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LETHAL PHOTO-OXIDATIVE DAMAGE IN HIGH LIGHT IS OVERCOME IN A SUPPRESSOR STRAIN DEFICIENT IN THE ASSEMBLY OF THE LIGHT HARVESTING COMPLEX*

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Strains of *Chlamydomonas reinhardtii* lacking the *PsaF* gene or containing the mutation K23Q within the N-terminal part of PsaF are sensitive to high light (>400 μE m^{-2} s^{-1}) under aerobic conditions. *In vitro* experiments indicate that the sensitivity to high light of the isolated photosystem I (PSI) complex from wild type and from PsaF mutants is similar. *In vivo* measurements of photochemical quenching and oxygen evolution show that impairment of the donor side of PSI in the PsaF mutants leads to a diminished linear electron transfer and/or a decrease of photosystem II (PSII) activity in high light. Thermoluminescence measurements indicate that the PSII reaction center is directly affected under photo-oxidative stress when the rate of electron transfer becomes limiting in the PsaF-deficient strain and in the PsaF mutant K23Q. We have isolated a high light-resistant PsaF-deficient suppressor strain that has a high chlorophyll a/b ratio and is affected in the assembly of light-harvesting complex. These results indicate that under high light a functionally intact donor side of PSI is essential for protection of *C. reinhardtii* against photo-oxidative damage when the photosystems are properly connected to their light-harvesting antennae.

Photosynthesis is driven by light. Photons are absorbed by pigments of the light harvesting chlorophyll-carotenoid-protein complexes that are associated with the membrane embedded photosynthetic reaction centers. Excitation energy can be transferred to the reaction centers where it induces charge separation across the membrane. This leads to a series of electron transfer reactions resulting in oxidation of water and the production of chemical energy in the form of ATP and NADPH, which are used for CO_2 fixation. If the absorption of light exceeds the capacity of photosynthetic energy consumption, over-reduction of the electron transport carriers and increase of the lifetime of excited states in the light-harvesting-antennae occur. Over-reduction of the electron carriers can lead to photo-oxidative damage by reactive oxygen species. O_2 can be directly reduced by PSI[1] (1) generating superoxide anion radical (O_2^-) and other reactive oxygen species. Increase in the lifetime of excited singlet chlorophyll increases the probability of triplet chlorophyll formation, which can react with O_2 to produce $^1O_2$ (2, 3). Reactive oxygen species cause photo-oxidative damage, especially to PSII, which is considered to be the primary target for photoinhibition (4–6). Thus, mechanisms that balance energy input through photochemistry with energy consumption through CO_2 assimilation and other metabolic pathways are essential for plant survival.

Mechanisms that balance energy input and consumption can be divided into short and long term responses. Short term responses include nonphotochemical dissipation of excess energy (7, 8) and reduction of energy transfer to PSII through state transition, a process that leads to a redistribution of excitation energy to PSI by a reorganization of the antennae (9–11). In *Chlamydomonas reinhardtii* the absorbance cross-sections of the two photosystems are nearly balanced in state I and change to 0.15 for PSII and 0.85 for PSI in state II (11). In algae long term responses may result in a reduction of the PSII antenna size (12–16). Alternatively, photoautotrophs could adapt by enhancing their electron-consuming sinks through a selective increase in the capacity of CO_2 assimilation or photorespiration (17). In contrast, if electron consuming processes are diminished or blocked, the organisms become highly sensitive to light (18, 19). This can also be observed in mutants of C. *reinhardtii* that have reduced amounts of PSI or that are deficient in PSI (20). Comparison of the light sensitivity of mutants of *C. reinhardtii* deficient in PSII, the cytochrome b_6/f complex, or PSI has revealed that PSI-deficient mutants have the highest sensitivity,[2] suggesting that among the photosynthetic electron transfer complexes PSI is the most important in providing protection against photo-oxidation.

The PSI complex functions as a light-driven oxidoreductase that transfers electrons from plastocyanin or cytochrome c_6 to ferredoxin. Whereas the reducing side of PSI is remarkably conserved between prokaryotic and eukaryotic organisms, there are differences in the oxidizing side. One major difference concerns the PsaF subunit that is partly exposed to the lumenal space of the thylakoids. The PsaF-deficient mutant of *C. reinhardtii*, 3bF, is drastically impaired in electron transfer from both plastocyanin and cytochrome c_6 to PSI (21, 22). In

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[1] The abbreviations used are: PSI, photosystem I; PSII, photosystem II; LHC, light-harvesting complex; LHCP, proteins of the LHC; TL, thermoluminescence; PAGE, polyacrylamide gel electrophoresis; Chl, chlorophyll; Mops, 4-morpholinepropanesulfonic acid.

[2] K. Redding and J. D. Rochaix, unpublished results.

contrast, the specific deletion of the *psaF* gene in cyanobacteria does not affect photoautotrophic growth (23). The *in vivo* measured electron transfer rate between cytochrome c_{553} and PSI is the same as in the wild type (24). These differences can be explained by the presence of a N-terminal domain in the algal and plant-type PsaF that is responsible for binding of cytochrome c_6 or plastocyanin to PSI (25, 26). Site-directed mutagenesis of the PsaF subunit from *C. reinhardtii* reveals that the eukaryotic protein has a specific recognition site for the binding of plastocyanin and cytochrome c_6 that is missing in the cyanobacterial protein. Lys^{16-23} of PsaF appear to play a crucial role in the electrostatic interaction with both electron donor proteins (27). However, the PsaF-deficient strain grows photoautotrophically with doubling times comparable with wild type at light intensities of 60 μE m^{-2} s^{-1} (20).

Here we show that the PsaF-deficient strain and the PsaF-K23Q mutants are sensitive to high light (>400 μE m^{-2} s^{-1}) under aerobic conditions. *In vitro* experiments indicate that the stability of isolated PSI complexes from wild type and PsaF mutants are comparable in high light. We have isolated a PsaF-deficient suppressor strain that is resistant to high light. This strain has a high Chl a/b ratio and is deficient in functional LHCI and II. Biochemical analysis of thylakoid membranes isolated from this strain indicates that the phenotype can be best explained by a defect in assembly of the proteins of the light-harvesting complex (LHCP). *In vivo* photoinhibition experiments and thermoluminescence measurements show that PSII function is impaired at high light intensities in the PsaF-deficient strain and in two independent K23Q transformants, but not in strains containing PsaF or in the PsaF suppressor strain. This indicates that under high light a functionally intact oxidizing side of PSI is essential for protection of *C. reinhardtii* from photo-oxidation when PSI is properly connected to the antennae.

