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Isolation and Characterization of Photoautotrophic Mutants of *Chlamydomonas reinhardtii* Deficient in State Transition*

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In photosynthetic cells of higher plants and algae, the distribution of light energy between photosystem I and photosystem II is controlled by light quality through a process called state transition. It involves a reorganization of the light-harvesting complex of photosystem II (LHCII) within the thylakoid membrane whereby light energy captured preferentially by photosystem II is redirected toward photosystem I or *vice versa*. State transition is correlated with the reversible phosphorylation of several LHCII proteins and requires the presence of functional cytochrome b_6f complex. Most factors controlling state transition are still not identified. Here we describe the isolation of photoautotrophic mutants of the unicellular alga *Chlamydomonas reinhardtii*, which are deficient in state transition. Mutant *stt7* is unable to undergo state transition and remains blocked in state I as assayed by fluorescence and photoacoustic measurements. Immunocytochemical studies indicate that the distribution of LHCII and of the cytochrome b_6f complex between appressed and nonappressed thylakoid membranes does not change significantly during state transition in *stt7*, in contrast to the wild type. This mutant displays the same deficiency in LHCII phosphorylation as observed for mutants deficient in cytochrome b_6f complex that are known to be unable to undergo state transition. The *stt7* mutant grows photoautotrophically, although at a slower rate than wild type, and does not appear to be more sensitive to photoinactivation than the wild-type strain. Mutant *stt3-4b* is partially deficient in state transition but is still able to phosphorylate LHCII. Potential factors affected in these mutant strains and the function of state transition in *C. reinhardtii* are discussed.

State transition has been originally described as a process whereby organisms performing oxygenic photosynthesis respond to changes in the spectral quality of light by changing the relative sizes of their photosystem I (PSI)[1] and photosystem II (PSII) antennae (for review, see Ref. 1). This leads to a redistribution of excitation energy between these two photosystems, which optimizes the photosynthetic yield. The reorganization of the antennae involves the displacement of the mobile fraction of a peripheral antenna complex, the light-harvesting antenna of photosystem II (LHCII), within the thylakoid membrane. During transition from state I to state II induced by PSII light (650 nm), the mobile fraction of LHCII is displaced from the PSII-enriched grana region to the PSI-enriched stromal lamellae. In the presence of PSI light (700 nm), the reverse migration of LHCII from PSI to PSII occurs. The redistribution of excitation energy between the photosystems results in changes in room temperature fluorescence with high and low fluorescence levels in state I and II, respectively, and in drastic alterations of the fluorescence emission spectrum at low temperature. As a consequence of this redistribution of energy between the two photosystems, state transition regulates the ratio between cyclic and linear electron flow and thereby modulates the level of ATP in the cells (2).

At the molecular level, state transition is controlled by the redox state of the plastoquinone (PQ) pool (3). Reduction of plastoquinone to plastoquinol (PQH_2) induces state II, whereas its oxidation induces state I. Transition from state I to state II correlates with the phosphorylation of a subset of the LHCII proteins on their stromal exposed N-terminal ends (4, 5). This has led to the proposal that a redox-sensitive kinase (LCHII kinase) could control state transition by phosphorylation of the antenna molecules (1). However attempts to isolate this kinase have not been successful (6–9), although several kinases tightly associated with the thylakoid membrane have been recently identified (10, 11).

Changes in fluorescence yield in response to changes in the PQ redox state can be observed readily in the unicellular alga *Chlamydomonas reinhardtii* (12), and several important observations concerning state transition have been made with this organism. In particular, an actual increased sensitization of PSI by LHCII has been observed in state II in this microalga (13). As for higher plants, a correlation between state transition and protein phosphorylation has been reported for *C. reinhardtii* (12, 14, 15). The major phosphoproteins are CP29, CP26, LHCP11, LHCP13 and LHCP17. CP29 and CP26, corresponding to P9 and P10 in the nomenclature of Chua and Bennoun (16), are part of the minor antenna of photosystem II, whereas LHCP11, LHCP13 and LHCP17 are members of the LHCII protein family (17). *In vivo*, state II to state I and state I to state II transitions can be blocked by the phosphatase inhibitor sodium fluoride and by the kinase inhibitor staurosporine, respectively (18), suggesting that phosphorylation of LHCII is indeed involved in the control of state transition.

The analysis of mutants of *C. reinhardtii* deficient in cytochrome b_6f activity has shown that absence of this complex

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[1] The abbreviations used are: PSI, photosystem I; PSII, photosystem II; LHCII, light-harvesting complex of PSII; PQ, plastoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TAP, Tris acetate-phosphate medium; PAGE, polyacrylamide gel electrophoresis; μE, microEinstein.

prevents transition from state I to state II as well as its associated phosphorylation events (19). It therefore appears that the cytochrome b_6f complex is involved in the regulation of state transition, most probably by controlling the LHCII kinase activity through plastoquinol-binding to the Qo site of the protein complex (20–22). Whereas deficiencies in the activity of PSII or ATP synthase have no direct effect on state transition (2, 19), PSI-deficient mutants do not display fluorescence changes under conditions of state transition (13). However, LHCII protein phosphorylation and migration still occur in these mutants in contrast to cytochrome b_6f-deficient mutants.

To gain new insights into the molecular mechanisms underlying state transition in *C. reinhardtii*, we have isolated and characterized mutants unable to change the fluorescence yield in response to changes in the redox state of the plastoquinone pool. Among the state transition mutants isolated, two were able to grow photoautotrophically. One of these mutants, *stt7*, is blocked in state I and displays major alterations in LHCII protein phosphorylation. It is able to grow photoautotrophically, although at a slower rate than wild type, accumulates normal levels of functional cytochrome b_6f, photosystem I and photosystem II complexes, and does not appear to be more sensitive to photoinactivation than wild type. The other mutant, *stt3-4b*, is partially deficient in state transition but is still able to phosphorylate LHCII under state II conditions.

