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Thermostability and Photostability of Photosystem II in Leaves of the *Chlorina-f2* Barley Mutant Deficient in Light-Harvesting Chlorophyll *a/b* Protein Complexes

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The chlorophyll-*b*-less *chlorina-f2* barley mutant is deficient in the major as well as some minor light-harvesting chlorophyll-protein complexes of photosystem II (LHCII). Although the LHCII deficiency had relatively minor repercussions on the leaf photosynthetic performances, the responses of photosystem II (PSII) to elevated temperatures and to bright light were markedly modified. The *chlorina-f2* mutation noticeably reduced the thermostability of PSII, with thermal denaturation of PSII starting at about 35°C and 38.5°C in *chlorina-f2* and in the wild type, respectively. The increased susceptibility of PSII to heat stress in *chlorina-f2* leaves was due to the weakness of its electron donor side, with moderate heat stress causing detachment of the 33-kD extrinsic PSII protein from the oxygen-evolving complex. Prolonged dark adaptation of *chlorina-f2* leaves was also observed to inhibit the PSII donor side. However, weak illumination slowly reversed the dark-induced inhibition of PSII in *chlorina-f2* and cancelled the difference in PSII thermostability observed between *chlorina-f2* and wild-type leaves. The mutant was more sensitive to photoinhibition than the wild type, with strong light stress impairing the PSII donor side in *chlorina-f2* but not in the wild type. This difference was not observed in anaerobiosis or in the presence of 3-(3,4-dichlorophenyl)- 1,1-dimethylurea, diuron. The acceptor side of PSII was only slightly affected by the mutation and/or the aforementioned stress conditions. Taken together, our results indicate that LHCII stabilize the PSII complexes and maintain the water-oxidizing system in a functional state under varying environmental conditions.

In higher plant chloroplasts, photoreduction of $NADP^+$ is driven by the tandem operation of PSII and PSI. The multi-subunit PSII complex spans the thylakoid membrane and functions as a water-plastoquinone oxidoreductase, oxidizing water at the lumen side of the membrane and reducing plastoquinone at the stromal side (Hansson and Wydrzynski, 1990). PSII is composed of the CCII, which is surrounded by a series of LHCII (Thornber et al., 1992). LHCIIb is the major chlorophyll-protein complex that exists as a trimer in vivo and binds approximately 40% of the chlorophyll in the thylakoid membranes and 65% of the PSII-chlorophylls (Kühlbrandt, 1994; Paulsen, 1995). LHCIIb serves as an additional and extensive light-harvesting system, transferring the absorbed light energy to P680 via the minor chlorophyll antennae. Aside from this primary light-harvesting role in photosynthesis, LHCIIb fulfills various regulatory functions related to the long- and short-term adaptation of plants to their light environment. For example, in shade-adapted plants light absorption is optimized by increasing the size of the peripheral LHCIIb complex, whereas long-term adaptation to elevated light intensities is accompanied by a decreased light-absorption capacity of PSII (Melis, 1991). LHCIIb is also involved in thylakoid membrane stacking (Barber, 1980) and in the rapid regulation of light energy distribution between the two photosystems through the state-transition phenomenon (Allen, 1992). Recently, the major as well as the minor PSII antennae have been implicated in the protection of PSII against photoinhibition. Acidification of the thylakoid lumen in strong light is believed to induce structural changes in the PSII antennae, which induce thermal energy dissipation, thus reducing energy delivery to the sensitive reaction center (Horton et al., 1994; Ruban et al., 1996). These structural changes are presumably favored by the light-induced conversion of the carotenoid violaxanthin (an accessory pigment of the PSII antennae) to zeaxanthin.

The attachment of LHCIIb to the PSII complex is fragile and is rapidly disrupted at elevated temperatures (Armond et al., 1978, 1980; Sundby et al., 1986). However, the T_c is influenced by the environmental conditions. For instance, T_c is noticeably increased when leaves are pre-exposed to moderately elevated temperatures (Havaux, 1993a). The chlorophyll-fluorescence and light-absorption characteristics of such preheated leaves are reminiscent of the fluorescence and absorbance changes that accompany the violaxanthin-to-zeaxanthin conversion in leaves exposed to strong light (Havaux and Tardy, 1996). This finding has led

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Abbreviations: CC, core complex; CCII, chlorophyll-*a*-containing reaction center core of PSII; F_m, maximal level of chlorophyll fluorescence; F_o, initial level of chlorophyll fluorescence; F_v/F_m, ratio of variable chlorophyll fluorescence to the maximal fluorescence level which is a measure of the maximal quantum yield of PSII photochemistry; LHCII, light-harvesting complex of PSII; LHCIIb, major LHCII; O-I_1-I_2-m, polyphasic rise of chlorophyll fluorescence in supersaturating white light; P680, reaction center pigment of PSII; Q_A and Q_B, primary and secondary (quinone) electron acceptor of PSII; $t_{1/2}$, half-time of the I_1-I_2 fluorescence induction phase; T_c, temperature at which PSII starts to denature.

to the suggestion that the conformation of the PSII antennae could influence the heat stability of PSII. A recent in vitro study of subchloroplast preparations showed an apparent correlation between the level of PSII thermoresistance and the amount of LHCIIb associated with PSII in the preparations (Shutilova et al., 1995).

In this study, we compared the in vivo responses of PSII to changes in light and temperature in leaves of two barley genotypes: the wild type (cv Plaisant) and the pale-green *chlorina-f2* mutant. The latter genotype is one of the most investigated pigment mutants and is devoid of chlorophyll *b*, causing a complete loss of LHCIIb, the major light-harvesting chlorophyll antenna of PSII (Thornber and Highkin, 1974). Mature *chlorina-f2* leaves have intact CCII units (in terms of polypeptide composition) (Preiss and Thornber, 1995). The effect of the lack of chlorophyll *b* on the minor LHCIIs is less clear. Krol et al. (1995) have found that *chlorina-f2* PSII lacks LHCIId (CP24) and has strongly reduced amounts of LHCIIa (CP29). In contrast, in another study (Preiss and Thornber, 1995) the amount of the minor LHCIIs was strongly reduced but not to zero. The PSII light-harvesting antenna size of *chlorina-f2* chloroplasts has been estimated to be only 20% of that of wild-type plants, whereas PSI antennae are much less affected (Ghirardi et al., 1986). The results presented here show that the LHCII deficiency in *chlorina-f2* chloroplasts was associated with a marked reduction of the in vivo photostability and thermostability of PSII. Our data suggest that this effect is due to the weakness of the PSII electron donor side when the LHCII system is strongly reduced. Therefore, LHCII has an important stabilizing function in PSII.

