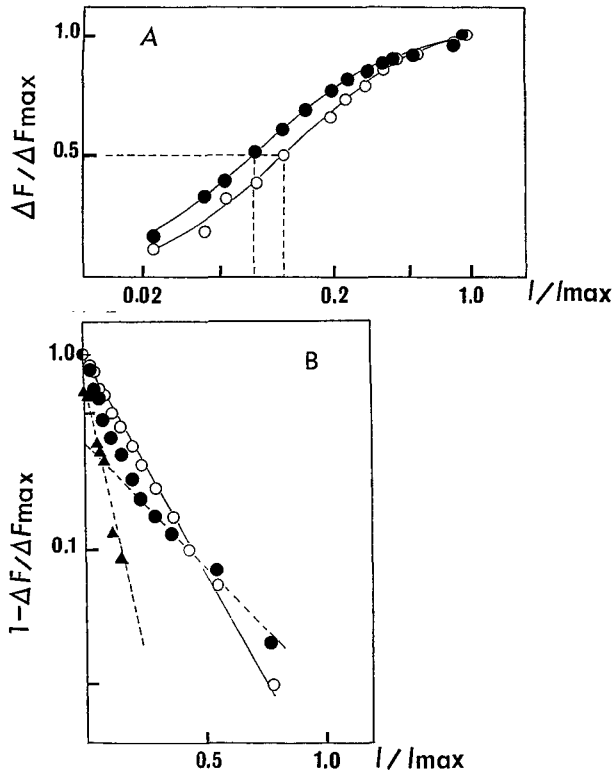

Figure 8. A, Relative increase of PSII fluorescence upon a single turnover flash (ΔF/ΔFmax versus I/Imax, I being the intensity of the flash). B, Semilog plot analysis (complement of ΔF/ΔFmax versus I/Imax). Filled and open symbols represent data obtained with wild-type and mutant wheat leaves, respectively. The analyses were repeated at least three times, and the plots show typical results.

induce photoinhibition (Habash and Baker, 1990; Wijk et al., 1993). (e) Phosphorylation of thylakoids mediated by light-induced kinase activity decreases the fast phase (Telfer et al., 1983; Hodges et al., 1987). It causes destabilization of the anionic semiquinone Q_B^- (Hodges et al., 1987). (f) A certain fraction (less than 30%) of PSII reaction centers has been shown to be unable to transfer electrons to the secondary quinonic acceptor plastoquinone (Graan and Ort, 1986; Chylla and Whitmarsh, 1989; Guenther and Melis, 1990; Ort and Whitmarsh, 1990; Sinclair and Spence, 1990; Sinclair et al., 1991; Lavergne and Leci, 1993). (g) Fluorescence induction curves of DCMU-treated grana particles show at least two phases (Hodges et al., 1985; Hodges and Barber, 1986).

Biochemical evidence of PSII core phosphorylation heterogeneity has been presented recently (Giardi et al., 1991, 1992; Giardi, 1993). So far, the knowledge concerning the properties of differently phosphorylated isolated PSII core populations can be summarized as follows. (a) In grana particles four differently phosphorylated PSII core populations showing different degrees of affinity for photosynthetic herbicides are present (Giardi et al., 1992). (b) These PSII core populations also show differing abilities to transfer electrons from DPC to DCPIP. The most phosphorylated PSII population (**d**), which accounts for about 30% of the total PSII core in light-adapted plants, is not active in electron transfer (Giardi et al., 1991, 1992). (c) The relative distribution of these populations is partially regulated by light, the most phosphorylated PSII core population (**d**) being almost absent in dark conditions (Giardi et al., 1992). (d) The four PSII core populations show differing sensitivity to photoinhibitory conditions when the interconversion of the populations is blocked (Giardi, 1993).

In the present study we demonstrate that the relative distribution of the phosphorylated PSII core populations is partially modified by the content of Mg^{2+}, the presence of herbicides, and the presence of MAIM during extraction of thylakoids (Table I), conditions that are known to alter the activity of light-induced protein kinase.

Under dark conditions at least two differently phosphorylated PSII core populations are always present, indicating the existence of a phosphorylation component that is never completely reversed. This occurrence is in accordance with the observation that radioactivity is still found in the core proteins CP43 and 8 kD after dark incubation (data not shown; Giardi et al., 1994). This is also in agreement with recent observations that at least two kinetically distinguishable classes of phosphoprotein phosphatase reactions are present in the thylakoid membrane (Silverstein et al., 1993a). It is also observed that the dim light present in the laboratory during extraction of dark-adapted plants is able to induce phosphorylation of PSII core polypeptides (Table I).