EXPERIMENTAL PROCEDURES

Strains and Media—The *C. reinhardtii* mutants *FuD6*, *FuD7*, and *H13* have been described previously (23–25). These mutants are deficient in the accumulation of cytochrome b_6f, PSII, and PSI complex, respectively. *arg7* is auxotrophic for arginine due to a mutation in the *ARG7* gene, which encodes argininosuccinate lyase (EC 4.3.2.1) (26, 27). *cw15* is a cell wall-deficient mutant (28). The *arg7*; *cw15 mt-* strain contains both mutations. Wild-type and mutant strains were grown as described by Harris (28). If necessary, the enriched medium (TAP) and the high salt minimal medium were solidified with 2% Bacto agar (Difco) and supplemented with 10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (ICN Biochemicals).

Insertion Mutagenesis by Transformation—Nuclear transformation of cell wall-deficient *C. reinhardtii* cells was carried out according to the protocol of Kindle (29). *C. reinhardtii arg7; cw15 mt-* cells were grown in TAP containing 110 μg ml^{-1} arginine until a cell density of ~2.5 × 10^6 cells ml^{-1} was reached. Cells were pelleted at room temperature and resuspended in TAP to a final concentration of 10^8 cells ml^{-1}. 300 μl of cells, 300 mg of sterile glass beads, and 1.5 μg of plasmid pARG7Φ3 (30, 31) were mixed in a glass tube and vortexed for 15 s at maximal speed. Transformed cells were plated on TAP plates containing 10^{-6} M DCMU. Finally, plates were incubated at 25 °C under low light (6 μE m^{-2} s^{-1}) until isolated colonies appeared. The transformation efficiency was approximately 200 colonies/μg of DNA. No colonies were obtained when the transformation was performed in the absence of plasmid DNA indicating that the reversion rate of the *arg7* mutation was negligible under the conditions used.

State Transition Measurements—A qualitative measurement of state transition was obtained by growing *C. reinhardtii* cells on TAP plates supplemented with 10^{-6} M DCMU. State I (leading to high fluorescence at room temperature) was obtained by oxidation of the plastoquinone pool by illumination of the plates with white light (60 μE m^{-2} s^{-1}) for 15 min. State II (leading to low fluorescence at room temperature) was induced by reducing the plastoquinone pool by flushing the plates with nitrogen in the absence of light (2, 32). These conditions prevent ATP synthesis by either photosynthesis or respiration but stimulate instead the glycolytic pathway (Pasteur effect) and lead to an increase in the stromal NADPH concentration and ultimately to the reduction of the plastoquinone pool, presumably via a NADPH/plastoquinone oxidoreductase (33) or a succinate dehydrogenase (34). Fluorescence pictures of the whole plate corresponding to state I and state II were recorded, quantified and stored using a device similar to that described by Fenton and Crofts (35) and subtracted numerically. The differential fluorescence signal of each colony provides a measure of state transition.

State transition can be measured quantitatively using fluorescence at room temperature. *C. reinhardtii* cells in liquid culture were shaken vigorously in the absence of light for at least 15 min to oxidize the plastoquinone pool (state I). DCMU at a final concentration of 10^{-5} M was added to the culture 1 min before fluorescence was measured using the plant efficiency analyzer device (Hansatech Ltd., King's Lynn, England). State II was induced by adding glucose and glucose oxidase or sodium azide (250 μm) to the sample to inhibit mitochondrial respiration and to reduce the plastoquinone pool. Cells were incubated for 20 min in the absence of light before the second fluorescence measurement was performed. Finally, state transition was expressed as the relative difference of maximal fluorescence in both conditions: $[F_m(ox) - F_m(red)]/F_m(ox)$.

Fluorescence Transients—Fluorescence transients were measured on whole cells grown on plates TAP (± arginine) as described previously (35).

Immunoblot Analysis—Frozen cell pellets were resuspended in 55 mM Tris-HCl, pH 7, 2% SDS, and 0.5 mM EDTA. Cells were lysed for 15 min at 37 °C before the lysate was centrifuged for 2 min in an Eppendorf centrifuge. The supernatant containing solubilized proteins was stored at −70 °C.

Protein concentration was determined using the bicinchoninic acid protocol (36). Equal amounts of proteins were separated by SDS-PAGE as described (37). Western blotting and ECL detection were carried out according to the manufacturer's protocol (Amersham Pharmacia Biotech).

Measurements of 77 K Fluorescence Emission Spectra—Cells were harvested in mid-exponential phase by low speed centrifugation and resuspended in TAP medium at 10^7 cells/ml. Oxidation of the plastoquinone pool to reach state I was achieved by illuminating the cells for 20 min in the presence of 10^{-5} M DCMU. Reduction of the plastoquinone pool to favor state II was obtained by incubating the cells for 20 min in the dark in the presence of 20 mM glucose and 2 mg ml^{-1} glucose oxidase. Under these conditions oxygen is consumed, and mitochondrial and chloroplast respiration are inhibited. Reactions were stopped by the addition of 20 mM NaF and rapid cooling to 4 °C. Cells were then pelleted, washed once in TAP medium, and used for 77 K fluorescence. Cells in state I or state II were resuspended in TAP medium at 5 × 10^6 to 2 × 10^7 cells/ml and frozen in liquid nitrogen. Low temperature fluorescence emission spectra were recorded using a Jasco FP-750 spectrofluorimeter.

Photoacoustic Spectroscopy—The spectral dependence of the quantum yield of photochemistry in the red region (630–705 nm) was measured as described by Delosme *et al.* (13, 38) in the first microsecond following a monochromatic laser flash of very low energy.