MATERIALS AND METHODS

Two barley (*Hordeum vulgare* L.) genotypes were used: the wild type (cv Plaisant) and a chlorophyll-*b*-less mutant (*chlorina-f2*). Plants were grown in a greenhouse. The experiments were performed on young, fully expanded leaves (usually the second leaf of plants at age 3–5 weeks). We have confirmed by HPLC analysis of leaf pigments that leaves of our mutant have no chlorophyll *b*. Chlorophyll fluorescence measurements in the presence of DCMU revealed that they have a dramatically reduced PSII antenna size, and nondenaturing fractionation of thylakoid membranes show that they are completely deficient in LHCIIb (data not shown).

Heat and Light Treatments

Detached leaves placed on moist filter paper were warmed for 15 min at a constant temperature in darkness or in white light (100 μmol m^{-2} s^{-1}) as previously described (Havaux, 1993b): the leaf sample (plus filter paper) was placed on a block of metal, the temperature of which was adjusted by circulation of water from a thermostatted water bath. Leaf temperature was monitored with a tiny thermoresistor (PT100, Mimco, Aston, France). A light source (KL1500, Schott-Glaswerke, Mainz, Germany) equipped with a flexible light guide was used to illuminate the leaves during heat stress. Strong light stress was imposed using halogen metal halide lamps (Osram, Molsheim, France) equipped with two heat-reflecting filters (Schott-Glaswerke) and two heat-absorbing filters at 23°C in air on detached leaves placed on moist filter paper. Light stress in anaerobiosis was imposed in a thermostatted closed cell flushed with nitrogen gas containing 750 μg g^{-1} CO_2. Oxygen was monitored with an oxygen cell (91360, ABISS, Villemoisson, France). In some experiments, rectangular leaf pieces were infiltrated with 50 μM DCMU for 30 min in darkness prior to light stress. Light- and heat-treated leaves were adapted at 23°C in darkness for 15 min prior to chlorophyll fluorescence measurements. Photon flux densities (400–700 nm) and far-red light irradiance were measured with a millivolt meter (Li-185B, Li-Cor, Lincoln, NE) equipped with a quantum sensor (Li-190SB, Li-Cor) and a pyranometer sensor (Li-200SB, Li-Cor) respectively.

Chlorophyll Fluorescence

Chlorophyll fluorescence emission from the upper surface of the leaves was measured in modulated light with a fluorometer (PAM, Walz, Effeltrich, Germany). F_o was elicited with a dim red (655 nm) light modulated at 600 Hz (PAM-2000, Walz) or 1.6 kHz (PAM-101, Walz), and was measured with a photodiode at wavelengths higher than 700 nm. F_m was induced by a 800-ms pulse of intense white light (>4000 μmol m^{-2} s^{-1}). The maximal quantum yield of PSII photochemistry was calculated in dark-adapted leaves as $(F_m - F_o)/F_m = F_v/F_m$. The temperature dependence of the apparent F_o level was measured by increasing or decreasing the leaf temperature at a rate of 1°C min^{-1} or −1°C min^{-1} (unless specified otherwise). Leaf temperature was monitored with a thermistor thermometer (YSI, Yellow Springs, OH). The kinetics of chlorophyll fluorescence induction in supersaturating white light (9000 μmol m^{-2} s^{-1}) and fluorescence relaxation after a single-turnover flash were recorded with a computer using software (DA100, Walz) previously described (Havaux, 1993b). The multiple-turnover white light was supplied by a light source (KL1500E, Schott) equipped with a fast electronic shutter (Uniblitz versus 14-S2-S0, Vincent Associates, Rochester, NY) and the single-turnover flashes were produced by a discharge xenon lamp (XF-103/XST-103, Walz). The kinetics of chlorophyll-fluorescence relaxation were also measured in DCMU-poisoned leaves (50 μM) illuminated with a 2-s pulse of red light (40 μmol m^{-2} s^{-1}; PAM-102, Walz).

Photosynthetic Oxygen Evolution

The rate of photosynthetic oxygen evolution (absolute values) by leaf discs (diameter of 1.6 cm) was measured at 23°C using a Clark-type O_2 electrode (Hansatech LD2/2, King's Lynn, UK). White light (KL1500, Schott) was transmitted onto the leaf samples using a flexible light guide and the adaptor (LD/WA, Hansatech). CO_2 was generated in the closed chamber by a bicarbonate/carbonate buffer. Gross O_2 evolution was estimated as net photosynthesis-dark respiration.

The quantum yield of O_2 evolution (relative values) was estimated in modulated light with the photoacoustic method (Malkin and Canaani, 1994). The experimental setup has been described elsewhere (Havaux, 1993b). Leaf discs 1 cm in diameter were placed in the closed photoacoustic cell and illuminated with white light (30 μmol m^{-2} s^{-1}) modulated at 20 Hz. The maximal fluence rate of the white background light used to saturate photochemistry was 4000 μmol m^{-2} s^{-1}.

Polypeptide Profile of PSII

PSII-enriched membranes were prepared by the method of Bassi et al. (1985) with some modifications: the membranes were not washed with EDTA and the ratios of Triton X-100 to chlorophyll were reduced to half the values used in Bassi's original method. We observed that the degree of Mg^{2+}-induced restacking after EDTA washing was different in heat-stressed and unstressed membranes. The EDTA step was then eliminated to obtain comparable membrane preparations. Consequently, the resulting preparations of *chlorina-f2* membranes are not typical PSII particles because they contain appreciable amounts of the coupling factor. This is not problematic in this study because the extrinsic proteins of the water-oxidizing system of PSII were clearly observable. The polypeptide composition of the PSII membranes was analyzed by SDS-PAGE with a 15%-acrylamide separating gel and a 4%-acrylamide stacking gel containing 5.5 M urea. Thirty micrograms of proteins was loaded per lane. The proteins were visualized by staining with Coomassie brilliant blue and the polypeptide profiles were scanned (EASY-Store system, Herolab, Wiesloch, Germany). The abundance (intensity × area of the bands) of the proteins was determined (EASY analysis software, Herolab), with CP43 as a reference.