EXPERIMENTAL PROCEDURES

Strains and Media—*C. reinhardtii* wild type and mutant strains were grown as described (28). Tris acetate phosphate medium (TAP) or high salt medium (HSM) were solidified with 2% Bacto agar (Difco) and supplemented with 75 μg/ml emetine (Sigma) when required.

Nucleic Acid Techniques—Procedures for the preparation of recombinant plasmids and DNA sequencing were performed as described (29). *Escherichia coli* DH5α was used as bacterial host.

Nuclear Transformation and Analysis of Transformants—Nuclear transformation of the *C. reinhardtii* light-resistant PsaF-deficient suppressor was performed according to Ref. 27. Cells were co-transformed with 2 μg of DNA of the p2.9F1 plasmid, containing the *PsaF* gene and 1 μg of DNA of the cry1–1 plasmid (30). After transformation the cells were diluted into 10 ml of TAP-N medium lacking ammonium chloride and incubated under low light (5 μE m^{-2} s^{-1}) for 3 days. The cells were then concentrated by centrifugation at 2500 $\times$ *g*, resuspended in 0.5 ml of TAP-N, and plated on TAP plates supplemented with 75 μg/ml emetine. The plates were incubated under low light (5 μE m^{-2} s^{-1}) until colonies appeared. Growing colonies were restreaked on fresh TAP/emetine plates and characterized.

Isolation of Plastocyanin—The isolation of plastocyanin followed published procedures (31) with modifications as described in Ref. 22. The concentrations of plastocyanin was determined spectroscopically using an extinction coefficient of 4.9 mM^{-1} cm^{-1} at 597 nm for the oxidized form of plastocyanin (32).

Growth Rate Measurements and in Vivo Tests—Doubling times of the mutants were determined from four independent measurements. Growth tests were initiated by spotting 15 μl of log phase cultures onto agar plates. Anaerobiosis of agar plates was established using the BioMérieux (Marcy-l'Etoile, France) Generbag Anaerobiosis system according to the manufacturer's instructions.

Isolation of Thylakoid Membranes and of the PSI Complex—The isolation of thylakoid membranes purified by centrifugation through a sucrose step gradient and the isolation of PSI particles were as described (22, 32, 33). Chl concentrations were determined according to Ref. 34. Protein determination in solution was done by the bicinchoninic acid method (Sigma) according to the manufacturer's instructions.

SDS-PAGE and Western Analysis—After electrophoretic fractionation by SDS-PAGE (15.5% T, 2.66% C) according to Ref. 35 the proteins were electroblotted onto nitrocellulose and incubated with antibodies as described (36). Immuno-detection was carried out according to Ref. 22. For N-terminal amino acid sequencing proteins were blotted onto polyvinylidene difluoride membranes (36).

Green PAGE—Thylakoids were solubilized with a 10:1 (w/w) ratio of dodecyl maltoside/chlorophyll prior to electrophoresis. Deriphat-PAGE (7.5% T) was performed according to Ref. 37.

Measurements of PSII Excitation Pressure (1 − q_P)—For determination of F_m and F_m', samples were subjected to saturating light pulses of 0.7 s at 1-min intervals and illuminated continuously with a nonactinic modulated light using a Hansatech fluorimeter. q_P was calculated from the fluorescence data as $(F_m' - F)/(F_m')$ (38). For this experiment cells were grown in minimal medium and were used for the experiment when they had reached a cell density of $\sim 2 \times 10^6$ cells/ml.

Oxygen Evolution—Oxygen evolution was measured with a Clark electrode (Hansatech). For illumination of the sample, the sample housing was connected through fiber optics with a halogen lamp (Hansatech). Light intensities could be adjusted through a set of neutral filters with different transmission values.

Gross Oxygen Evolution and Oxygen Uptake—Gross oxygen evolution and oxygen uptake was measured at 25 °C using a system described previously (39). Actinic light was provided by a slide projector (Braun) with heat filter. The light intensity was adjusted using neutral density filters. The rates of oxygen evolution and uptake were calculated according to Ref. 39.

Thermoluminescence—Thermoluminescence (TL) was measured as described previously (40). TL was charged by giving a single turnover flash at −5 °C followed by rapid freezing to −20 °C. The TL signal was then recorded during warming to 70 °C with a heating rate of 20 °C/min. If samples were subjected to photoinhibitory treatment, the samples were dark adapted at 20 °C for 2 min before starting the TL measurements. No cryoprotectants were added for the TL measurements.

Flash Absorption Spectroscopy—Flash-induced spectroscopy was carried out using a single beam spectrophotometer (Occam Technology). The measuring light, provided by a quartz halogen lamp was filtered through a 817 nm (5 nm full width at half-maximum) interference filter and passed through a cuvette containing 500 μl of the sample with an optical path length of 1 cm. The signal from the detecting photodiode (1 cm^2) was amplified with an electrical bandwidth ranging from direct current to 60 MHz. Signals were recorded with a Tektronic Oscilloscope THS 710 (2500 channels) and transferred to a computer interface analog signal capture board (8 bit, 100 MHz). Flash excitation was provided by a xenon flash (2 μs full width at half-maximum). 16 individual signals were averaged for each trace at a repetition rate of 0.2 Hz.

RESULTS

PsaF-deficient Strains Are Sensitive to High Light—To determine whether the PsaF-deficient mutant grows under high light, growth tests were performed on TAP and HSM at light intensities of 60 and 400 μE m^{-2} s^{-1} under aerobic or anaerobic conditions (Fig. 1). Two different PsaF-deficient strains were used, the 3bF strain (21) and one of its progeny, $6a^+$, derived from a back-cross of 3bF to wild type. Immunoblot analysis using PsaF- and PsaD-specific antibodies revealed that these strains do not express PsaF but have normal levels of PsaD, another subunit of PSI (Fig. 1). As control strains, we used a rescued 3bF strain (RPsaF), that was selected on emetine-containing plates after co-transformation with two plasmids containing the nuclear PsaF gene and the Cry1 gene (which confers cryptopleurine and emetine resistance; Ref. 30), respectively, and two strains with mutations in the N-terminal domain of PsaF, K23Q and K30Q (27). These strains express between 50 and 100% of PsaF compared with the wild type as determined by immunoblotting with PsaF-specific antibodies (Fig. 1).

