Impaired respiration discloses the physiological significance of state transitions in *Chlamydomonas*

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State transitions correspond to a major regulation process for photosynthesis, whereby chlorophyll protein complexes responsible for light harvesting migrate between photosystem II and photosystem I in response to changes in the redox poise of the intersystem electron carriers. Here we disclose their physiological significance in *Chlamydomonas reinhardtii* using a genetic approach. Using single and double mutants defective for state transitions and/or mitochondrial respiration, we show that photosynthetic growth, and therefore biomass production, critically depends on state transitions in respiratory-defective conditions. When extra ATP cannot be provided by respiration, enhanced photosystem I turnover elicited by transition to state 2 is required for photosynthetic activity. Concomitant impairment of state transitions and respiration decreases the overall yield of photosynthesis, ultimately leading to reduced fitness. We thus provide experimental evidence that the combined energetic contributions of state transitions and respiration are required for efficient carbon assimilation in this alga.

cyclic electron flow | photosynthesis | energetic metabolism

State transitions (ST) are a short-term photosynthetic acclimation process that controls the reversible association of the photosystem II (PSII) antenna protein complex (LHCCI) with either PSII (in state 1) or photosystem I (PSI) (in state 2) (1–3). This process relies on the reversible LHCCI phosphorylation involving the membrane-bound protein kinase Stt7-STN7, which recently has been identified in *Chlamydomonas reinhardtii* and *Arabidopsis thaliana* (4, 5). Phosphorylation changes lead to the migration of a fraction of the antenna between the PSII-enriched membrane domains and the PSI-enriched membrane domains within the thylakoids in plant and algal chloroplasts (3, 6).

STs first were observed in unicellular green algae and originally were described as a mechanism linking the redox poise of the intersystem electron carriers to changes in the absorption capacity of the photosystems (3). Reduction of the plastocyanine (PQ) pool upon increased PSII sensitization activates the kinase via the cytochrome *bcf* complex (7). Conversely, upon increased PSI sensitization, PQH₂ oxidation inactivates the kinase. P,


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effect in wild-type cells, suggesting that efficient photosynthetic growth requires respiration in the absence of ST.

This observation prompted us to use a genetic approach to explore further the interplay between ST and respiration. We resorted to *Chlamydomonas* mutants defective in mitochondrial activity (*dum*) (19) to generate a double mutant impaired in both ST and mitochondrial respiration. To this end, we crossed the *dum* mutant (lacking both complex I and III activities) with the *stt* mutant (lacking ST) to obtain *dum*/*stt* double mutants. We then compared these clones with their parental strains for growth capacity in photoautotrophic and mixotrophic conditions. Light intensity was either 50 or 400 μmol photons m⁻² s⁻¹.

**Functional Characteristics of *dum*/*stt*.** To understand which factors limit the growth rate of *dum*/*stt* in photoautotrophic conditions, we further characterized its photosynthetic properties. Recent data have suggested that ST may contribute to long-term acclimation via the regulation of gene expression (9, 10). In particular, it has been suggested that ST may modulate the relative amounts of active PSI and PSII in plants acclimated to either state 1 or state 2 (20). To test possible changes in the reaction center (RC) stoichiometry in *dum* cells, which are acclimated to state 2 (18), and in *stt* cells, which are, in principle, locked in state 1 (5), we used a spectroscopic approach based on the amplitude of the light-induced electrochromic shift (ECS). This technique has been used successfully to evaluate the PSI/PSII stoichiometry in freshwater green algae (21). As shown in Fig. 2, very minor differences were seen between the different strains, suggesting that the absence of ST has no significant effect on the RC stoichiometry, at least in *Chlamydomonas* (1).

We next assessed the balance in light energy distribution between the 2 photosystems that should be affected by the inability of *stt* cells and of *dum*/*stt* to perform ST. We first evaluated the PSI antenna in cells that were dark-adapted for 30 min after being grown photoautotrophically in low light (i.e., conditions in which the growth phenotype of *dum*/*stt* cells is maximum). To this aim, we measured the rate of chlorophyll fluorescence induction from open (F₀) to closed (Fₚ) PSI centers in the presence of 3-(3’,4’-dichlorophenyl)-1,1-dimethylurea (DCMU). This parameter, which is related quantitatively to the absorption cross-section of this photosystem (22) was 2 times slower in *dum*/*stt* than in *dum*/*stt* and wild-type cells. In addition, illumination of *dum* in the presence of DCMU for 10 min (a treatment known to promote transition to state 1 via oxidation of the PQ pool) (17, 18) increased its rate of fluorescence induction to values close to those measured in the other strains, but such treatment had no effect on *dum*/*stt* cells (Fig. 2C). Consistent with this finding, the ratio of PSI/PSII fluorescence emission at 77 K was higher in *dum*/*stt* than in the other strains in aerobic conditions.