RESULTS

Photosynthetic Characteristics of Unstressed *Chlorina-f2* Leaves

Photosynthetic O_2 evolution was monitored in barley leaf discs with the photoacoustic method (Malkin and Canaani, 1994). Figure 1 shows that the *chlorina-f2* mutation was accompanied by a reduction in the maximal quantum yield for O_2 evolution (approximately −15%) and that the shape of the light-saturation curve of photosynthesis was changed, with the O_2 quantum yield decreasing less rapidly with increasing photon flux density in the mutant. These observations were confirmed by O_2-evolution measurements with a conventional Clark-type O_2 electrode at a high CO_2 concentration. O_2 evolution in limiting light (<500 μmol m^{-2} s^{-1}) was lowered in *chlorina-f2* leaves compared with wild-type leaves (data not shown), and O_2 evolution in the mutant was less rapidly saturated by increasing light intensity and reached a significantly higher value in strong, almost saturating light (approximately 4000 μmol m^{-2} s^{-1}): 0.118 μmol O_2 min^{-1} cm^{-2} versus 0.095 μmol O_2 min^{-1} cm^{-2}. It is possible that this enhancement of O_2 evolution in chlorophyll-*b*-less leaves could be related to a compensatory increase in their PSII content (Ghirardi et al., 1986; Ghirardi and Melis, 1988).

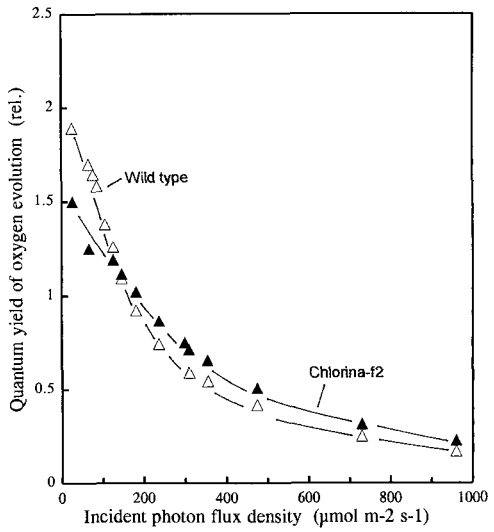

Figure 1. Light dependence of the relative quantum yield of photosynthetic O_2 evolution measured in leaves of wild-type barley (△) and *Chlorina-f2* mutant (▲) with the photoacoustic method. Different photon flux densities of background white light were added to the modulated white light (30 μmol m^{-2} s^{-1}, 20 Hz).

The maximal quantum yield for PSII photochemistry was measured by chlorophyll fluorometry (F_v/F_m ratio) in dark-adapted leaves suddenly illuminated with a 1-s flash of intense white light (9000 μmol m^{-2} s^{-1}): the barley mutant exhibited a small decrease (−6%) in the PSII quantum yield from 0.79 to 0.74, thus confirming previous results (e.g. Simpson et al., 1985; Leverenz et al., 1992). A close analysis of the kinetics of chlorophyll fluorescence induced by the intense white light revealed some interesting features of chlorophyll-*b*-less PSII. As previously described (Neubauer and Schreiber, 1987; Schreiber and Neubauer, 1987; Strasser et al., 1995), the fluorescence rise kinetics in supersaturating light displayed three distinct phases (termed O-I_1, I_1-I_2, and I_2-m, according to the nomenclature of Neubauer and Schreiber). The first (O-I_1) phase corresponds to the complete reduction of Q_A (Neubauer and Schreiber, 1987; Strasser et al., 1995), whereas the intermediate (I_1-I_2) phase has been attributed to the release of a fluorescence-quenching mechanism controlled by the electron donor side of PSII (Schreiber and Neubauer, 1987). The last phase (I_2-m) presumably corresponds to the release of the "static" fluorescence quenching by the oxidized plastoquinone pool (Vernotte et al., 1979). Treatments that primarily affect components at the PSII donor side have been demonstrated to change the secondary fluorescence rise to I_2: preillumination of the sample with single-turnover saturating flashes induced period-4 oscillations of the I_1-I_2 amplitude, and deactivation of the water-splitting enzyme system by various chemicals or by heat stress suppressed the I_1-I_2 phase (Schreiber and Neubauer, 1987; Havaux, 1993b). Therefore, the I_1-I_2 fluorescence phase is a useful indicator of the water-splitting activity, although the exact mechanism of this donor-side-

dependent quenching remains to be established (for hypothetical mechanisms, see Schreiber and Neubauer, 1987; Schreiber and Kreiger, 1996).

The O-I_1-I_2-m fluorescence transients induced by an intense white light (9000 μmol m^{-2} s^{-1}) are presumably identical to the O-J-I-P transient rise in red light reported by Strasser et al. (1995), although this has not been proven. The most prominent change associated with the *chlorina-f2* mutation was a slowdown of the I_1-I_2 phase: $t_{1/2}$ was around 7 ms in wild-type leaves and almost 11 ms in mutant leaves (see $t_{1/2}$ at time 0 in Fig. 2A). The reduction of $t_{1/2}$ in *chlorina-f2* was dependent on the dark-adaptation time, with $t_{1/2}$ substantially increasing with increasing length of the dark period (Fig. 2A). For instance, after 18 h in darkness, $t_{1/2}$ increased to 17 ms. It was also observed that the I_1-to-I_2 induction phase could be re-accelerated by illuminating leaves. When *chlorina-f2* leaves were illuminated after 5.5 h of dark adaptation, $t_{1/2}$ decreased from 15 to 11 ms within 3 h. In parallel, dark adaptation of *chlorina-f2* leaves caused a decrease in the photoacoustically measured quantum yield of O_2 evolution, which was slowly reversed in the light (Fig. 2B). Therefore, one can conclude that the electron donor side of PSII, as monitored by the I_1-to-I_2 fluorescence rising phase, was somewhat

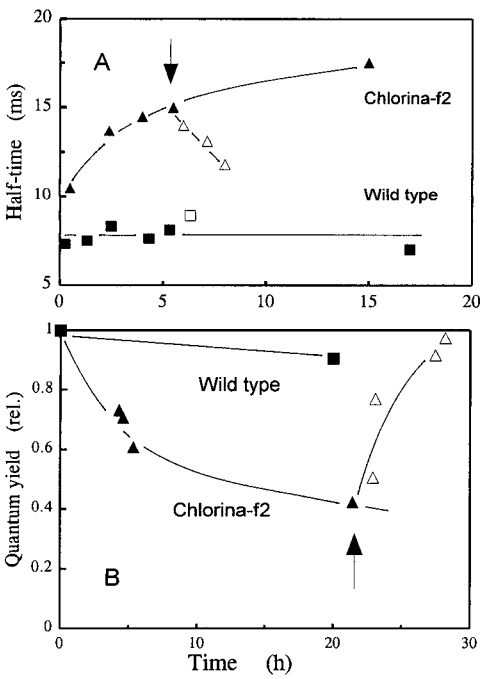

Figure 2. A, Effect of dark adaptation (closed symbols) on the half-time of the I_1-I_2 phase of the chlorophyll-fluorescence induction in barley leaves suddenly illuminated with a supersaturating white light (9000 μmol m^{-2} s^{-1}). Wild type, □ and ■; *Chlorina-f2*, △ an ▲; open symbols, dark-adapted leaves were illuminated with a white light (150 μmol m^{-2} s^{-1}) at the time indicated by the arrow. B, Relative quantum yield of photosynthetic oxygen evolution in barley leaves (wild type, ■; *chlorina-f2* mutant, △ and ▲) adapted to darkness (closed symbols) and subsequently exposed to white light (150 μmol m^{-2} s^{-1}, △). Photoacoustic measurements were performed with a modulated white light of 70 μmol m^{-2} s^{-1}.