The control and the PsaF-deficient strains 3bF and $6a^+$ grow on minimal medium at a light intensity of 60 μE m^{-2} s^{-1}. At a higher light intensity of 400 μE m^{-2} s^{-1}, both strains 3bF and $6a^+$ die on HSM in the presence or absence of copper, whereas the control strains survive. Copper deficiency is known to lead to replacement of plastocyanin by cytochrome c_6 in *C. rein-*

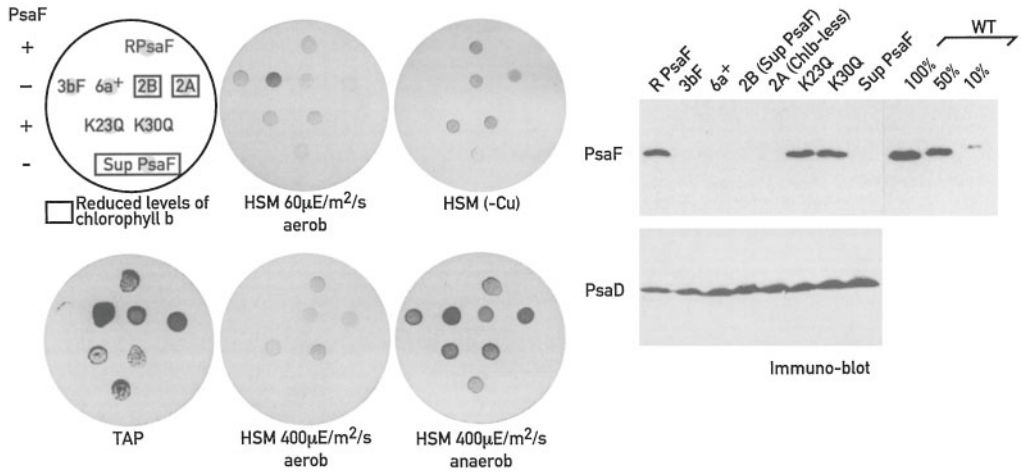

FIG. 1. **PsaF-deficient strains with a low Chl a/b ratio are unable to grow on minimal medium under high light.** *Left panel*, growth tests using *C. reinhardtii* strains under aerobic and anaerobic conditions and light intensities of 60 and 400 μE $m^{-2}s^{-1}$. The strains were: the PsaF-deficient mutants 3bF and $6a^+$; the PsaF site-directed mutants K23Q-17 and K30Q-22; the 3bF strain rescued with *PsaF,* RPsaF; two high light-resistant suppressors SupPsaF and 2B (a progeny containing the suppressor mutation from a cross between $6a^+$ and SupPsaF); and a Chlb-less and PsaF-deficient mutant 2A (a progeny from a cross between $9a^+$ (lacking PsaF) and CC1355 (lacking Chl b)). Photosynthetic function is dispensable on TAP but not on HSM medium. The poor growth of 3bF on TAP medium under high light is most likely due to the fact that this strain is cell wall-deficient and more sensitive to adverse growth conditions than $6a^+$. *Right panel*, total cell extracts (3 μg of Chl) were fractionated by 12% T SDS-PAGE. The immunoblot was decorated with anti-PsaF and anti-PsaD antibodies.

hardtii (31). Growth of the K23Q mutant, which is strongly impaired in electron transfer between plastocyanin and PSI, on HSM under high light is slower in the presence than in the absence of copper. This observation could be explained by the fact that cytochrome c_6 is a more efficient electron donor to the mutated PSI complex than plastocyanin (27). The PsaF-deficent strains 3bF and $6a^+$ are resistant to a light intensity of 400 μE m^{-2} s^{-1} when grown on TAP under aerobic or on minimal medium under anaerobic conditions.

To screen for high light-resistant suppressors of 3bF, cells were plated on HSM and illuminated with continuous light of 400 μE m^{-2} s^{-1}. Out of 3×10^7 cells plated, 1–5 colonies were obtained. When this experiment was repeated with a higher light intensity (1000 μE m^{-2} s^{-1}), no suppressor strains were recovered. Among the isolated high light-resistant mutants, 19 out of 20 had a high Chl a/b ratio (>10). One of these suppressors was analyzed more thoroughly (see below). As shown in Fig. 1 this suppressor strain (Sup PsaF), as well as one of its progeny, 2B, derived from a back-cross with $6a^+$, survives under conditions where the PsaF-deficient strains 3bF and $6a^+$ die. The same was obtained when a PsaF- and Chl b-deficient strain, 2A, derived from a cross between strain CC1355 (*cbn1*-deficient) and $6a^+$, was tested on minimal medium in high light. The immunoblot in Fig. 1 verifies that the strains SupPsaF, 2B, and 2A are deficient in PsaF. These results indicate that high light tolerance in PsaF-deficient strains correlates with a deficiency in Chl b.

Genetic Characterization of the 3bF Suppressor Strain—To determine whether the phenotype of light resistance is due to a chloroplast or nuclear mutation and cosegregates with a high Chl a/b ratio, the suppressor strain Sup PsaF was crossed with the PsaF-deficient strain $6a^+$. The Chl a/b ratio and light sensitivity of the resulting progeny was determined. As shown in Table I, the segregation of progeny with a low and high Chl a/b ratio is close to 1:1. None of the PsaF-deficient progeny with a low Chl a/b ratio survived light intensities above 400 μE m^{-2} s^{-1}, whereas most of the progeny with a high Chl a/b ratio did. This suggests that the suppressor mutation is nuclear encoded. To confirm these results, we performed an additional cross between one of the progeny, $15B^-$, resistant to a light intensity of 1000 μE m^{-2} s^{-1}, and $6a^+$. Again, all progeny with a low Chl a/b ratio died in high light. In contrast, nearly all of the progeny with a high Chl a/b ratio survived the light stress. This indicates that the high Chl a/b ratio phenotype cosegregates with the suppression of the light sensitivity and that the differences in light sensitivity are most likely due to differences in the genetic background. The suppressor strains revert with a frequency of nearly 80% when kept under low light. Revertants have a low Chl a/b ratio, comparable with 3bF.