### Fig. 1. Impact of inhibition of the mitochondrial respiratory chain on growth of ST-deficient strain. Cells were cultivated at 50 μmol photons m⁻² s⁻¹ in mixotrophic (TAP) or photoautotrophic (Min) conditions in the absence or presence (¾ M) of the mitochondrial respiratory inhibitor myxothiazol (5 μM). (A) Drops of cell suspensions (0.01 nm A₆₀₀) were plated on solid media, and growth was estimated after 3 to 5 days. (B) Doubling time in liquid cultures. Error bars indicate standard deviation of the mean of 3 independent measurements. (C) The experimental procedure is the same as in A. Photoautotrophic (Min) conditions. Light intensity was either 50 or 400 μmol photons m⁻² s⁻¹.

### Fig. 2. Comparative analysis of the photosynthetic features of the *dum*/*stt* mutant. (A) PSI/PSI ratios. Changes in the amplitude of the fast phase of the ECS signal (at 520–545 nm) upon excitation with a saturating laser flash in the presence or absence of PSI inhibitors DCMU (20 μM) and hydroxyamine (1 mM) were used to assess PSI and PSII stoichiometry. (B) Relative antenna size in PSII, as evaluated form fluorescence induction kinetics. Curves were normalized to the same value of variable fluorescence to allow a better comparison. (C) Effect of preillumination on PSII relative antenna size in *dum* cells and *dum*/*stt* cells. Solid symbols, dark adaptation for 20 min under strong agitation; open symbols, exposure to 50 μmol m⁻² s⁻¹ for 10 min in the presence of DCMU (10 μM); downwards triangles, *dum* cells; upwards triangles, *dum*/*stt* cells. (D) PSI antenna size as evaluated from the light saturation curve for P₇₀₀ oxidation in intact cells. The curves were acquired by exposing the cells to flashes of laser light of varying intensity and measuring the resulting P₇₀₀ oxidation. Traces were measured in the presence of hydroxyamine (1 mM) and DCMU (20 μM) to prevent PSI charge separation. Symbols are as in B.
conditions, but it decreased to similar values when illuminated in the presence of DCMU (Table S1). Together, these data confirm that the dum22 stt7–9 mutant was mostly in a state 1 condition, because it lacked the Stt7 kinase, whereas the dum22 mutant was in state 2. We next assessed changes in PSI antennae by measuring the light saturation curve for oxidation of the primary electron donor of PSI (P700) (23). Dark-adapted cells placed in aerobic conditions were exposed to a laser flash (duration 5 ns) of variable intensity, and the light-induced oxidation of P700 was estimated from the amplitude of the 4-μs component of the decay-associated spectra of P700+ (24). As shown in Fig. 2D, a larger P700 oxidation was observed in dum22 cells upon excitation with limiting light intensities. In agreement with previous functional (13) and biochemical (14) studies in wild-type cells, this increased P700 oxidation indicates that the 2-fold decrease in PSII absorption capacity caused by state 2 acclimation is accompanied by a similar increase in the PSI antenna size.

Increasing the PSI absorption capacity at the expense of PSII, as observed in dum22, should lead to unbalanced excitation of the 2 photosystems at limiting photon flux. To evaluate this possibility, we measured the redox state of P340 in steady-state continuous illumination. Again, experiments were conducted in conditions in which growth was affected mostly in the dum22 stt7–9 mutant, i.e., photoautotrophic growth in low light. Furthermore, we added an artificial PSI electron acceptor, methyl viologen (MV, 2 mM), to avoid any kinetic limitation by the PSI acceptor side and/or to avoid accumulation of reduced ferredoxin and NADPH, which may lead to re-injection of electrons into the inter-system electron carriers through CEF (25).

Fig. 3A shows typical kinetics of P340 oxidation in wild-type, stt7–9, dum22, and dum22 stt7–9 using continuous illumination of limiting intensity (50 μmol photons m−2 s−1) or saturating intensity (800 μmol photons m−2 s−1). These traces were used to estimate the light-saturation profiles of P340 oxidation in the 4 strains. As shown by the large difference in these profiles (Fig. 3B), photon absorption was unbalanced in favor of PSI in dum22 cells when compared to the 3 other strains, consistent with the transfer of light-harvesting antenna from PSII to PSI. Addition of the PSI inhibitor DCMU to MV-treated cells largely enhanced P340 oxidation (Fig. S3), confirming that most of the electrons delivered to P340 were of PSII origin. However, in the presence of both chemicals we noted an incomplete oxidation of P340 at low light intensities in all strains. We tentatively attribute this DCMU-insensitive electron pathway to the NADPH dehydrogenase (NDH)-mediated chlororespiratory pathway (26, 17).