Table I. *Half-time of the dark reoxidation of reduced Q_A^- in DCMU-poisoned barley leaves (control and chlorina-f2) before and after 4-h adaptation to darkness*

Q_A was reduced by a 2-s pulse of red light (40 μmol m^{-2} s^{-1}). Data are mean values ± SD. Numbers in parentheses indicate the number of independent experiments.

Treatment	Half-Time (s)
Wild type	
Before	1.02 ± 0.06 (6)
After dark adaptation	1.24 ± 0.11 (5)
Chlorina-f2	
Before	1.54 ± 0.15 (4)
After dark adaptation	2.72 ± 0.19 (4)

altered in the mutant and that this alteration was amplified in darkness.

This conclusion is supported by the data in Table I. Q_A was reduced in DCMU-treated leaves with a pulse of red light and the rate of Q_A^- reoxidation in the dark was estimated from the kinetics of chlorophyll-fluorescence decay. In DCMU-treated leaves, reduced Q_A is oxidized by a recombination with positive charges at the PSII donor side. Table I shows the $t_{1/2}$ of charge recombination in barley leaves before and after long dark adaptation. The measured values are only apparent $t_{1/2}$ because, in reality, the chlorophyll-fluorescence decay is polyphasic (Chu et al., 1994). We observed that Q_A^- oxidation was slower in the mutant than in the wild type (approximately 1.5 s versus 1 s) and that this difference was further amplified after prolonged dark adaptation (2.7 s versus 1.2 s). These findings confirm that the *chlorina-f2* mutation affects the PSII donor side. The effect of the mutation on Q_A^- reoxidation kinetics is qualitatively similar to that reported for the Δ*psb*O strain of the cyanobacterium *Synechocystis*, which lacks the PSII extrinsic 33-kD polypeptide (Chu et al., 1994).

The acceptor side of PSII was investigated in barley leaves by measuring the kinetics of chlorophyll-fluorescence decay after a short flash (4 μs) of intense white light causing single turnover at PSII. Chlorophyll-fluorescence decay after a single-turnover flash is attributed to electron transfer from Q_A^- to Q_B (in the microsecond time scale), to the variable concentration of Q_A^-, which is in equilibrium with Q_B^- (in the millisecond time scale), and to a slow back-reaction of Q_A^- with the oxygen-evolving state S2 in the centers in which Q_A is not connected to Q_B and the plastoquinone pool (e.g. see Cao and Govindjee, 1990). The kinetics of the fluorescence decay in wild-type and *chlorina-f2* leaves were very similar and no significant effect of prolonged dark adaptation was noticed in the fluorescence decay kinetics of both types of leaves (data not shown).

Heat and Light Stresses

PSII is known for its vulnerability to elevated temperatures. The temperature at which PSII denatures corresponds to the temperature (T_c) at which the apparent F_o level of chlorophyll fluorescence starts to increase. It is generally believed that heat-induced increases in F_o mainly reflect the dissociation of LHCIIb from CCII (Schreiber and

Berry, 1977; Armond et al., 1978). Figure 3 shows the temperature dependence of chlorophyll fluorescence elicited by a dim red-light beam (corresponding to F_o at 25°C) in wild-type and *chlorina-f2* barley ("F-T curve"). In wild-type leaves (Fig. 3A), increasing temperature caused a 3-fold increase in the chlorophyll-fluorescence amplitude. The threshold temperature (T_c) above which chlorophyll fluorescence starts to increase averaged 38.5 ± 0.5°C. When leaf temperature was decreased back to 25°C, the fluorescence enhancement was largely reversible. In *chlorina-f2* leaves (Fig. 3B), the chlorophyll-fluorescence increase was comparatively small and showed little reversibility. The amplitude of the irreversible component of the fluorescence

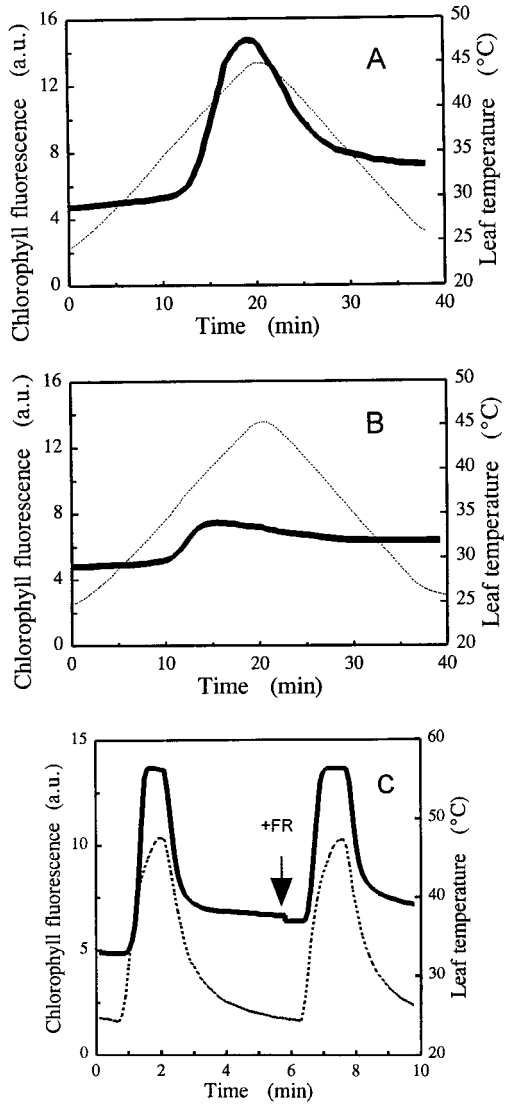

Figure 3. Chlorophyll-fluorescence emission (——) excited in barley leaves (A, wild type; B, *chlorina-f2*) by a dim modulated light during slow heating (from 25°C to 45°C) followed by slow cooling (from 45°C to 25°C). - - - - - -, Leaf temperature. C, Chlorophyll-fluorescence emission in wild-type barley leaves rapidly heated from 25 to 45°C and then suddenly cooled down to 25°C in the absence and the presence of far-red light (FR, 735 nm, 7 W m^{-2}).