TABLE I
Genetic characterization of the 3bF suppressor strain

The first cross was between the suppressor strain $SupPsaF^-$ and the PsaF-deficient strain $6a^+$. Two complete and 11 incomplete tetrads (three with three spores and eight with two spores) were obtained. The second cross was between one progeny from the first cross, $15B^-$ and $6a^+$. 12 incomplete tetrads were obtained (two with two spores and ten with one). The progeny were tested for their Chl a/b ratios and for growth at different light intensities on minimal medium.

		Percentage of surviving progeny at the light intensity indicated		
		$\mu E\ m^{-2}\ s^{-1}$	$\mu E\ m^{-2}\ s^{-1}$	$\mu E\ m^{-2}\ s^{-1}$
$6a^+ \times SupPsaF^-$		60	400	1000
Chl a/b ≈ 2	15	100	0	0
Chl a/b ≥ 8	18	100	83	66
$6a^+ \times 15B^-$				
Chl a/b ≃ 2	7	100	0	0
Chl a/b ≥ 8	7	100	100	86

LHCP of the 3bF Suppressor Strain Does Not Integrate Properly into the Thylakoid Membrane—It has been shown that Chl b-deficient mutants accumulate some LHCP (41–50). To determine whether the suppressor strain accumulates LHCP II, we performed a nondenaturating Deriphat-PAGE (37). Fig. 2 clearly shows that after fractionation of isolated thylakoid membranes with such a system, a prominent green band, LHCII, present in the 3bF control and in a revertant with a low Chl a/b ratio, is missing in the suppressor strain. In addition, a new Chl-containing band is present in this strain that migrates slightly slower than the free Chl band (indicated by *FC* in Fig. 2). Analysis of this new band revealed that it contains two polypeptides with molecular masses of 25 and 30 kDa that react with LHCP II-specific antibodies (data not shown). The same polypeptides were detected when the higher molecular mass band (*LHCII* in Fig. 2) present in the 3bF thylakoids was analyzed. N-terminal amino acid sequencing indicated that the

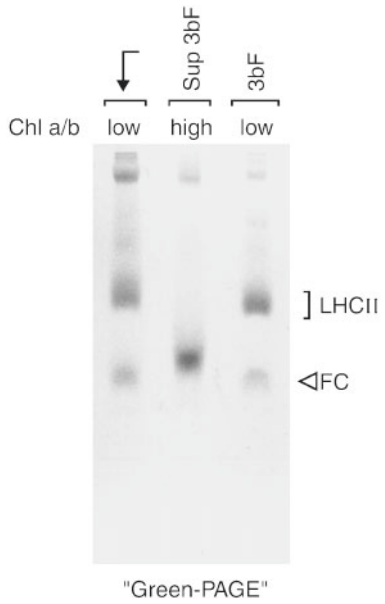

FIG. 2. **LHCPII-trimers are absent in thylakoids isolated from the high light-resistant suppressor strain SupPsaF.** Isolated thylakoid membranes from *C. reinhardtii* strains 3bF (PsaF-deficient), the suppressor strain SupPsaF, and a revertant derived from the high light-resistant suppressor (*left lane*) fractionated by 7.5% T Deriphat-PAGE are shown. 25 μg of chlorophyll was loaded in each lane. *FC*, free chlorophyll.

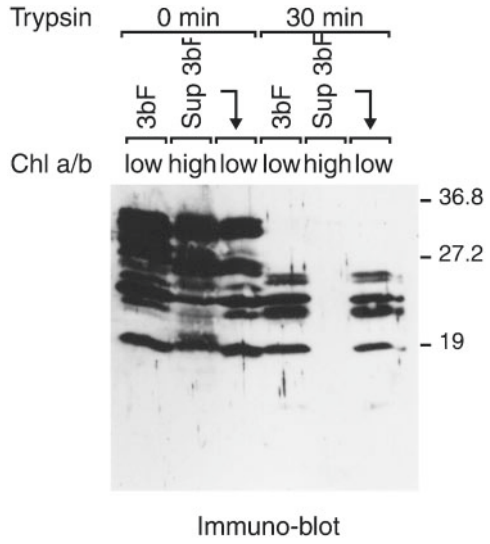

FIG. 3. **LHCP proteins are not properly integrated into thylakoid membranes of the high light-resistant suppressor strain SupPsaF.** Tryptic digestions of thylakoid membranes isolated from *C. reinhardtii* strains 3bF (PsaF-deficient), the suppressor strain SupPsaF, and a revertant that derived from the high light-resistant suppressor (indicated by *downward arrows*) are shown. 50 μg of protein were digested with 2 μg of trypsin for 30 min, and the reaction was stopped by addition of 2 mM phenylmethylsulfonyl fluoride. After addition of sample buffer and denaturation at 55 °C for 10 min, the samples were fractionated by 12% T SDS-PAGE. The immunoblot was decorated with the anti-LHCI antibodies 15, 15.1, 17.2, 18.1, and 22.1 (58). Size markers in kDa are indicated on the *right*.

larger polypeptide corresponds to LHCP II type I of *C. reinhardtii* (51) and that the smaller one contains the N-terminal sequence IEXYGPDRPKFLGPFR. Comparison with other LHCP II sequences shows that it corresponds to a LHCP II type II protein (52), which has not yet been identified in C. *reinhardtii*. Because LHCP II type I and II are known to form the LHCPII-trimer (52), this suggests that the green band missing in the suppressor strain and present in 3bF and the revertant is a LHCPII-trimer.

LHCP that are normally integrated into thylakoid membranes are largely insensitive to protease digestion (53–57). It has been shown that LHCP with alterations in their amino acid sequence that are able to associate with thylakoids are not necessarily properly integrated into the membrane based on their protease sensitivity (54, 56). To test the association and integration of LHCP with thylakoids isolated from the suppressor strain, its revertant and 3bF, tryptic digestions of isolated membranes were performed. Membranes prior to and after digestion were fractionated by SDS-PAGE and analyzed by immunoblotting. The blot shown in Fig. 3 was probed with a mixture of anti-LHCI antibodies directed against polypeptides 15, 15.1, 17.2, 18.1, and 22.1, which have also been shown to cross-react with the major LHCPII as well as with the CP24, 26, and 29 proteins from *C. reinhardtii* (58). Immunoblot analysis reveals that the majority of the LHCP detected in 3bF and the revertant can be isolated from thylakoids of the suppressor strain. Immunoblot analysis with single LHCI antibodies indicates that the LHCI subunit 17.2 is present at reduced levels in the thylakoid membranes from the suppressor strain (data not shown). However, whereas after digestion of the thylakoid membranes with trypsin comparable LHCP digestion fragments accumulate in the thylakoids of 3bF and the revertant, no fragments are observed in the membranes from the suppressor (Fig. 3). These results indicate that LHCI and II can associate with thylakoids isolated from the suppressor strain but that they do not integrate properly into the thylakoids to adopt a protease-resistant conformation.