Using the same procedure used to evaluate the rate of electron flow in DCMU-poised cells (see Table 1), we evaluated the maximum electron flow capacity of this pathway to be ~2 to 3 electrons/PSII/ in wild-type cells.

**Light-Dependent Plus Enhanced Light-Independent Plastoquinone Reduction Decreases Photosynthetic Yield at Low Light in dum22 stt7–9.** We next tested the overall photosynthetic activity by measuring oxygen evolution and fluorescence changes at different light intensities in wild-type, stt7–9, dum22, and dum22 stt7–9 cells in photoautotrophic conditions. In all strains, very similar maximum oxygen evolution rates were observed (Fig. 3C). However, a drastic inhibition of photosynthesis (65% to 75%) appeared in dum22 stt7–9 cells at limiting light intensities, whereas photosynthesis decreased by only ~35% in dum22 when compared with wild-type and stt7–9 cells (Fig. 3C, Inset). The reduced photosynthetic activity in dum22 stt7–9 cells also was accompanied by a drop in the quantum yield of linear electron flow as measured by the fluorescence parameter ΦPSII (Fig. 3D) (27).

This experiment reveals that, in *Chlamydomonas*, efficient photosynthesis cannot take place in an ST-impaired mutant when respiration is inhibited. However, the mere inhibition of ST is insufficient to modify photosynthesis when the stromal redox pressure is low, because of sustained respiratory activity, as shown by the lack of phenotype in stt7–9 cells. Thus, the rationale for the inhibition observed in dum22 stt7–9 cells should be found in 2 processes that develop with changes in the redox poise of the stromal compartment when respiration is inhibited. First, both the dum22 cells and dum22 stt7–9 mutants display a high level of non-photochemical reduction of the PQ pool because of the Pasteur effect associated with their respiratory defect (see introduction). This large electron reservoir for PSI turnover is paralleled by a higher PSI light-harvesting capacity only in dum22 cells, because they undergo a transition to state 2; the dum22 stt7–9 mutant, in contrast, is locked in state 1 and keeps a small PSI antenna. It therefore seems that the rate of PSI-driven oxidation of the PQ pool in the dum22 stt7–9 mutant cannot cope with the rate of PSII-driven photochemical reduction combined with enhanced rates of stromal-driven non-photochemical reduction; as a result, reducing equivalents accumulate among the intersystem electron carriers (Fig. 3B and D), and overall photosynthetic activity (Fig. 3C) and biomass production decrease. Still, the enhanced reduction pressure from the stroma would be expected to enhance CEF (25). However, the impaired transition to state 2 in dum22 stt7–9 mutants should lead to a diminished CEF in light-limiting conditions because the PSI antenna are smaller in dum22 stt7–9 mutants than in dum22 cells. To test this hypothesis, we assessed the efficiency of CEF.
by measuring the redox changes of P$_{700}$ (using the approach in Fig. 3A) in the presence of the sole PSI inhibitor DCMU. When PSI activity is prevented, the rate of P$_{700}$ re-reduction after illumination allows the rate of CEF to be determined (28). The flow rate of this process (i.e., the number of electrons transferred per PSI per unit of time) was evaluated as $k \times [P_{700}^{+}]/([P_{700}^{+}]+[P_{700}])$, where $k$ is the P$_{700}$ re-reduction rate after full oxidation by 10 s of saturating light and ([P$_{700}^{+}]+[P_{700}$]) is the oxidation level at any light intensity. Table 1 shows that although the relative efficiency of CEF decreased at low light (50 μmol m$^{-2}$ s$^{-1}$) in all strains, the CEF rate was much higher in $dum22$ cells that were locked in state 2 than in the other strains that remained in state 1.

Because CEF should contribute significantly to ATP synthesis upon illumination, we compared the steady-state cellular ATP levels. Table 1 shows that, despite changes in CEF and the overall photosynthetic activity, no differences in ATP levels were seen between $dum22$ cells and $dum22 stt7−9$ mutant cells in either mixotrophic or photoautotrophic conditions in the light. As expected, because of their respiratory deficiencies, the 2 strains showed ATP depletion upon dark incubation and a slightly lower ATP content in the light than seen in their wild-type and $stt7−9$ counterparts.