In Vitro Phosphorylation—The membrane purification protocol was derived from the procedure described by Owens and Ohad (39) and Ohad *et al.* (40). *C. reinhardtii* cells grown in TAP (± arginine) to a concentration of 5–10 μg of chlorophyll/ml were pelleted at 4 °C, washed once with cold membrane buffer (50 mM Hepes-KOH, pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$) and resuspended in the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride to a concentration of 200–400 μg of chlorophyll/ml. Cells were broken by sonication at 4 °C and diluted 5-fold in cold membrane buffer. Unbroken cells were pelleted by a low speed centrifugation (1 min, 2,000 × g, 4 °C). The supernatant was then centrifuged at 10,000 × g for 15 min at 4 °C to pellet the membrane fractions. The pellet was resuspended carefully in membrane buffer using a paint brush and used immediately for *in vitro* phosphorylation.

Membrane fractions were resuspended in 100 μl of cold phosphorylation buffer (50 mM Hepes-NaOH, pH 7.6, 100 mM sorbitol, 5 mM $MgCl_2$, 5 mM NaCl) at a concentration of 0.1 μg of chlorophyll/ml and preincubated for 30 min at 4 °C in the dark. 10 μCi [γ-^{32}P]ATP (3,000 μCi nmol^{-1}) (Amersham Pharmacia Biotech) and 40 nmol of ATP were added to each sample, and labeling was carried out for 20 min at 25 °C in the presence of light (150 μE m^{-2} s^{-1}). Labeled membranes were washed twice in cold phosphorylation buffer with 1 mM phenylmethylsulfonyl fluoride and 20 mM sodium fluoride, resuspended in loading buffer, and fractionated by SDS-PAGE using 12% (w/v) acrylamide gels containing 6 M urea. The gel was then stained with Coomassie Blue, dried, and subjected to autoradiography (37).

Thylakoid Membrane Purification and Immunoblotting Using Polyclonal Antibodies to Phosphothreonine—Cells incubated under state I or state II conditions were resuspended in 25 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 0.3 M sucrose, and 1 mM phenylmethylsulfonyl fluoride, and broken with glass beads under vigorous shaking. Thylakoids were then isolated by the flotation procedure described by Chua and Bennoun (16). Thylakoid polypeptides were separated by SDS-PAGE using 12% (w/v) acrylamide gels containing 6 M urea and transferred to nitrocellulose filters. Phosphoproteins were detected using rabbit polyclonal antibodies to phosphothreonine (Zymed Laboratories Inc.).

TABLE I
Growth properties of state transition mutants

A sample of 10 μl from cell cultures of the different strains was spotted on agar plates and grown under the light indicated. Growth was monitored after 1 week. +, normal growth; (+), reduced growth; *, no confluent growth; −, no growth; [y], yellow colonies; p, partially deficient.

Medium	Light	*stt2*	*stt3*	*stt3–4b*	*stt5*	*stt7*	*FuD6*	*arg7; cw15*	Wild type
	$\mu E\ m^{-2}\ s^{-1}$								
TAP + Arg	6	+	(+)	+	+	+	+	+	+
TAP	0	ND[a]	(+)[y]	+	ND	+	+	—	+
TAP	6	+	(+)	+	+	+	+	—	+
TAP	60	ND	(+)*	+	ND	+	+	—	+
TAP	600	ND	—	+	ND	+	—	—	+
High salt minimal medium	60	—	(+)*	+	—	+	—	—	+
State transition		—	—[p]	—[p]	—	—	—	+	+

[a] ND, not determined.

In Vivo ^{33}P -Labeling of Thylakoid Membranes—Cells were labeled as described by Delepelaire and Wollman (14) except that [^{33}P]orthophosphate (specific activity 2,500–3,500 Ci mmol^{-1}) at 1 μCi ml^{-1} was used. Thylakoid membrane proteins were fractionated by electrophoresis on an 8 M urea 12–18% polyacrylamide gel.

Immunocytochemical Study—Preparation of *C. reinhardtii* cells for electron microscope immunoanalysis and counting of the gold granules over appressed and nonappressed thylakoid membranes were as described (41).

RESULTS

Isolation of State Transition Mutants—Transformation of *C. reinhardtii arg7; cw15* cells with plasmid pARG7.8Φ3 restores argininosuccinate lyase activity through random insertions of plasmid sequences into the nuclear genome (30, 31). Using this approach, 4,500 transformants were isolated by selection on acetate-containing medium (TAP) lacking arginine. To identify state transition mutants among the transformants, the fluorescence yield of each colony was compared under conditions that lead to the oxidation or reduction of the plastoquinone pool (see "Experimental Procedures"). After subtraction of the fluorescence images of the cells in state I and state II, wild-type colonies give rise to strong signals, whereas the signals originating from colonies deficient in state transition are nearly undetectable. Using this differential fluorescence screen, four mutants deficient in state transition were isolated. These strains were named *stt2, stt3, stt5*, and *stt7* (for state transition-deficient mutants).

Growth and Fluorescence Properties—Growth of the four isolated mutants was tested under different conditions (Table I). As expected from the selection used, all four state transition mutants grow on medium lacking arginine in contrast to the original recipient strain *arg7; cw15*. This correlates with the presence of sequences from the transformation vector inserted in their genome (data not shown).

Transformants *stt2* and *stt5* are unable to grow in the absence of acetate (Table I) indicating that they are deficient in photosynthetic activity. In contrast, *stt3* and *stt7* are able to grow photoautotrophically. Because one possible function of state transition that has been proposed is to provide some protection against PSII damage under high light (42), we tested the growth properties of *stt7* and *stt3* under different light regimes (Table I). Growth of *stt7* is unaffected even under high light (600 $\mu E\ m^{-2}\ s^{-1}$). In contrast, *stt3* grows significantly slower and is sensitive to high light (600 $\mu E\ m^{-2}\ s^{-1}$) Compared with the *arg7*; *cw15* strain, *stt3* has also acquired a *yellow in the dark* phenotype (Table I), suggesting a deficiency in light-independent chlorophyll synthesis.