To further test whether the LHCI complex is associated with PSI in the suppressor strain, PSI complexes from wild type, 3bF, different revertants of the suppressor strain, the suppressor strain, and RPsaF, a suppressor strain expressing PsaF, were isolated. Fig. 4 shows the Coomassie staining and an immunoblot analysis of different PSI preparations fractionated by SDS-PAGE, using the anti-LHCI antibodies, 15, 15.1, 17.2, 18.1, and 22.1, and PsaF-specific antibodies. The enriched PSI complexes isolated from wild type, 3bF, and a revertant with a Chl a/b ratio of 2 show protein patterns that are comparable with published *C. reinhardtii* PSI preparations (22, 59). However, in strains with high Chl a/b ratios, the amount of LHCI that can be isolated jointly with PSI declines. No LHCI was detected by immunoblot analysis with PSI particles isolated from the suppressor strain whether or not it contained PsaF. This shows that LHCI is not physically connected with PSI or that its association with PSI is considerably weakened in the suppressor strain and confirms our previous interpretation that LHCI is not properly integrated into the thylakoid membrane. It further shows that integration of PsaF into PSI is not dependent on the presence of LHCI.

Fluorescence emission spectra of thylakoids from the suppressor strains at 77 K are different to spectra obtained with thylakoids from 3bF (data not shown). Whereas the spectrum measured in the 3bF strain resembles that in the wild type, the spectrum in the suppressor strains exhibit a single peak at 672 nm, but no contribution at 715 nm, the wavelength at which the LHCI/PSI peak is expected (60), confirming that LHCI and PSI are not functionally connected. It appears, therefore, that one possible reason for the resistance of the suppressor strain to high light is the functional disconnection between reaction centers and LHCP, so that even at high light intensities the rate of light absorption is not sufficient to drive an over-reduction of the electron transfer components.

The PsaF-deficient PSI Complex Is as Stable as PSI from Wild Type When Incubated under High Light in Vitro—One reason for the pronounced light sensitivity of the PsaF-defi-

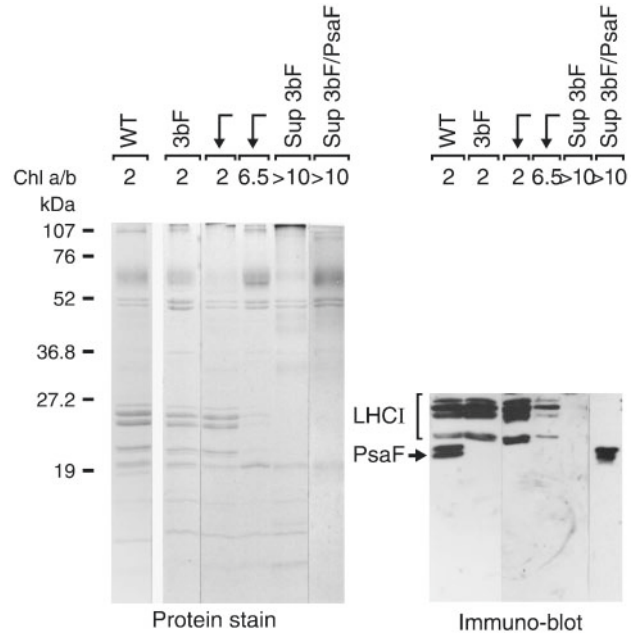

FIG. 4. **PSI isolated from the high light-resistant suppressor strain SupPsaF is not connected to LHCPI.** PSI particles from wild type, the suppressor strain SupPsaF, a rescued SupPsaF strain expressing the PsaF protein, and different revertants derived from the high light-resistant suppressor with different Chl a/b ratios (indicated by *downward arrows*) were isolated, and the proteins were fractionated by PAGE. 2 μg of Chl were loaded on each lane. The immunoblot was probed with anti-PsaF and anti-LHCI antibodies 15, 15.1, 17.2, 18.1, and 22.1.

cient strain 3bF could be that PSI is unstable in high light. This was tested *in vitro*: isolated PSI particles were incubated under a continuous light intensity of 2500 μE m^{-2} s^{-1} for 150 min in the presence of 5 μM plastocyanin as electron donor and 0.1 mM methyl viologen as electron acceptor. Reduced methyl viologen produces H_2O_2 so that resistance of PSI to active oxygen species can also be tested. Fig. 5 (*A* and *B*) shows the absorbance transients at 820 nm induced by a xenon flash for PSI particles from wild type and PsaF-deficient strains prior to and after 150 min of illumination. Prior to the high light treatment, $P700^+$ was reduced with a half-life of 4 ms. In the 3bF strain, the rereduction of $P700^+$ is slower than 400 ms as reported previously (22). After 150 min of high light treatment, the amount of PSI that can perform stable charge separation declined to 69% in the wild type and 82% in the PsaF-deficient strains, respectively. This decrease is due to partial photoinactivation of PSI. The fraction of wild type PSI that is reduced with a half-time of 4 ms decreases from 80 to 60% after illumination (Fig. 5*C*). Thus, it appears that the PsaF-deficient PSI is slightly more stable in the presence of high light and methyl viologen than is wild type PSI. This indicates that the cause for the elevated light sensitivity of the PsaF-deficient strain is not due to the instability of PSI.