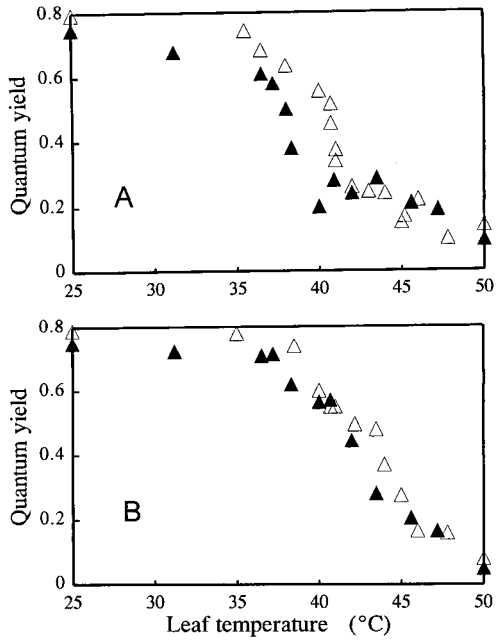

Figure 4. Maximal quantum yield of PSII photochemistry (F_v/F_m) in barley leaves (wild type, △; *chlorina-f2*, ▲) pretreated for 15 min at different elevated temperatures in darkness (A) or in white light (B) (100 μmol m^{-2} s^{-1}).

change was roughly similar in *chlorina-f2* and the wild type. The T_c for PSII denaturation in *chlorina-f2* was determined to be 34.8 ± 1.1°C, indicating that the thermal stability of PSII is lower in this genotype than in the wild type. Figure 3C shows that temperature-dependent LHCIIb disconnection/reconnection from/to CCII was almost instantaneous and was insensitive to PSI-exciting far-red light. This latter observation excludes the possibility that the fluorescence increase was due to a massive accumulation of Q_A^- in the dark. The F_o enhancement in wild-type leaves was rapidly reversible, even after a "long" exposure of around 20 min at 45°C (data not shown).

In Figure 4, we have measured the maximal quantum yield (F_v/F_m) for PSII photochemistry in barley leaves exposed for 15 min to different temperatures. When the temperature treatment was imposed in darkness (Fig. 4A), the temperature dependence curve of F_v/F_m in *chlorina-f2* leaves was shifted by about 3°C toward lower temperatures compared with wild-type leaves, thus confirming the differential heat resistance of PSII in the two types of plants. When leaves were treated in the light (Fig. 4B), PSII was more resistant to heat stress (+2.5°C in the wild type and +4.5°C in the mutant compared with the PSII thermostability in darkness) and the difference in PSII thermotolerance between wild-type and *chlorina-f2* leaves was strongly reduced.

Barley leaves were exposed for 30 min to high photon flux densities (Fig. 5A), causing an appreciable reduction of the PSII photochemical efficiency. The photoinhibition of PSII was more pronounced in *chlorina-f2* than in wild-type leaves. For instance, a 30-min treatment at 2500 μmol m^{-2} s^{-1} reduced F_v/F_m by about 10% in wild-type leaves and

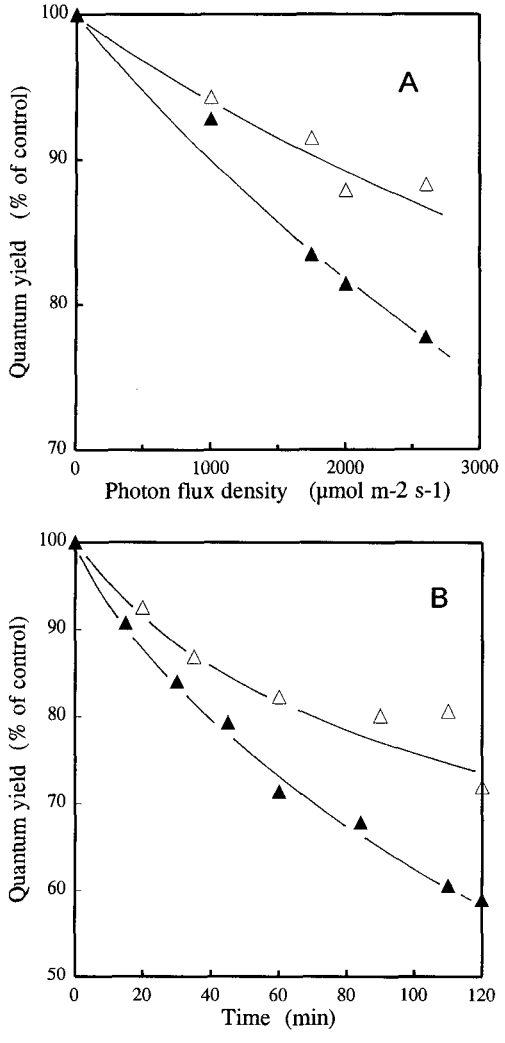

Figure 5. Maximal quantum yield of PSII photochemistry (F_v/F_m) in barley leaves (wild type, △; *chlorina-f2*, ▲) pre-exposed for 30 min to different photon flux densities of white light (A) and to a photon flux density of 2000 µmol m^{-2} s^{-1} for various periods of time (B).

20% in *chlorina-f2* leaves. Figure 5B, which shows the time course of PSII photoinhibition in mutant and wild-type leaves exposed to a strong white light of 2000 µmol m^{-2} s^{-1}, clearly confirms the decreased photostability of PSII in *chlorina-f2*. No loss of pigments was noticed during the photoinhibitory light treatment (data not shown). Inhibition of photosynthesis during exposure of *chlorina-f2* leaves to a photon flux density that was not (or was less) photoinhibitory for wild-type barley was previously observed by Leverenz et al. (1992), but not by Cleland and Melis (1987). These discrepancies could be due to differences in the growth conditions and/or the stage of chloroplast development (Preiss and Thornber, 1995). As shown in Table II, the photoinhibition enhancement observed in chlorophyll-*b*-less plants was dependent on the presence of O_2. Indeed, when light stress was imposed in anaerobiosis, photoinhibition of PSII in *chlorina-f2* was alleviated and coincided with the photoinhibition level measured in the wild type. In Figure 6, leaves were infiltrated with 50 µM DCMU

Table II. *Effect of strong light stress (2000 µmol m^{-2} s^{-1} for 2 h at 23°C) in air or in anaerobiosis (730 µg g^{-1} CO_2 in nitrogen gas) on F_v/F_m in wild-type and chlorina-f2 barley leaves*

Data are mean values ± SD. Numbers in parentheses indicate the number of experiments.