The pattern of four phosphorylated PSII core populations appears to be ubiquitous in normal higher plants, whereas it differs in a Chl-deficient mutant of wheat (Fig. 6) that does not show a certain functional PSII heterogeneity in vivo (Figs. 7 and 8; Table IV). In a previous work, the mutant of wheat was observed to have a slightly higher Chl *a*/*b* ratio, and a reduced quantity of PSI component measured as Chl/P700 ratio. This last result is consistent with the slightly higher F_v/F_m ratio observed in the mutant compared to the wild-type wheat leaves. The PSII component, measured as content of Q_B, was only marginally reduced compared to the wild type. The mutant maintained a high capacity for electron transfer and a high rate of CO_2 assimilation at saturating light intensities (Di Marco et al., 1989).

In this study we also show that the mutant contains a PSII polypeptide composition qualitatively similar to that of the wild type except for the presence of possible CP47 and CP43 breakdown products, and a depletion in a membrane-bound protein 64 kD attributed to LHCII kinase (Coughlan and Hind, 1986). Moreover, it lacks the normal pattern of four phosphorylated PSII core populations usually observed in higher plants (Fig. 6; Tables I–IV). In particular, a PSII core population with a pI and Suc gradient position close to population b is absent. It shows a preponderant PSII population e (more than 80% in the dark). Therefore, the whole phosphorylation pattern is changed in the mutant.

The main differences observed were the absence of light-induced phosphorylation of CP43 and D_2 (which could be correlated to the lack of two main phosphorylated popu-

lations) (Figs. 1–7). These experimental observations on the existence of differently phosphorylated PSII cores isolated by IEF are in accordance with the following considerations: five proteins (CP43, D_2, D_1, 9 kD, and 8 kD) are observed to be phosphorylated in the wild type so that in a PSII core, one, two, three, or four, etc., proteins are phosphorylated, resulting in a pattern of four main populations composed of PSII cores with slightly different charge and pI values, in which two populations are preponderant. In the mutant only two proteins are phosphorylated (D_1 and 8 kD), so that the phosphorylation combinations are fewer than those possible for the wild type, and result in a pattern of only three populations, in which one is preponderant.

The phosphorylation process is regulated by steric and energetic factors (Bennett, 1991; Allen, 1992), and some PSII core populations seem to be the most favored. Experimentally we observed that population a of the wild type and population e of the mutant mainly contain 8-kD phosphoprotein (Table III). A possible explanation for the altered phosphorylation in the mutant is that the depletion in both LHCII and LHCII kinase influence the phosphorylation of the other PSII polypeptides, the action of the kinases being mutually interactive by charge and steric factors. It is known that the introduction of phosphate may sufficiently modify the topography of the membrane surface so as to inhibit the interaction of a protein kinase with its target proteins (Lin et al., 1982; Owens and Ohad, 1982; Bennett, 1991; Allen, 1992). It has recently been shown that multiple kinases are present and that different types can be responsible for phosphorylating LHCII or PSII core polypeptides (Silverstein et al., 1993b). Our present results indicate that the kinase(s) for D_2 and CP43 are probably different from the kinase(s) responsible for phosphorylation of the D_1 and 8-kD proteins. Moreover, the presence of some LHCII phosphorylation in the mutant seems to suggest that the studied 64-kD polypeptide attributed to the LHCII kinase (Coughlan and Hind, 1986) is possibly not the only kinase responsible for phosphorylating the main PSII antennae. However, due to the lack of information concerning the kinases acting on PSII proteins, the real reason for the altered mutant phosphorylation pattern is at present unknown. Despite the partial absence of phosphorylation, the structure of the mutant is still defined by appressed and nonappressed regions (Table II; data not shown). The phosphorylation of the CP43 and D_2 polypeptides does not seem to be essential for PSII electron transfer activity, since the mutant, in which the phosphorylation of these proteins is absent, maintains a high capacity for electron transfer (Di Marco et al., 1989). It has been suggested that phosphorylation has a protective effect on degradation of the D_1 protein due to strong illumination (Rintamäki et al., 1995).

Under physiological light it is known that the D_1 protein undergoes a rapid cycle of degradation and repair (Mattoo et al., 1989; Satoh, 1992); which could be a reason for the heterogeneity of PSII (Guenther and Melis, 1990). The unusual presence of possible CP47 and CP43 breakdown products in the mutant (Fig. 2) indicates that perhaps the turnover of the PSII core is altered in this genotype, in accordance with the idea that phosphorylation is involved in PSII turnover (Elich et al., 1992, 1993) and with the suggestion that in vivo PSII heterogeneity is also related to this cycle (Guenther and Melis, 1990).

In conclusion, a correlation between the PSII core phosphorylation and the functional heterogeneity described by PSII variable fluorescence seems to exist. We suggest that some of the observations on the functional heterogeneity above could be explained by the finding of differently phosphorylated PSII core populations.

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