**Discussion**

In plants, ST is regarded as a regulatory process of photosynthesis that optimizes linear electron flow by a proper balance of energy distribution between the 2 photosystems (3). In microalgae such as *Chlamydomonas*, STs have been proposed to tune the efficiency of CEF and re-equilibrate the ATP/NADPH stoichiometry for CO$_2$ assimilation. Conversely, our results argue for a bioenergetic utilization is fully optimized, alternative processes must contribute to the generation of an "extra" ATP (32), and also through the reduction of molecular oxygen by the Mehler reaction or the activity of plastid terminal oxidase (33). Mitochondria also can provide extra ATP while consuming reducing equivalents exported from the chloroplast via the malate or the tricarboxylic acid cycle (34).

Table 1. Relative cyclic electron flow (CEF) and cellular ATP content in wild-type, $stt7−9$, $dum22$, and $dum22 stt7−9$ cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>CEF</th>
<th>ATP TAP Dark</th>
<th>ATP TAP h$_{v}$</th>
<th>ATP Min h$_{v}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.35</td>
<td>66.2 ± 6.8</td>
<td>100</td>
<td>93.0 ± 9.1</td>
</tr>
<tr>
<td>$stt7−9$</td>
<td>0.37</td>
<td>55.9 ± 6.1</td>
<td>102.3 ± 12.3</td>
<td>83.8 ± 20.4</td>
</tr>
<tr>
<td>$dum22$</td>
<td>0.63</td>
<td>3.0 ± 1.3</td>
<td>69.0 ± 10.0</td>
<td>69.0 ± 10.0</td>
</tr>
<tr>
<td>$dum22 stt7−9$</td>
<td>0.35</td>
<td>1.8 ± 2.1</td>
<td>65.7 ± 6.5</td>
<td>71.2 ± 2.7</td>
</tr>
</tbody>
</table>

CEF was evaluated as $-k \times [P_{700}^{+}]/([P_{700}^{+}]+[P_{700}])$ after illumination in the presence of DCMU (20 μM), where $k$ represents the P$_{700}$ re-reduction rate after steady-state illumination of saturating intensity. CEF rates at low light (50 μmol m$^{-2}$ s$^{-1}$) are normalized to the maximum value (typically 12.5 ± 0.1 s$^{-1}$ in wild-type cells). Standard deviation is relative to at least 3 replicates. ATP is expressed in percentage of the wild-type control (107 ± 12 nmol/mg Chlorophyll-1). Cells were fixed after 3 h in the dark or after continuous illumination with low light (50 μmol m$^{-2}$ s$^{-1}$).

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Our present study, which shows no differences in photosynthesis (Fig. 3) or growth (Fig. 1) between the wild-type cells and the $stt7−9$ mutant impaired in ST, suggests that the mere inhibition of ST is not sufficient to impair biomass production by photosynthesis. Conversely, our results argue for a bioenergetic recruitment of mitochondria in relieving photosynthesis in the
absence of ST-driven CEF by providing extra ATP for photosynthesis. The bioenergetic contribution of mitochondria to photosynthesis-driven metabolism is exemplified by the lower intracellular ATP levels in photoautotrophic conditions under low light when respiration is blocked (in *dum22* cells), even if the PSI turnover and growth rate are not compromised. Of note, the ATP level reached upon exposure of *dum22* cells to low light is very close to that measured in the wild-type cells in the dark, suggesting that in low light photosynthesis in *dum22* cells still is capable of sustaining cell growth, although it is ATP limited. Thus, STs in the absence of respiration promote enough CEF-driven ATP synthesis for growth but place the cells in an ATP-limiting situation. Conversely, our observation that the ATP levels in *dum22* *stt7–9* cells cannot be decreased further below the levels seen in *dum22* cells (Table 1) strongly suggests that below a threshold level, ATP becomes limiting for photosynthetic growth. This possibility is in agreement with previous data (35). Thus, the limited energy supply in the double mutant at low light probably is consumed in intracellular metabolism at the expense of biomass production and cell division. Ultimately, this limitation leads to a decrease in growth rate.

In conclusion, our study provides evidence for the contribution of the 2 major bioenergetic pathways to supply photosynthesis with the extra ATP required for carbon assimilation in *Chlamydomonas*. Oxidative phosphorylation in mitochondria seems to play a prominent role both in the dark-to-light transition, as previously proposed for vascular plants (36), and in steady-state, light-limited conditions. CEF, which is boosted in state 2 conditions, also contributes to this process and supplies ATP in the absence of energy supplied by mitochondria. The intimate relationship between the cellular respiratory capacity and the ability to modulate the PSI absorption cross-section through STs results in a tight interplay between CEF and respiration. This interplay seems to be particularly effective in *Chlamydomonas*. It provides these unicellular organisms with a very high photosynthetic flexibility in both ATP generation and electron transfer capacity in highly reducing conditions. This flexibility certainly is one of the major metabolic features enabling this alga to acclimate successfully to rapidly changing environmental conditions.