Measurements of fluorescence transients provide a noninvasive means to monitor the activity of the photosynthetic complexes in the thylakoid membrane. It can be seen in Fig. 1 that the fluorescence transients of *stt7, arg7;* of *cw15* and wild type are nearly undistinguishable indicating that the photosynthetic complexes PSII, cytochrome b_6f, and PSI function at a similar rate in these strains. The fluorescence transients of *stt2* and *stt5* are clearly different from that of wild type (Fig. 1) and resemble those of mutants deficient in cytochrome b_6f activity such as *FuD6* (23). This raises the possibility that cytochrome b_6f is also affected in mutants *stt2* and *stt5*. In the case of *stt3*, the fluorescence transient indicates a defect in the electron transport chain.

Accumulation of Photosynthetic Complexes—To determine the accumulation of the photosynthetic complexes in the state transition mutants, immunoblot analysis was performed using antibodies directed against the polypeptides PsaA (PSI), cytochrome *f* (cytochrome b_6f complex), and D1 (PSII). The *stt2* and *stt5* mutants contain no detectable amount of cytochrome *f* indicating that they lack a functional cytochrome b_6f complex (Fig. 2). The absence of the cytochrome b_6f complex explains the growth phenotype, the fluorescence transients, as well as the lack of state transition in these transformants. Because the relationship between cytochrome b_6f deficiency and state transition has been extensively studied in *C. reinhardtii* (13, 19), these mutants were not characterized further.

As expected from its growth phenotype and its fluorescence transient, *stt7* accumulates all tested photosynthetic complexes to normal levels (Fig. 2). In *stt3*, cytochrome *f* accumulates normally, but the amount of PSI-A is considerably diminished and accumulation of the D1 protein is reduced (Fig. 2). To test whether the phenotypes of the mutants *stt7* and *stt3* are due to single mutations and whether these mutants are tagged, several backcrosses with a wild-type strain were performed. Because the *arg7; cw15* strain used for transformation crosses poorly, only incomplete tetrads were recovered. In the case of *stt7*, analysis of the progeny from the second backcross revealed that the mutation does not cosegregate with the signals arising from hybridization of the DNA from this progeny with a probe from the transformation vector (data not shown). The half-times of growth on high salt minimal medium of progeny with and without the *stt7* mutation were measured to be 10.6 ± 1.0 h and 8.4 ± 0.4 h, respectively (three independent measurements). The corresponding value for wild type was 7.2 ± 0.6 h. The *stt7* mutation thus slightly decreases the growth rate. Upon crossing *stt3* to wild type, the state transition phenotype of *stt3* could be segregated from the PSI deficiency, and the state transition mutant was named *stt3-4b*. In this case, analysis of 15 progeny revealed a cosegregation between the *stt3* mutation and the hybridization signal obtained with the probe from the transformation vector indicating that this mutant is most likely tagged.

State Transition in stt7 and stt3-4b—Quantitative state transition measurements on strain *stt7* show that the fluorescence yield at room temperature under oxidizing conditions is slightly lower than under reducing conditions, whether the

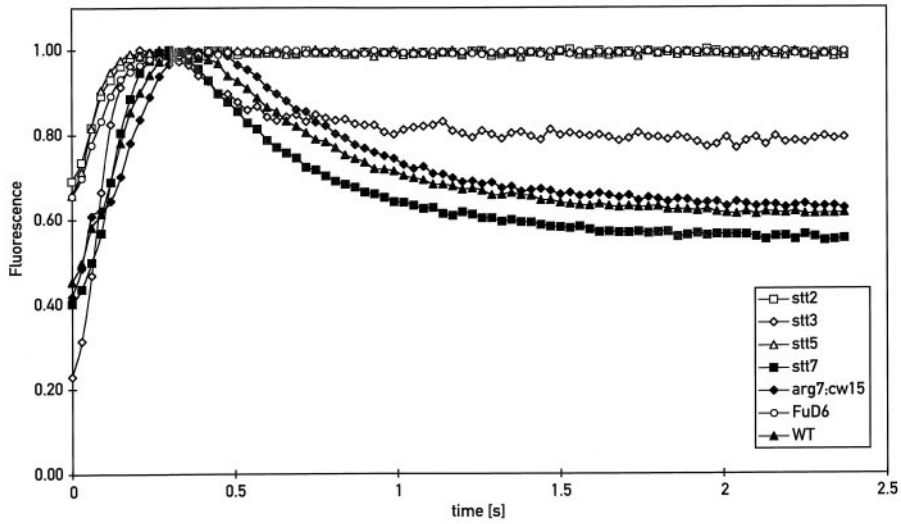

FIG. 1. **Normalized fluorescence transients of wild-type and state transition mutants.** Cells were grown in TAP medium and dark adapted for 1 min before each measurement. The transients were normalized to F_{max}.

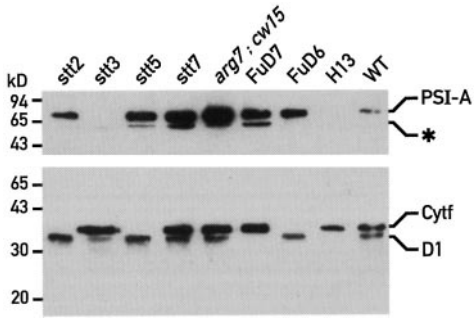

FIG. 2. **Immunoblot analysis of state transition mutants.** 10 μg of protein extracts from cells grown under low light (6 μE m^{-2} s^{-1}) were analyzed by immunoblotting using antibodies directed against PsaA, cytochrome *f*, and the D1 protein. The band marked by a *star* is labeled unspecifically with the PsaA antibody. *Fud7*, *H13*, and *Fud6* are mutants deficient in PSII, PSI, and cytochrome b_6f complex. Although the PSI-A subunit is not detectable in *stt3* on this blot, this mutant is only partially defective in PSI and is able to grow photoautotrophically but at a slower rate than wild type.

latter were obtained by an incubation in anaerobic conditions (Table II) or upon treatment with the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (not shown). The state transition value of *stt7* is thus slightly negative ($(F_m(ox) - F_m(red))/F_m(ox)$, Table II). Small negative state transition values are also observed for the cytochrome b_6f-deficient control strain *FuD6* (Table II), which are interpreted as an absence of state transition with a small quenching of fluorescence under state I conditions due to oxidized plastoquinone (43). The wild-type and *arg7; cw15* control strains, however, yield positive values characteristic of a functional state transition (Table II). Thus, comparison of *stt7* with control strains shows that this strain is affected in state transition to the same extent as cytochrome b_6f-deficient mutants. The *stt3-4b* mutant was only partially deficient in state transition (Table II).