A Functionally Efficient Donor Side of PSI Protects against Photo-oxidation—As seen in Fig. 1, PsaF-deficient strains do not grow on minimal medium in high light. However, the mutant K23Q survives under these conditions, although its electron transfer between plastocyanin and PSI is strongly impaired. The different PsaF site-directed mutants were grown in liquid culture in minimal medium under high and low light, and their doubling times were determined (Table II). At low light (60 μE m^{-2} s^{-1}) the doubling times of the suppressor strain and 3bF are comparable. As recently shown, the growth rates of the rescued strain and the different site-directed mutants are also comparable (27). However, at high light (1000 μE m^{-2} s^{-1}) differences become apparent. The growth rates of mutant K16Q and of two independent K23Q mutants are significantly diminished relative to low light conditions. In contrast, the rescued strain and mutant K30Q and K12P are less

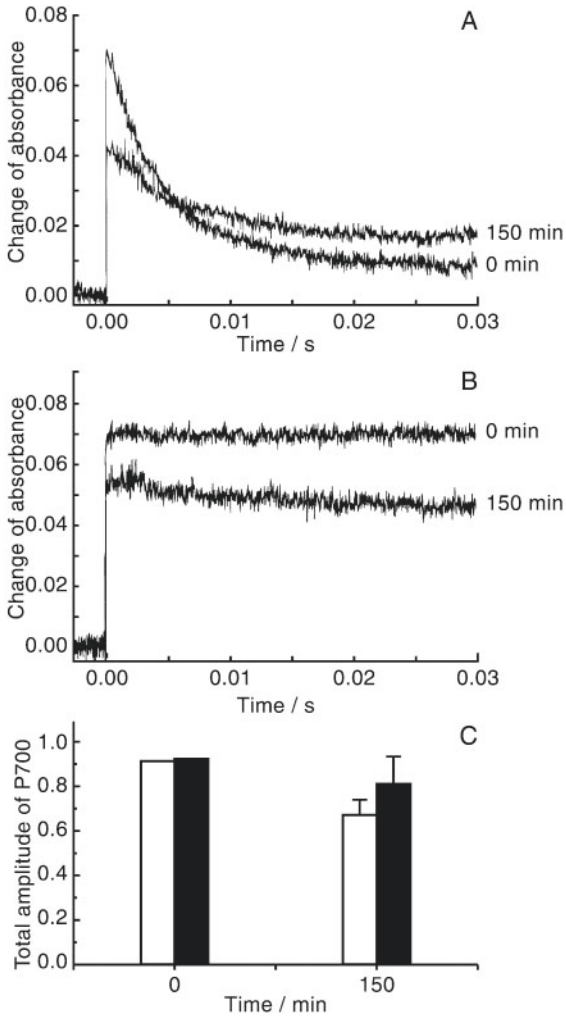

FIG. 5. **The PSI complex isolated from the PsaF-deficient strain 3bF is stable in high light.** Photoinactivation of PSI isolated from wild type (*A*) and the PsaF-deficient strain 3bF (*B*) of *C. reinhardtii* is shown. PSI particles were incubated at a light intensity of about 2500 μE m^{-2} s^{-1} at 25 °C. The amount of PSI capable of charge separation was determined as ΔA at 820 nm after averaging 32 xenon flashes before and after 150 min of illumination. The cuvette contained PSI particles at a concentration of 90 μg Chl/ml in 0.05% (w/v) β-dodecyl maltoside, 10 mM $MgCl_2$, 5 μM plastocyanin, 30 mM Mops (pH 7.0), 1 mM sodium ascorbate, 0.1 mM methyl viologen, and 0.2 mM diaminodurene. *C*, the mean values of ΔA from three independent experiments are shown for wild type (*open bars*) and the PsaF-deficient strain (*shaded bars*).

TABLE II
Doubling times of the PsaF mutants

The doubling times are indicated in hours. 3bF, PsaF-deficient strain; RpsaF, rescued 3bF strain; SupPsaF, suppressor strain. The other mutants are site-directed mutants of PsaF obtained by transformation of 3bF (27); the numbers in parentheses identify the individual transformants. Growth experiments (n = 4) were performed in liquid minimal medium.

	60 $\mu E\ m^{-2}\ s^{-1}$	1000 $\mu E\ m^{-2}\ s^{-1}$
3bF	12.6 ± 3.9	ND
RpsaF	5.8 ± 1.0	8.3 ± 1.7
K23Q (17)	7.8 ± 2.1	29.9 ± 3.9
K23Q (30)	ND	27.2 ± 5.4
K16Q (9)	5.8 ± 0.9	20.6 ± 5.6
K30Q (22)	7.3 ± 3.8	12.8 ± 5.6
K12P (5)	7.8 ± 2.1	16.9 ± 3.7

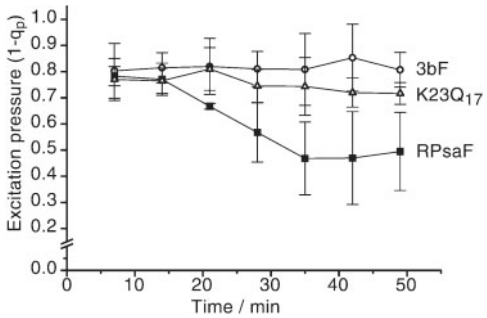

FIG. 6. **Excitation pressure ($1 - q_P$) remains high in strains with an impaired oxidizing side of PSI in high light.** After 15 min of illumination with far red light, cells of the following strains were exposed to 1000 μE m^{-2} s^{-1} of light. ■, 3bF rescued with *PsaF*; ○, 3bF; △, K23Q-17. The mean values and standard deviations from three (RPsaF and K23Q-17) or four measurements (3bF) are shown.

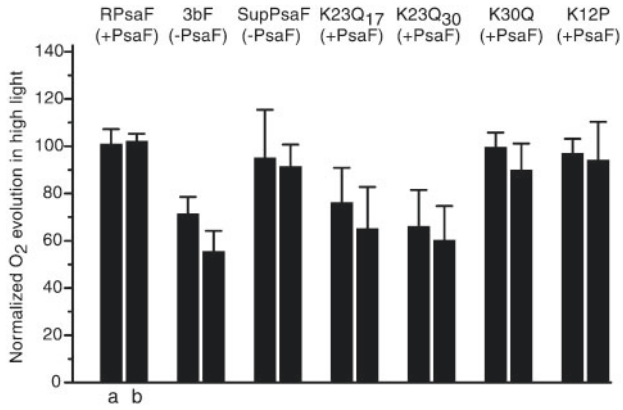

FIG. 7. **Oxygen evolution is diminished in strains with an impaired oxidizing side of PSI in high light.** Oxygen evolution was measured in whole cells with an oxygen electrode (Hansatech). Samples were illuminated for 6 min at each light intensity. The cells were grown in minimal medium and were used for measurement when they had reached a cell density of about 2×10^6 cells/ml. The panel shows the oxygen evolution rates at 1800 and 2500 μE m^{-2} s^{-1} as a percentage of the mean values of the rates at 500, 700, and 1000 μE m^{-2} s^{-1}. The mean values from three to five independent experiments are shown. *Left column* (*a*), 1800 μE m^{-2} s^{-1}; *right column* (*b*), 2500 μE m^{-2} s^{-1}.

affected. *In vitro* measurements of electron transfer between plastocyanin and PSI isolated from these different PsaF mutants have shown that the mutations K23Q and K16Q have the strongest effects on the electron transfer rates (27), suggesting that the intactness of the donor side of PSI is important for growth of *C reinhardtii* in high light.