**Materials and Methods**

**Strains and Growth Conditions.** The *Chlamydomonas* wild-type strain used in this work is derived from the 137T strain (cc-1373 of the Duke University). The *Chlamydomonas dum22* strain is a deletion mutant lacking the left telomere, the cob gene, and part of the nd6 gene (19). *stt7–9* is a clone allelic to *stt2*, which can be crossed easily, unlike the original strain (gift from J.-D. Rochaix). The double mutant *dum22* stt7–9 was obtained by crossing the *dum22* mt mutant with a *stt7–9* mt mutant using standard procedures (see supplemental data for further information). We isolated 7 *dum22*stt7–9 clones (Fig. 5G) and further analyzed 2 of them (A1, J1) for their photosynthetic features. Cells were cultivated routinely at 50 μmol photons m⁻² s⁻¹ in mixotrophic (TAP) or photoautotrophic (Min) conditions.

**Spectroscopy.** Cells were harvested during exponential growth (2·10⁶ cells/mL) and were resuspended at a concentration of 10⁷ cells/mL in minimum medium with the addition of 20% (v/v) Ficoll to prevent cell sedimentation. In vivo kinetics measurements were performed at room temperature with 2 different setups. Steady-state P700 oxidation kinetics and RC stoichiometries were measured using a LTS spectrophotometer (Biologic, France). Continuous light was provided by a red source (630 nm), which was switched off transiently while measuring P700 absorption changes at 705 nm. PSI and PSII content was estimated spectroscopically from changes in the amplitude of the fast phase (100 μs) of the ECS signal (at 520–545 nm) upon excitation with a saturating laser flash. The ECS spectral change linearly follows the number of light-induced charge separations within the reaction centers (37). Thus, the PSI contribution can be calculated from the decrease in the signal amplitude upon the addition of DCMU (20 μM) and hydroxylamine (1 mM) that irreversibly block PSII charge separation once the sample has been pre-illuminated (21). Conversely, PSI was estimated as the fraction of the signal that was insensitive to these inhibitors. The light-saturation profile of P700 oxidation (Fig. 2D) was measured with a second setup with a time resolution of 10 ns. Actinic flashes were provided by a dye laser at 600 nm, the intensity of which was changed using neutral filters; detecting flashes were provided by an optical parametric oscillator. The extent of P700 oxidation was evaluated from the 900 ns to 20 μs phase of absorption changes at 430 to 460 nm, in line with previously recorded spectra of the 4-μs component of the decay-associated spectra of P700⁺ in wild-type cells (24). Fluorescence inductions were measured in the interval between the pre-illumination flash and the green LED source (520 nm), and fluorescence was detected in the near IR region. PSI antenna size was evaluated by the rate of fluorescence induction in the presence of the PSI inhibitor DCMU. In the presence of this inhibitor, an average of 1 photon per PSI center is absorbed at time t (25). This parameter was estimated for every fluorescence induction trace to evaluate the number of absorbed photons. Fluorescence emission spectra at 77 K were recorded using a LS 508 spectrophotofluorometer (PerkinElmer). Excitation was at 440 nm, and emission was detected at 685 nm (PSII) and 715 nm (PSI). Spectra were corrected for the wavelength-dependent photomultiplier response.

**Oxygen Evolution and Fv/Fm.** Oxygen evolution and the quantum yield of PSII in the light (Fv/Fm) (27) were measured simultaneously using a Clark electrode connected to a modulated fluorometer (type MFMS, Hansatech Instruments) in Min liquid medium supplemented with 5 mM NaHCO₃. Fv/Fm was calculated as (Fm'–F)/Fm′, where Fm′ is the maximum fluorescence emission level induced by a pulse of saturating light (=5,000 μmol of photons m⁻² s⁻¹), and F is the steady-state level of fluorescence emission. Chlorophyll concentration was adjusted to 5 μg/mL.

**ATP Measurements.** ATP was extracted as in (17) using 5% HClO₄. Samples were centrifuged at 10,000 × g, and a volume of the supernatant was diluted in 0.5 M Tris-Acetate, pH 7.75 (1/300, vol/vol). Determination of ATP content was made using the Enliten luciferase/luciferin kit (Promega) with a Lumat LB9501 apparatus (Berthold).

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