Fluorescence emission spectra of intact *C. reinhardtii* cells at 77 K resolve two peaks, which correspond to light re-emitted from PSII at 684 nm and PSI in the 705–715 nm region (12). In wild type, the ratio of fluorescence yield between the two peaks ($F_{PSII/PSI}$) depends on the redox state of the plastoquinone pool (Table II, Fig. 3). As reported previously (12), this ratio is higher in state I than in state II, reflecting the different light energy distribution by the antenna toward the two photosystems. For the cytochrome b_6f-deficient mutants, the fluorescence ratios do not change significantly between state I and state II, and the fluorescence emission spectra resemble those of wild-type cells in state I (Fig. 3). However we noted an enrichment in fluorescence emission around 700 nm of presently unknown origin. A typical state I spectrum was also observed with *stt7* cells with a high F_{PSII}/F_{PSI} ratio under both redox conditions (Table II, Fig. 3). Thus the *stt7* cells appear to be blocked in state I with the LHCII antenna preferentially connected to photosystem II as observed for the cytochrome b_6f-deficient mutants. The low temperature fluorescence emission spectrum of the *stt3-4b* mutant reveals an enrichment in fluorescence emission around 710 nm, which does not change appreciably during state transition. Alterations in the long wavelength emission peaks at 77 K has been reported previously (44) in mutants with modified PSI-LHCI content.

Photoacoustic measurements provide another means to determine the distribution of the antenna pigments between the two photosystems during state transition (38). The quantum yield spectrum in the red region was measured for the wild-type, *stt3-4b*, and *stt7* strains (Fig. 4). The observed variations in the quantum yield reflect differences in the efficiency of excitation transfer from the various pigments to the reaction center. The PSI + PSII spectrum was determined to obtain a reference spectrum for the state of connection of the light-harvesting antenna when the two reaction centers are active. The PSI spectrum was established by blocking PSII activity by preillumination in the presence of hydroxylamine and DCMU (38). The PSI spectrum of wild-type cells in state I shows a significant drop in quantum yield below 680 nm, which corresponds to the LHCII absorbance region. This indicates that the chla/b antenna is connected mostly to PSII centers. The PSI spectrum in state II reveals that this complex is considerably more sensitized by the LHCII-associated pigments indicating that a significant fraction of the LHCII antenna is connected to PSI. In striking contrast, the quantum yield spectrum of PSI of the *stt7* mutant is nearly identical in state I and state II (Fig. 4). A trough near 650 nm is apparent, which indicates that LHCII is disconnected from PSI, a characteristic feature of state I. The results obtained with *stt3-4b* confirm that this mutant still displays state transitions but of limited amplitude. There is only a modest increase in the quantum yield of PSI in state II in comparison to state I.

Migration of LHCII to Nonappressed Thylakoid Regions Is Impaired in the stt7 Mutant—Previous studies have revealed extensive changes in the distribution of cytochrome b_6f complex and LHCII between the appressed and the nonappressed thylakoid membrane regions during state transition (41). To further confirm that the lateral redistribution of the mobile part of the LHCII antenna from PSII in the appressed regions to PSI

TABLE II
Fluorescence properties of state transition mutants and control strains

	stt7	*stt3–4b*	*arg7;cw15*	*FuD6*	Wild type
Room temperature fluorescence					
F_m (*ox*)	100	100	100	100	100
F_m (*red*)	106	79	71	103	62
State transition	−6%	21%	29%	−3%	38%
Low temperature fluorescence					
$F_{684/705}$ (*ox*)	1.54	0.61	1.35	1.01	1.48
$F_{684/705}$ (*red*)	1.57	0.53	0.9	0.96	1.07
State transition (77K)	−2%	13%	33%	5%	28%
F_v/F_m	0.83	0.63	0.79	0.48	0.82
Chlorophyll a/chlorophyll b	2.30	1.80	2.71	ND[a]	2.31

All values are an average of at least three independent measurements. Room temperature fluorescence, state transition is defined as the value $(F_m(ox) - F_m(red))/F_m(ox)$ expressed as percentage. Fluorescence at low temperature, state transition (77K) is defined as the ratio $F_{684/705}(ox)/F_{684/705}(red)$ expressed as percentage. F_v/F_m, cells were pregrown in TAP under low light (6 μE m^{-2} s^{-1}) and dark-adapted for 1 min before the measurements that were performed in the presence of 10^{-5} M of DCMU.

Chlorophyll a/chlorophyll b, cells were grown in TAP; chlorophyll was measured in acetone:water 80:20 using the coefficients of Porra *et al.* (45).

[a] ND, not determined.

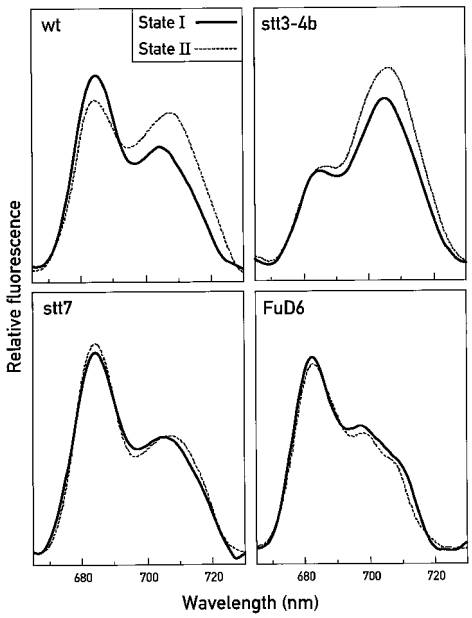

FIG. 3. **77 K fluorescence emission spectra of whole cells.** 77 K emission spectra of wild type wt, *stt3-4b*, *stt7*, and *Fud6* cells were measured under state I or state II conditions. *Full lines* represent cells incubated in light in the presence of 10^{-5} M DCMU (state I). *Dotted lines* represent cells incubated in the dark in the presence of glucose and glucose oxidase (state II).