To obtain further insights into the light sensitivity of the PsaF mutants, the PSII excitation pressure ($1 - q_P$) was determined at a light intensity of 1000 μE m^{-2} s^{-1} (Fig. 6). ($1 - q_P$) provides a measure of the relative reduction state of PSII and reflects changes in the redox state of the thylakoid intersystem electron transport chain (61). The excitation pressure remained high in the PsaF mutants 3bF and K23Q-17 (Fig. 6). The same result was obtained with the K23Q-30 mutant strain. In contrast, in the rescued PsaF strain the excitation pressure was significantly lower, especially after prolonged light exposure. These results are expected because the limitation in electron transfer in the PsaF mutants in high light will lead to increased reduction of the plastoquinone pool and of Q_A, the primary stable electron acceptor of the PSII reaction center.

To measure the photosynthetic activity of the different strains, oxygen evolution was measured with an oxygen electrode at different light intensities. Light saturation curves of oxygen evolution of the suppressor strain SupPsaF, 3bF, the rescued strain RPsaF and the PsaF mutants K23Q-17, K23Q-30, and K12P-5 revealed that for all strains the half-maximal rates of oxygen evolution are found at light intensities between 100 and 200 μE m^{-2} s^{-1} (data not shown). In the case of the PsaF-deficient strain 3bF, and the mutants K23Q-17 and K23Q-30 (which are two independently isolated transformants with the same PsaF mutation) the rates of oxygen evolution at 1800 and 2500 μE m^{-2} s^{-1} are significantly lower than the rates at 500, 700, and 1000 μE m^{-2} s^{-1} only (Fig. 7). This suggests that PSII activity is impaired or that limitations in linear electron transfer diminish oxygen evolution in these strains. Interestingly, such a decline is not observed for the suppressor strain, although its PSI donor side is less functional than that of the K23Q mutants. We verified that the diminished rate of oxygen evolution in the 3bF strain under high light is due to a decline in PSII activity or an impairment of the photosynthetic electron transfer rather than to an increase of oxygen uptake by measuring gross oxygen evolution and uptake using mass spectrometry (data not shown).

To determine whether the decreased photosynthetic electron transfer activity in the PsaF-deficient strain under light stress is directly due to an impairment of PSII activity, we performed TL measurements. Fig. 8 shows thermoluminescence curves from RPsaF (*left panel*), 3bF (*middle panel*), and K23Q-17 (*right panel*) strains. TL was excited by a single turnover flash

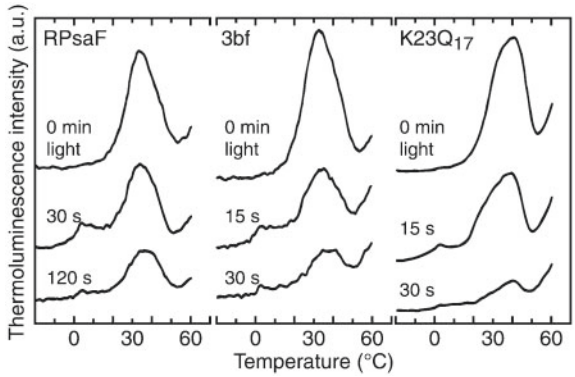

FIG. 8. **The susceptibility of PSII toward light is increased in mutants affected at the donor side of PSI.** Thermoluminescence curves from strains RPsaF (*left panel*), 3bF (*middle panel*), and K23Q17 (*right panel*). Chl concentration was about 5 μg/ml. Photoinhibitory illumination was performed with white light (I = 1400 μE m^{-2} s^{-1}).

at −5 °C. Nonphotoinhibited cells of the RPsaF and 3bF strains show a band at 32 °C (the B-band), arising from charge recombination between the PSII redox components S_2 and Q_B^- (62). Algae from the K23Q-17 strain gave rise to a more complex, broader signal extending from 32 to 40 °C (Fig. 8). After a short photoinhibitory illumination with white light (I = 1400 μE m^{-2} s^{-1}), the intensity of the B-band decreased, and a low intensity band appeared at approximately 5 °C, most likely a Q-band arising from charge recombination between S_2 and Q_A^- (62). After photoinhibitory illumination, the rate of loss of TL intensity was higher in the 3bF and K23Q-17 strains than in the RPsaF strain. This indicates that the susceptibility of PSII toward light is increased after deletion or alteration of the PsaF subunit of PSI.

TL studies have previously been performed with photoinhibited thylakoid membranes of higher plants (63–65), on pea leaves (66), and also with cells of *C. reinhardtii* (67). In thylakoid membranes of higher plants, the intensity of the B-band was reduced after photoinhibitory illumination, and no shift in the maximal emission temperature of the B-band was seen in agreement with the data shown in Fig. 8. However, Ohad *et al.* (67) reported a different phenomenon; in their experiments the B-band was decreased in intensity and shifted by 15 °C toward a lower temperature after the photoinhibitory treatment. This

decrease in the peak temperature of the B-band was observed after a much longer period of photoinhibitory illumination than the one used here. This decrease of the B-band was interpreted to be related to an alteration of the D1 protein. This phenomenon does not appear to occur after the short times of illumination (maximum 2 min) used in the present study. However, the differences observed could also be explained by the fact that Ohad *et al.* (67) used cells grown under heterotrophic conditions, whereas the measurements performed in this study were performed with cells grown photoautotrophically.

DISCUSSION

In previous studies we have shown that PsaF is essential for efficient electron transfer between plastocyanin or cytochrome c_6 and PSI in *C. reinhardtii* and that the N-terminal domain of PsaF forms a precise recognition site for binding of both donors (22, 27). We have also shown that the introduction of the N-terminal part of PsaF from *C. reinhardtii* into PSI from *Synechococcus sp.* enables efficient interaction between the cyanobacterial PSI and the algal donors (26). It is, however, not clear why this binding site within the N-terminal part of PsaF evolved, because the electron transfer between PSI and the cytochrome b_6/f complex is not limiting for linear photosynthetic electron transfer under moderate light conditions, even in the absence of PsaF (21).