Treatment	Wild Type	*Chlorina-f2*
Before	0.79 ± 0.01 (9)	0.77 ± 0 (7)
After strong light stress		
In air	0.57 ± 0.03 (9)	0.45 ± 0.08 (9)
In anaerobiosis	0.51 ± 0.06 (9)	0.56 ± 0.05 (7)

before light stress. Herbicide-induced block of the electron flow from Q_A^- to Q_B results in over-reduction of Q_A in strong light, leading to "acceptor side photoinhibition" (Barber and Andersson, 1992; Prasil et al., 1992; Aro et al., 1993). Although DCMU exacerbated PSII photoinhibition at 2000 µmol m^{-2} s^{-1}, the PSII of *chlorina-f2* leaves was not more photoinhibited than the PSII of wild-type leaves; in fact, *chlorina-f2* tended to be slightly less inhibited than wild-type plants.

Photoinhibition of PSII in *chlorina-f2* leaves was associated with a noticeable inhibition of the PSII donor side, as indicated by the long $t_{1/2}$ (around 60 ms) of the I_1-I_2 phase of chlorophyll-fluorescence induction (Table III). In contrast, strong light had no effect on $t_{1/2}$ in wild-type barley leaves, suggesting that PSII photoinhibition in these leaves is of a different nature than that occurring in *chlorina-f2* leaves. As expected (Nash et al., 1985; Havaux, 1993b; Enami et al., 1994), the PSII donor side, as monitored by the $t_{1/2}$ value, was also destroyed by heat stress and this effect was alleviated by the presence of light during the heat treatment. However, whether heat stress was imposed in the light or in the dark, electron donation to PSII suffered less in wild-type plants than in chlorophyll-*b*-less plants. Chlorophyll-fluorescence measurements after a single-turnover flash revealed that the reducing side of PSII in *chlorina-f2* barley was relatively unaffected by heat or strong light (data not shown). The only noticeable change was a slight slowdown of the intermediate component of

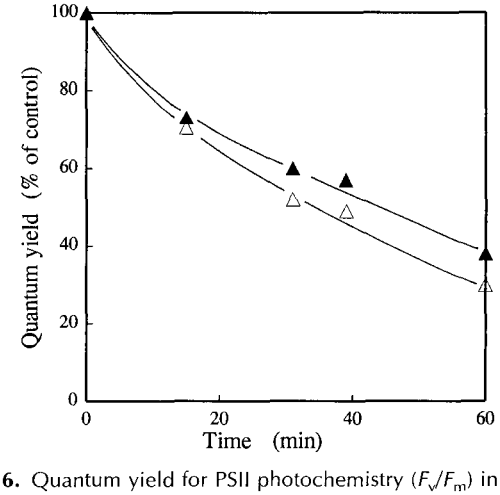

Figure 6. Quantum yield for PSII photochemistry (F_v/F_m) in DCMU-poisoned barley leaves (wild type, △; *chlorina-f2*, ▲) during strong light stress (2000 µmol m^{-2} s^{-1}).

Table III. *F_v/F_m and $t_{1/2}$ in supersaturating white light (9000 μmol m^{-2} s^{-1}) in barley leaves (wild type and chlorina-f2) pre-exposed to a strong light treatment (2000 μmol m^{-2} s^{-1} for 55 min) or heat stress (38 or 40.5°C for 15 min in the dark or in white light of 100 μmol m^{-2} s^{-1})*

Data are mean values ± SD. Numbers in parentheses indicate the number of independent experiments.

Treatment	Wild Type	*Chlorina-f2*
After strong light stress		
F_v/F_m	0.72 ± 0.03 (4)	0.62 ± 0.01 (4)
Half-time (ms)	8.3 ± 0.8 (3)	59.9 ± 21.9 (3)
After 38°C in the dark		
F_v/F_m	0.71 (2)	0.54 (2)
Half-time (ms)	94 (2)	330 (2)
After 40.5°C in the dark		
F_v/F_m	0.32 ± 0.04 (3)	0.26 ± 0.03 (3)
Half-time (ms)	502 ± 28 (3)	602 ± 30 (3)
After 40.5°C in the light		
F_v/F_m	0.55 ± 0.05 (3)	0.45 ± 0.01 (3)
Half-time (ms)	118 ± 11 (3)	382 ± 17 (3)

fluorescence decay (in the millisecond time range), indicating that the Q_A^-/Q_B^- equilibrium constant was somewhat changed.

PSII-enriched membranes isolated from chlorophyll-*b*-less and wild-type barley leaves were analyzed for their polypeptide composition using SDS-PAGE (Fig. 7). In the case of *chlorina-f2*, the membrane preparations were contaminated by the coupling factor and are not typical PSII particles (see "Materials and Methods"). Lanes 1 and 2 show that exposure of wild-type leaves to heat stress (39°C for 15 min in darkness) caused a substantial decrease in two bands with apparent molecular masses of 21 and 17 kD. These bands correspond to the 17- and 23-kD extrinsic proteins of the water-oxidizing apparatus of PSII (Murata and Miyao, 1985; Peter and Thornber, 1991). No significant loss of the 33-kD extrinsic PSII protein was observed. The identity of the extrinsic proteins of PSII was checked by treating PSII particles with alkaline Tris (Yamamoto et al., 1981; Peter and Thornber, 1991) (data not shown). Heat stress had a very different effect on the polypeptide profile of *chlorina-f2* PSII (lanes 4 and 5). A selective reduction of the proteins in the 30- to 35-kD region was observed. These bands include the 33-kD extrinsic PSII protein and a minor LHCII (Peter and Thornber, 1991). Using an image analysis system, we determined more precisely that the 33-kD extrinsic protein of *chlorina-f2* PSII was reduced by around 40% after heat stress. The 33-kD protein is assumed to be a Mn stabilizer (Murata and Miyao, 1985) and, therefore, it is likely that heat-induced removal of the 33-kD protein was accompanied by the release of Mn atoms. In contrast, the 23- and 17-kD PSII proteins were apparently not reduced in the mutant after heat stress. However, considering the intrinsic instability of the O_2-evolving system of PSII in *chlorina-f2* (Fig. 2), one cannot exclude that these extrinsic proteins were partially lost during the membrane preparation from unstressed leaves, thus masking their release under heat-stress conditions. One can conclude from the data shown in Figure 7 that the assembly of the water-oxidizing side of PSII is unstable at high temperature, thus confirming previous observations (Nash et al., 1985; Enami et al., 1994), and that heat stress affected very differently the PSII assemblies in *chlorina-f2* and wild-type barley. Dissociation of the 33-kD extrinsic protein of the water-splitting system from the PSII complexes is probably an important factor involved in the loss of PSII photochemistry in heat-stressed *chlorina-f2* leaves.