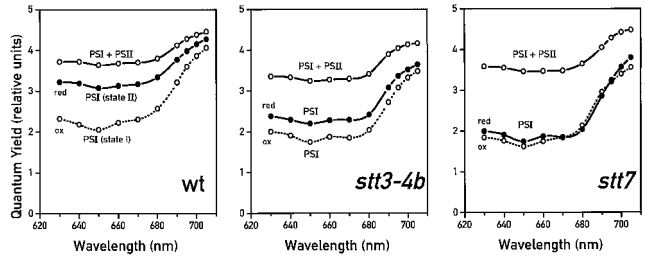

FIG. 4. **Quantum yield spectrum of photochemical charge separation measured by photoacoustic spectrometry.** *Open circles*, the cells were fixed in "state I" conditions by addition of 0.3 mM benzoquinone following complete oxidation of the plastoquinone pool. *Solid circles*, "state II" conditions (plastoquinone pool fully reduced under anaerobic conditions). The PSI + PSII spectrum under state II conditions (not shown) was practically the same as under state I conditions. The PSI spectrum was measured after a preillumination of a few seconds in the presence of 40 μM DCMU and 2 mM hydroxylamine, which block the PSII centers in an inactive state. *ox* and *red* refer to the redox state of the plastoquinone pool.

TABLE III
Distribution of LHCII and cytochrome b_6f complex between the nonappressed and appressed thylakoid membrane regions

The antibodies used were against the polypeptide P11 of LHCII and cytochrome *f* and the Rieske protein. dn/da, density of antibody-linked gold granules on the nonappressed and appressed thylakoid membrane regions.

Strain	dn/da	
	α-LHCII	α-cytochrome *f* + Rieske
Wild-type state I	0.25 ± 0.07	0.44 ± 0.06
Wild-type state II	0.81 ± 0.17	0.82 ± 0.17
stt7 state I	0.27 ± 0.06	0.54 ± 0.24
stt7 state II	0.32 ± 0.06	0.61 ± 0.05
stt3–4b state I	0.22 ± 0.05	0.66 ± 0.20
stt3–4b state II	0.54 ± 0.13	0.83 ± 0.09

in the nonappressed thylakoid membrane regions is impaired during state transition in the mutants *stt7* and *stt3-4b*, an immunocytological study was performed. Cells from the wild type and from the mutants were examined under state I and state II conditions by immunoelectron microscopy using antibodies against LHCII. The distribution of gold particles was measured over selected areas in which appressed and nonappressed thylakoid membranes could be clearly identified. From these measurements the ratio of densities of label dn/da between the nonappressed and appressed regions could be determined. As shown in previous reports (13, 41) and confirmed in this study (Table III) the ratio dn/da was found to be significantly higher in wild-type cells in state II than in state I because of the displacement of a portion of LHCII from the appressed to the nonappressed regions during a state I to state II transition. In contrast, no significant change of dn/da was found between state I and state II for *stt7* (Table III). A modest increase of this ratio was observed for the *stt3-4b* mutant in state II relative to state I, confirming that this mutant is only partially deficient in state transition (Table III). It was of interest to perform the same analysis with cytochrome *f* and Rieske protein antibodies, as it has been reported that the cytochrome b_6f complex is also redistributed between the appressed and nonappressed regions during state transition (41). The results shown in Table III indicate that whereas the dn/da ratio for the cytochrome b_6f complex of the wild type is higher in state II than in state I, the corresponding difference is much reduced in *stt3-4b*, and there is no significant change in dn/da for *stt7* under the same experimental conditions.

Phosphorylation of LHCII Proteins—In *C. reinhardtii*, as well as in higher plants, state transition correlates *in vivo* with phosphorylation of a specific subset of LHCII proteins (4, 12, 14). To examine the phosphorylation patterns of the LHCII polypeptides of the mutants, *in vivo* protein pulse-labeling with

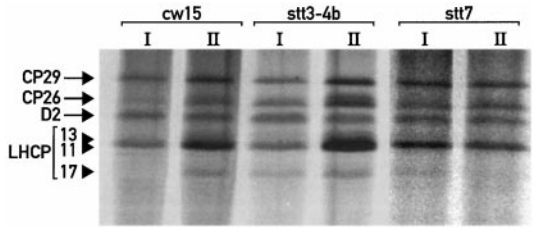

FIG. 5. **Autoradiogram of *in vivo* ^{33}P-labeled antenna polypeptides.** State I was induced by incubating cells in darkness under strong aeration, and state II in the presence of 20 mM glucose and 2 mg/ml glucose oxidase, respectively. Cells were labeled with [^{33}P]orthophosphate as described under "Experimental Procedures." Thylakoid polypeptides were fractionated by PAGE on an 8 M urea 12–18% gradient gel and autoradiographed.

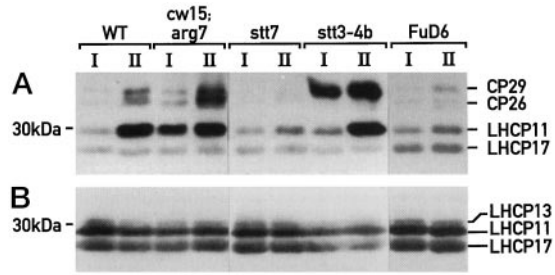

FIG. 6. **Levels of phosphothreonine in the LHCII proteins.** Thylakoids from cells in state I or state II were isolated and the proteins were separated by SDS-PAGE using 12% polyacrylamide gels containing 6 M urea and transferred to nitrocellulose and immunodecorated with antibodies against phosphothreonine (*A*) and LHCP11 (*B*).