Light Sensitivity of the PsaF-deficient Mutants under Aerobic Conditions—Our study demonstrates that deletion of PsaF or mutations within the N-terminal domain of PsaF that impair electron transfer between plastocyanin and PSI have strong effects on the growth properties of the *C. reinhardtii* strains in high light on plates or in liquid medium. The *in vivo* electron transfer between the cytochrome b_6/f complex and $P700^+$ was measured to be 1.2 ms in the PsaF-deficient strain 3bF, 20 times slower than in the wild type (21). However, it is still faster than the rate-limiting step of linear electron transfer, which involves the concerted reduction of cytochrome *f* and cytochrome b_H by plastoquinol oxidation with a half-life of 10–15 ms (68). Under high light conditions in which the plastoquinone pool is fully reduced, this half-life is accelerated to 1.2 ms as measured in intact *Chlorella* cells (69). Taken together, these results indicate that under these conditions the electron transfer between plastocyanin and the PsaF-deficient PSI may become limiting, so that over-reduction of the electron transfer components and overexcitation of the antennae result in the production of reactive oxygen species. The fact that the PsaF-deficient strain survives under anaerobic conditions in high light indicates indeed that the production of reactive oxygen species under high light in aerobic conditions is most likely responsible for the cell damage. Because the stability of PSI isolated from wild type and the PsaF-deficient strain is comparable in high light, an instability of PSI in the absence of PsaF cannot explain the light sensitivity of this strain. The constant high PSII excitation pressure (Fig. 6) and the decrease in oxygen evolution (Fig. 7) observed for the PsaF-deficient strain and the K23Q mutants suggests that electron transfer between cytochrome b_6/f complex and PSI becomes limiting when the donor side of PSI is impaired.

PSII Is Photoinhibited When the Donor Side of PSI Is Impaired—The results of the TL measurements directly indicate that the decreased photosynthetic activities in the PsaF-deficient strain and K23Q mutants are due to an impairment of PSII upon photo-oxidative stress. The low intensity TL band at 5 °C suggests that in high light not only $S_2Q_B^-$ but also some $S_2Q_A^-$ charge recombination takes place. It has been suggested that in the presence of reduced Q_A charge recombination reactions occur that can lead to the formation of a long-lived chlorophyll triplet state and consequently to the production of $^1O_2$, which is believed to be responsible for photodamage (70, 71). The redox state of Q_A depends on a balance between light absorption by the antennae and the redox state of the PQ pool (15, 72). This balance is compromised in the PsaF-deficient strains, leading to over-reduction of Q_A, but partially restored in the suppressor strain by lowering the excitation energy input through the antennae. However, it has been shown that in *C. reinhardtii* strains unable to photo-oxidize the PQ pool and therefore unable to grow photoautotrophically, photoinactivation of PSII is slower as compared with wild type (73, 74). This discrepancy with our data could be explained by the fact that linear electron transfer from PSII to PSI is needed to observe a decline in PSII activity when the donor side of PSI becomes limiting.

High Light-resistant Suppressor Strain with Misassembled Antennae—The light-harvesting capacity of photosynthetic cells can be adapted to avoid over-excitation of the antennae. However, in the case of the PsaF-deficient strain such an adaptation mechanism is not sufficient to protect the strain from photo-oxidative damage under high light. Our suppressor screen has identified a strain in which the LHC polypeptides are not properly integrated within the thylakoid membrane and not functionally connected to the reaction centers, based on the following observations. In this suppressor strain LHC trimers are absent (Fig. 2). The LHC polypeptides are highly sensitive to mild protease digestion of thylakoid membranes (Fig. 3), and it has not been possible to isolate the PSI reaction center stably associated with LHCI. Thus a large part of the excitation energy cannot reach the reaction center.

We have found that the high light sensitivity of the PsaF-deficient mutant can also be suppressed by a mutation affecting Chl b synthesis. Loss of Chl b is known to prevent the correct insertion of the major LHCII polypeptides from *C. reinhardtii* into the thylakoid membrane (75). Analysis of the chlorophyll b-deficient barley *chlorina-f2* mutant revealed that only a few LHC polypeptides, LHCb1, LHCb6, and LHCa4, require Chl b for protease-stable integration into the thylakoid membrane (49, 50). In contrast, all LHC polypeptides appear to be protease-sensitive in the suppressor strain characterized here, suggesting that the nuclear mutation that suppresses the phenotype of 3bF affects most likely a factor that is important for integration of the LHCI and LHCII polypeptides into the thylakoids of *C. reinhardtii*. Immunoblot analysis has shown that the chloroplast SRP54 protein, which is believed to play a role in post-transcriptional transport of LHCPII (76), is present in the suppressor strain (data not shown), indicating that the mutation affects another component of the assembly machinery. Thus, our suppressor selection scheme opens the door for a genetic dissection of LHC assembly.

Our results indicate that the presence of a functional antenna requires an intact donor side of PSI for protection of *C. reinhardtii* from photo-oxidative damage in high light. It is thus possible that the development of light-harvesting systems for PSI and PSII in eukaryotic organisms demanded improvement of the donor side of PSI, in particular with regard to its interaction with its electron donors. This may have resulted in the evolution of the recognition site within the N-terminal part of PsaF that is essential for efficient electron transfer between PSI and plastocyanin and cytochrome c_6 under certain stress conditions such as high light. Such a feature appears to be found uniquely in eukaryotic photosynthetic organisms. We also suggest that plastocyanin displaced cytochrome c_6 as electron donor for PSI during evolution, because it is slightly more efficient in reducing $P700^+$ than the heme-containing protein (22) and should therefore provide a better protection against photo-oxidative stress.

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Limitation in Electron Transfer in Photosystem I Donor Side Mutants of *Chlamydomonas reinhardtii* : LETHAL PHOTO-OXIDATIVE DAMAGE IN HIGH LIGHT IS OVERCOME IN A SUPPRESSOR STRAIN DEFICIENT IN THE ASSEMBLY OF THE LIGHT HARVESTING COMPLEX

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