DISCUSSION

The present study reveals that PSII is more vulnerable to heat stress (Figs. 3 and 4) and to strong light stress (Fig. 5) when its LHCII system is strongly reduced. Using chlorophyll fluorometry and biochemical analysis of thylakoid membranes, our study suggests that the decreased stability of LHCII-deficient PSII is ascribable to its oxidizing side (Fig. 7; Table III). Exposure of *chlorina-f2* leaves to bright light was also observed to induce a selective inhibition of the electron donor side of PSII that was not observed in the wild type (Table III). In fact, even under favorable temperature conditions and in the absence of strong light stress, the oxidizing side of PSII appeared to be less efficient and less stable in *chlorina-f2* than in the wild type (Fig. 2). In contrast, the reducing side of PSII was unaffected or only slightly affected by heat, bright light, or prolonged dark adaptation. Therefore, one of the main conclusions of this study is that the chlorophyll *a*/*b*-protein complexes have a structural and stabilizing role, possibly allowing the maintenance of the assembly and the conformation of the PSII electron donor side (in particular, the attachment of the 33-kD protein) for optimal oxygen evolution under varying conditions.

This conclusion is in agreement with previous in vitro studies of thylakoid preparations from chloroplasts grown under intermittent light (Jahns and Junge, 1992, 1993), which demonstrated that chlorophyll *a*/*b*-binding proteins modify the pH and Ca^{2+} dependence of the rate of O_2 evolution and modulate the kinetics and the stoichiometry of proton release from water oxidation. Chlorophyll-

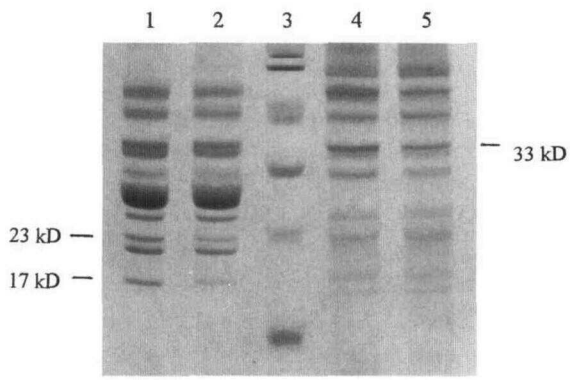

Figure 7. SDS-PAGE of PSII-enriched membranes from wild type and *chlorina-f2* barley. Lanes 1 and 2, Wild-type membranes before and after heat stress (39°C for 15 min in darkness), respectively; lanes 4 and 5, *chlorina-f2* membranes before and after heat stress, respectively; lane 3, molecular weight markers (from top to bottom: 94, 67, 43, 30, 20, 14.4 kD). F_v/F_m after heat stress was 0.73 ± 0.01 in the wild type and 0.36 ± 0.07 in the mutant.

protein complexes could stabilize the active conformation of the water oxidation complex and maintain high local concentrations of Ca^{2+} and Cl^-, essential cofactors of the water-splitting reactions. Consequently, shielding/unshielding of the water-oxidizing system could explain the differential stability of PSII observed in *chlorina-f2* and wild-type barley leaves exposed to various environmental conditions. Shutilova et al. (1995) observed that the decreased thermostability of the O_2-evolving complex of PSII in various subchloroplast preparations was correlated with a decrease in the content of the light-harvesting complex and an increase in the accessibility of the O_2-evolving complex to hydrophilic electron acceptors. Furthermore, Peter and Thornber (1991) showed that when LHCIIb subunits were depleted from PSII complexes, dissociation of the extrinsic 33-kD protein and conversion of CCII dimers into their monomer forms were strongly increased during purification of O_2-evolving PSII complexes and nondenaturing electrophoretic fractionation of thylakoid membranes, thus indicating less-stable assembly of the PSII complex.

Heat Stress

Thermal denaturation of PSII has been shown to involve two main phenomena: a dissociation of LHCIIb from the PSII core complex (Armond et al., 1980; Sundby et al., 1986) and a loss of the water-splitting activity attributed to the release of extrinsic PSII proteins, Mn atoms, and Cl^- anions (Krishnan and Mohanty, 1984; Nash et al., 1985; Havaux, 1993b; Enami et al., 1994; Takeushi and Thornber, 1994). Accordingly, the decreased thermostability of PSII in *chlorina-f2* was associated with a weaker association of the 33-kD protein with the PSII complex (Fig. 7). In spinach and pea leaves, heat denaturation of PSII has been shown to be alleviated by light of low or moderate intensity (Weis, 1982; Havaux et al., 1991). This finding is confirmed here in leaves of *chlorina-f2* and wild-type barley (Fig. 4): light almost cancelled the difference in thermoresistance observed in darkness between the mutant and the wild type. The exact mechanism by which light protects PSII in vivo is unknown, but it is now clear that photoprotection does not require the presence of LHCIIb, in contrast to a recent study (Kobayashi et al., 1992). It has been hypothesized that the light-induced intrathylakoid acidification and related cation exchanges somehow stabilize the thylakoid membranes and maintain the PSII reaction centers in their normal conformation (Weis, 1982; Havaux et al., 1991). In fact, it is well known that purified PSII complexes that are destroyed by chemical removal of Mn and extrinsic proteins can be reassembled into active, O_2-evolving complexes by weak illumination in an appropriate ion and pH environment (Debus, 1992).

In vivo photoactivation of the water-oxidizing enzyme was also observed in leaves previously treated with hydroxylamine (Callahan and Cheniae, 1985). Considering the fact that heat stress selectively affects the water-splitting system of PSII and provokes the release of Mn^{+2} and extrinsic proteins (Nash et al., 1985; Enami et al., 1994; this study), we postulate that such photoactivation phenomenon occurred in vivo during the illumination of heated leaves, thus counterbalancing the heat-induced disassembly of the water-oxidation apparatus. It was also observed (see Fig. 2) that dark incubation of *chlorina-f2* leaves caused a progressive impairment of the PSII electron-donor side (although the effect was much less marked than during heat stress) and that weak light slowly reversed this inhibition. A similar phenomenon was reported in mutant strains of the cyanobacterium *Synechocystis* carrying deletions within a hydrophobic region of the chlorophyll-binding PSII protein CP47: the water-oxidizing complex became inactivated during prolonged dark incubation, and illumination reversed the inhibition (Gleiter et al., 1995). The time course of recovery of a fast I_1-I_2 phase in the fluorescence induction curve during illumination of pre-darkened *chlorina-f2* leaves is comparable to the photoactivation kinetics of the water-oxidizing enzyme measured in hydroxylamine-extracted leaves (Callahan and Cheniae, 1985).