[^{33}P]phosphate was performed. Thylakoid membranes were isolated from cells prelabeled for 90 min with [$^{33}P_i$]orthophosphate and then incubated for 20 min under state I and state II conditions in medium lacking isotope as described previously (12). Fig. 5 shows that there is a substantial increase in phosphorylation of the minor antenna complexes CP26 and CP29 as well as of LHCP11 in state II *versus* state I in the wild-type (cw15) strain and in the *stt3-4b* mutant. In addition LHCP13 and LHCP17 are labeled by [$^{33}P_i$]phosphate only in state II conditions in the wild type. In contrast no significant increase in phosphorylation is detectable in *stt7* during state transition.

Because phosphorylation of LHCII proteins occurs mostly at a threonine residue, phosphorylated LHCII proteins can be monitored with an antibody directed against phosphothreonine (46). Bands corresponding to CP26, CP29, LHCP11, and LHCP17 can indeed be detected by immunoblot analysis performed with this antibody on proteins from isolated thylakoid membranes of *C. reinhardtii* (Fig. 6*A*). It can be seen that in wild-type and in *arg7;cw15,* as well as in *stt3-4b* cells, the major antenna protein LHCP11 becomes more heavily phosphorylated in state II than in state I because the antenna complexes are present in similar amounts under both conditions (Fig. 6*B*). The same is true for CP29 and CP26 except for *stt3-4b* in which high levels of phosphothreonine are observed both in state I and state II. In the case of *FuD6*, protein phosphorylation *in vivo* is nearly independent of the redox state of the plastoquinone pool (Fig. 6*A*) thus confirming previous studies with cytochrome b_6f-deficient mutants (19). Moreover, the phosphorylation pattern corresponds to that observed for state I in wild type. As for *FuD6*, *stt7* shows a low phosphorylation pattern independently of the redox conditions (Fig. 6*A*) that cannot be attributed to differences in the accumulation of the substrate (Fig. 6*B*). Therefore, *stt7* is clearly affected in antenna protein phosphorylation *in vivo*, in a way that closely resembles the situation observed for cytochrome b_6f-deficient mutants.

The kinase activity associated with state transition is tightly associated with the thylakoid membranes (12). It is thus possible to reproduce the phosphorylation reaction *in vitro* with purified membrane fractions. Some of the antenna proteins are phosphorylated *in vitro* even if the PQ pool is oxidized or if the cytochrome b_6f complex is absent (12, 19). These proteins are P6 (PSII-C), CP29, CP26, and LHCP11. However, phosphorylation of LHCP13 and LHCP17 is specific to the transition from state I to state II (12). *In vitro* phosphorylation of these two LHCII subunits is not observed in *FuD6* nor in *stt7* under state II conditions (Fig. 7). Thus *stt7* is defective in the *in vitro* LHCII protein phosphorylation that is specifically associated to state transition in a similar way as observed for cytochrome b_6f-deficient strains. Taken together, the *in vitro* and *in vivo* antenna protein phosphorylation patterns as well as the anti-phosphothreonine immunoblot analysis concur that *stt7*, but

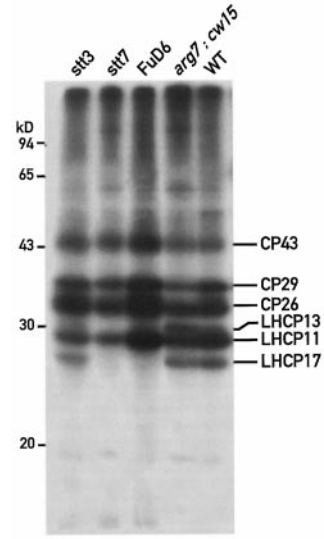

FIG. 7. **Autoradiogram of *in vitro* ^{32}P-labeled thylakoid polypeptides.** Membrane fractions of the different strains in state II were labeled *in vitro* with [γ-^{32}P]ATP (for details see "Experimental Procedures"). Proteins were fractionated on a 12% polyacrylamide gel containing 6 M urea and autoradiographed.

not *stt3-4b*, is unable to undergo an increased antenna protein phosphorylation upon state I to state II transition.

DISCUSSION

Here we have described a new method to isolate state transition mutants of *C. reinhardtii*, which is based on the large changes in room temperature fluorescence associated with state transition in this alga. The method does not depend on changes in the quality of light excitation that yield state transitions of limited amplitude (12), but it takes advantage of the possibilty to fully reduce the PQ pool *in vivo* by blocking respiration in darkness and depleting the intracellular ATP content (2, 12). The isolation of two mutants deficient in cytochrome b_6f activity using this screen confirms its validity, because these mutants are known to be unable to undergo state transition (19). The use of a genetic approach has the advantage of circumventing the problem associated with the potentially low abundance of the factors involved in state transition. It also opens the door for a dissection of the signal transduction pathway from the reduction of the plastoquinone pool to the displacement of the mobile part of the LHCII antenna. Finally it also offers the advantage that no *a priori* assumptions need to be made on the localization or the activity of these factors.

The *stt7* mutant isolated with our screen is defective in state transition and grows photoautotrophically, although at a reduced rate as compared with wild type. The state transition deficiency observed in this mutant is very similar to the one

described for cytochrome b_6f-deficient mutants: the cells are blocked in state I, and LHCII protein phosphorylation is affected both *in vitro* and *in vivo*. However *stt7* accumulates normal amounts of functional cytochrome b_6f complex. Therefore, this mutant allows one to study the state transition phenotype *per se* independently from any secondary effects due to impairment of electron transfer at the level of the cytochrome b_6f complex.