Heat-induced enhancement of chlorophyll-fluorescence emission in dim exciting light (F-T curves; see Fig. 3) is believed to be a direct manifestation of the physical separation of LHCIIb from the PSII core complex (Schreiber and Berry, 1977; Armond et al., 1978). Recently, this interpretation has been challenged and alternative explanations have been proposed (Briantais et al., 1996; Bukhov et al., 1990). Comparison of the F-T curves of chlorophyll-*b*-less and wild-type leaves (Fig. 3) clearly showed that the bulk of the fluorescence enhancement is dependent on the presence of a complete LHCII system. It has been also suggested that the "reduced size" PSII laterally migrates from the appressed regions of the thylakoid membrane to the nonappressed regions, leaving behind free LHCIIbs in the appressions (Sundby et al., 1986). Our data indicate that this phenomenon does not occur in vivo in barley leaves, because the LHCIIb-PSII dissociation was almost instantaneously reversible upon lowering leaf temperature, thus excluding slower, long-range diffusion of the PSII complexes. More probably, heat brought about a conformational change in PSII that interrupted energy transfer from the peripheral light-harvesting pigments to the core complex.

Light Stress

The *in vivo* PSII photochemistry of chlorophyll-*b*-less barley chloroplasts was much more rapidly inhibited by strong illumination than that of wild-type barley chloroplasts (Fig. 5). It is now well established that photoinhibition of PSII may involve multiple mechanisms depending on the experimental conditions and the functional status of the PSII complex. Current conceptions about photoinactivation of the PSII reaction center distinguish two different mechanisms, one induced from the acceptor side of the reaction center pigment and the other from the donor side (Barber and Andersson, 1992; Prasil et al., 1992; Aro et al., 1993). The acceptor-side-mediated photodamage occurs when the donor side of PSII is efficient and the plastoquinone pool becomes fully reduced in strong light. We induced this type of photoinhibition by blocking the electron

flow after Q_A with DCMU and observed that the PSII photostability was not lower in *chlorina-f2* than in wild-type barley (Fig. 6). Therefore, exacerbation of the PSII photoinhibition in *chlorina-f2* (in the absence of DCMU) must be due to another type of photodamage.

When donation of electrons to the PSII reaction center is deficient and cannot keep pace with electron withdrawal from the reaction center pigment P680, long-lived oxidizing radicals accumulate on the donor side, which in turn can cause inactivation of PSII electron transport. The latter inhibition is presumably caused by a functional disconnection of P680 from its primary electron donor Y_Z (Eckert et al., 1991). Although experimental evidence for donor-side-induced photoinhibition has mainly been obtained in photosynthetic samples in which the water-splitting apparatus has been deliberately impaired by chemical treatment or mutation (e.g. Theg et al., 1986; Cleland and Melis, 1987; Eckert et al., 1991; Mayes et al., 1991; Chen et al., 1992; Wang et al., 1992), there is at least one report correlating a reduction of the electron donation to PSII after stress (freezing temperatures) and an increase in the sensitivity to photoinactivation (Wang et al., 1992). Considering the weakness of the electron donor side of chlorophyll-*b*-less PSII, we suggest that increased sensitivity of *chlorina-f2* to photoinhibition is another example of in vivo donor-side photoinhibition. Accordingly, photoinactivation of PSII was accompanied by a marked impairment of the electron donation to PSII in *chlorina-f2* but not in the wild type (Table III). It was also observed that photoinhibition of *chlorina-f2* leaves was alleviated and became identical to that of wild-type barley when strong light stress was imposed in anaerobiosis (Table II). The oxygen dependency observed here is compatible with the work of Cheniae and co-workers, who observed that strict anaerobiosis slows the photoinhibition of PSII donor side components in hydroxylamine-extracted PSII membranes (Chen et al., 1992). Similarly, donor-side photoinhibition in PSII from the green alga *Chlamydomonas reinhartii*, as monitored by the photobleaching of carotenoids, did not occur in the absence of O_2 (Minagawa et al., 1996). Alternatively, it is also possible that the LHCII deficiency in *chlorina-f2* chloroplasts alters the controlled accessibility of water to the water-oxidizing catalytic site, thus favoring side reactions and formation of hydroperoxide (Wydrzynski et al., 1996).

We observed that the amplitude of the nonphotochemical quenching of chlorophyll fluorescence (in the steady state) was not reduced in *chlorina-f2* leaves and that the extent of the photoprotective conversion of violaxanthin to zeaxanthin in strong light was higher in *chlorina-f2* than in the wild type (data not shown), as shown in earlier works (e.g. Härtel et al., 1996). However, we do not know the precise localization of zeaxanthin in illuminated *chlorina-f2* leaves. Presumably, a large fraction of the xanthophyll-cycle pigments exists as free pigments in the thylakoid membrane lipid phase (Jahns and Krause, 1994). Some reports have suggested that zeaxanthin could preferentially bind to some minor LHCIIs (e.g. Bassi et al., 1993; Ruban et al., 1994). Therefore, one cannot exclude that the donor-side injury in *chlorina-f2* PSII might be somehow related to the absence of minor LHCIIs and of a zeaxanthin-related photoprotection mechanism in these proteins.

CONCLUSIONS

Recently, in a study of a chlorophyll-deficient mutant of cowpea, Habash et al. (1994) observed that a major loss of chlorophyll (−40%), LHCIIb in particular (−55%), does not necessarily have detrimental consequences for the photosynthetic productivity of plants. Similarly, Jenkins et al. (1989) previously reported that a large reduction of the chlorophyll content can occur in a yellow-green maize mutant without having much effect on photosynthesis. The present study of the *chlorina-f2* barley mutant confirms the validity of this conclusion. Indeed, a 50% decrease in chlorophyll (data not shown) and the complete suppression of LHCIIb (Fig. 7) had relatively limited effects on the fluorometrically measured quantum yield for PSII photochemistry and on the photoacoustically measured quantum yield of photosynthetic O_2 evolution in white light. In addition, the mutation did not reduce the light-saturated capacity for O_2 evolution. In contrast, the present work demonstrates that LHCII deficiency has dramatic repercussions on the stability of the electron donor side of PSII to heat stress, strong light, and prolonged dark adaptation. The increased sensitivity of PSII to elevated temperature and bright light could explain the rather wide range of F_v/F_m values (from around 0.7 to 0.84) available in the literature for chlorophyll-*b*-less barley (compare e.g. Simpson et al., 1985; Leverenz et al., 1992; Lokstein et al., 1993; Falk et al., 1994; and this study). Moreover, the instability of the PSII donor side under unfavorable environmental conditions and the slowly reversible reduction of the PSII photochemical efficiency in the dark are likely to be important factors involved in the strongly reduced growth rate of *chlorina-f2* compared with the wild type.

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