Based on the protein phosphorylation results *in vivo* (Figs. 5 and 6) and *in vitro* (Fig. 7), the *stt7* mutant is clearly deficient in the phosphorylation of antenna proteins that occurs upon a state I to state II transition. The s*tt7* mutant displays the same deficiencies in the antenna protein phosphorylation patterns as observed for cytochrome b_6f-deficient mutants. The low temperature fluorescence (Fig. 3) and photoacoustic measurements (Fig. 4) indicate that the chlorophyll a/b antenna remains mostly connected to PSII even under state II conditions. This is further confirmed by the electron microscopy analysis of the distribution of LHCII between the appressed and nonappressed thylakoid membrane regions. Whereas there is an important displacement of LHCII from the appressed to the nonappressed regions during a state I to state II transition in the wild type, this displacement is severely impaired in *stt7*. It is interesting to note that a similar lateral redistribution of the cytochrome b_6f complex in the thylakoid membrane, which occurs upon state transition in wild-type cells (41), is also abolished in *stt7*.

The phenotype of *stt7* indicates that the mutation affects an essential step in the regulation of state transition. Because state transition is measured on whole cells, we cannot exclude the possibility that the mutation acts indirectly. Mutations that would alter the ATP level by blocking glycolysis or mitochondrial respiration can, however, be excluded for two reasons. First, low ATP levels favor state II (2) and *stt7* is blocked in state I. Second, mutants unable to catabolize acetate do not grow in the absence of light in contrast to *stt7* (Table I). We can also exclude that the *stt7* mutation prevents the transition to state II only under anaerobic conditions because carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone is also unable to promote state II in *sst7.* The pronounced effect observed on state transition and the absence of any other major phenotype strongly suggest that the mutation in *stt7* acts specifically on state transition and not indirectly through a change in cellular metabolism. This is also supported by the *in vitro* phosphorylation experiment, which shows that the effect of the *stt7* mutation can be recapitulated with an isolated membrane fraction that contains mainly thylakoid membranes. Thus it is very likely that the factor altered by the mutation is associated with these membranes.

Assuming that LHCII kinase is indeed the master control in state transition, this enzyme could be mutated in *stt7*. This would agree with the phosphorylation results observed *in vitro* and *in vivo*. However, *stt7* could also be affected in a factor controlling state transition further upstream, *e.g.* in a factor associated with the thylakoid membranes that mediates the control of the cytochrome b_6f complex on state transition. This study has also offered the possibility to compare for the first time in Chlamydomonas the net changes in antenna phosphorylation, as probed with an antiphosphothreonine antibody, with the relative changes in phosphate groups on antenna proteins, probed with [^{33}P]orthophosphate, which also detects exchanges of phosphates on pre-existing phosphoresidues. We thus observed that ^{33}P-labeling of LHCP17, which is diagnostic of a transition to state II, does not correspond to a net increased phosphorylation of this antenna subunit but to a phosphate exchange on some of its residues. This finding is consistent with an earlier observation that only little dephosphorylation of LHCP17 was observed upon a reverse transition from state II to state I, whereas LHCP11, CP29, and CP26 were extensively dephosphorylated (47). This unexpected finding suggests either that some phosphotransfer process has promoted the displacement of some phosphate groups from LHCP17 to threonines on other antenna proteins thereby allowing their replacement on LHCP17 by the activated LHCII kinase, or that structural changes in the organization of the antenna proteins have triggered the turnover of phosphate groups on LHCP17. A similar process may occur with LHCP13 (compare Figs. 5 and 6).

We have not been able to detect any significant difference in the light sensitivity between the *stt7* mutant and the wild-type strain. This raises the question of the role of state transition in *C. reinhardtii*. State transition has been proposed to be involved in the control of distribution of excitation energy between the two photosystems through the reversible reduction of the PSII antenna and the concomitant increase in the size of the PSI antenna and *vice versa*. However because the absorption properties of the PSII and PSI antennae are similar, it is not clear whether a strong imbalance in energy distribution could occur in green algae under normal environmental light conditions. Several studies support the view that state transition is not only a light adaptation mechanism but that it also allows photosynthetic organisms to adapt to changes in cellular demand for ATP (2, 48, 49). In particular, an increase in the cellular need of ATP leads to state II, whereas the absence of ATP demand leads to state I. This view is further strengthened by the observation that upon transition from state I to state II the proportion of cytochrome b_6f complex associated with the PSI-enriched stromal lamellae is significantly increased and thereby favors cyclic electron flow and ATP synthesis at the expense of linear electron flow and NADPH production (41). It has been proposed that LHCII phosphorylation could participate in the protection against photoinhibition in cells exposed to excessive light (42). However, the LHCII kinase appears to be inactivated under photoinhibitory conditions in *C. reinhardtii* (50). Our results favor the idea that state transition provides no significant protection against photoinhibition because mutant *stt7* grows normally in high light, at least at 600 μE m^{-2} s^{-1} (Table I).

The original phenotype of the *stt3* mutant included both a partial deficiency in state transition and in PSI activity. By backcrossing this mutant to the wild type it has been possible to segregate these two phenotypes and to obtain the *stt3-4b* mutant, which displays normal PSI activity but is still partially deficient in state transition. This mutant appears to be deficient in a step that is further downstream than that affected in the *stt7* mutant. The *stt3-4b* mutant is able to phosphorylate the LHCII polypeptides in the same way as the wild type under state II conditions both *in vitro* and *in vivo*, although it is not able to display more than one-third of the transition to state II, as viewed by displacement of LHCII and the cytochrome b_6f complex or by changes in the fluorescence yields or PSI quantum yields. In addition, the low temperature fluorescence emission spectrum of *stt3-4b* shows some new features, with a significant increase in the 700-nm region, which may indicate some changes in the peripheral antenna organization. Thus the mobility of the phosphorylated antennae, but not the antenna phosphorylation process, is the most likely step to be altered in the *stt3-4b* mutant. Cloning of the mutated gene in the strains *stt7* and *stt3-4b*, as well as in other mutants affected in this process, will allow the identification of new components involved in state transition and thus provide new insights into its molecular basis in *C. reinhardtii* and in plants.

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