A Dual Strategy to Cope with High Light in *Chlamydomonas reinhardtii*[^1]

Guillaume Allorent[^a,b,c,d,1], Ryutarou Tokatsu[^a,1], Thomas Roach[^f,2], Graham Peers[^g], Pierre Cardol[^h], Jacqueline Girard-Bascou[^i], Daphné Seigneurin-Berry[^j, b, c, d], Dimitris Petroutos[^s, a, b, c, d], Marcel Kuntz[^a, b, c, d], Cécile Breyton[^i], Fabrice Franck[^k], Francis-André Wollman[^l], Krishna K. Niyogi[^l], Anja Krieger-Liszkay[^f], Jun Minagawa[^m], and Giovanni Finazzi[^n, a, b, c, d,3]

[^a]: Centre National Recherche Scientifique, Unité Mixte Recherche 5168, Laboratoire Physiologie Cellulaire et Végétale, F-38054 Grenoble, France
[^b]: Commissariat à l’Energie Atomique et Énergies Alternatives, l’Institut de Recherches en Technologies et Sciences pour le Vivant, F-38054 Grenoble, France
[^c]: Université Grenoble 1, F-38041 Grenoble, France
[^d]: Institut National Recherche Agronomique, Unité Mixte de Recherche 1200, F-38054 Grenoble, France
[^e]: Division of Environmental Photobiology, National Institute for Basic Biology, 444-8585 Okayama, Japan
[^f]: Commissariat à l’Energie Atomique et Énergies Alternatives Saclay, Institute of Biology and Technology-Saclay, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8221, Service de Bioénergétique, Biologie Structurale et Mécanisme, 91191 Gif-sur-Yvette cedex, France
[^g]: Department of Biology, Colorado State University, Fort Collins, Colorado 80523-1062
[^h]: Laboratoire de Génétique des Microorganismes Département des Sciences de la Vie, Université de Liège, B-4000 Liege, Belgium
[^i]: Unité Mixte de Recherche 7141, Centre National de la Recherche Scientifique/Université Pierre et Marie Curie Institut de Biologie Physico Chimique, F-75005 Paris, France
[^j]: Unité Mixte de Recherche 5075, Centre National de la Recherche Scientifique/Commissariat à l’Energie Atomique/Université Grenoble 1, Institut de Biologie Structurale, F-38054 Grenoble, France
[^k]: Laboratoire de Bioénergétique, Département des Sciences de la Vie, Université de Liège, B-4000 Liege, Belgium
[^l]: Howard Hughes Medical Institute, Department of Plant and Microbial Biology, University of California and Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720-3102

Absorption of light in excess of the capacity for photosynthetic electron transport is damaging to photosynthetic organisms. Several mechanisms exist to avoid photodamage, which are collectively referred to as nonphotochemical quenching. This term comprises at least two major processes. State transitions (qT) represent changes in the relative antenna sizes of photosystems II and I. High energy quenching (qE) is the increased thermal dissipation of light energy triggered by lumen acidification. To investigate the respective roles of qE and qT in photoprotection, a mutant (npq4 stt7-9) was generated in *Chlamydomonas reinhardtii* by crossing the state transition–deficient mutant (stt7-9) with a strain having a largely reduced qE capacity (npq4). The comparative phenotypic analysis of the wild type, single mutants, and double mutants reveals that both state transitions and qE are induced by high light. Moreover, the double mutant exhibits an increased photosensitivity with respect to the single mutants and the wild type. Therefore, we suggest that besides qE, state transitions also play a photoprotective role during high light acclimation of the cells, most likely by decreasing hydrogen peroxide production. These results are discussed in terms of the relative photoprotective benefit related to thermal dissipation of excess light and/or to the physical displacement of antennas from photosystem II.

INTRODUCTION

When photosynthetic organisms are exposed to light intensity overwhelming their photosynthetic capacity, they initiate a variety of mechanisms to protect their photosynthetic machinery from photodamage. All these processes are associated with photosystem II (PSII) and are collectively referred to as nonphotochemical quenching (NPQ) (Horton et al., 1996). This term comprises (1) state transitions (i.e., the reversible association of chlorophyll binding complexes with PSII [in state 1] and photosystem I [PSI; in state 2]) (Bonaventura and Myers, 1969; Allen, 1992); (2) high energy–dependent quenching (qE) (i.e., enhanced thermal energy dissipation occurring when the chloroplast is energized by a high rate of photosynthetic electron flow); and (3) photoinhibition, dismantling the photosystems photodamaged by high light.

State transitions are regulated by protein phosphorylation catalyzed by a membrane-bound kinase, STATE TRANSITION7.
(STT7 in *Chlamydomonas reinhardtii* or STN7 in higher plants; Rochaix, 2007). The kinase is activated in response to redox changes in the electron flow chain. Reduction of the plastoquinone (PQ) pool, either by unbalanced PSII/PSI activity (in favor of the former) or by the chlororespiratory chain, activates the kinase through a mechanism that requires plastoquinol, binding to the cytochrome 

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complex (Wollman, 2001). The phosphorylated light-harvesting complex II (P7-LHCCI) is disconnected from PSII and becomes a PSI antenna. The process is reversible because the kinase is inactivated by oxidation of the plastoquinol (i.e., when the PSI/PSI balance is modified in favor of the latter). This results in P7-LHCCI dephosphorylation by a phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010), which seems to have constitutive but low activity. Dephosphorylated LHCCI moves back to PSII, leading to state 1.

The second phenomenon is qE, the pH-dependent component of NPQ, which corresponds to an increased thermal deactivation of the PSI antenna. In higher plants and green algae, qE is related to the accumulation of two carotenoids (antheraxanthin and zeaxanthin), produced by deepoxidation of violaxanthin during to the accumulation of two carotenoids (antheraxanthin and zeaxanthin), produced by deepoxidation of violaxanthin during the so-called xanthophyll cycle (Yamamoto et al., 1962). The phosphorylated light-harvesting complex II (P7-LHCCI) is disconnected from PSII and becomes a PSI antenna. The process is reversible because the kinase is inactivated by oxidation of the plastoquinol (i.e., when the PSI/PSI balance is modified in favor of the latter). This results in P7-LHCCI dephosphorylation by a phosphatase (Pribil et al., 2010; Shapiguzov et al., 2010), which seems to have constitutive but low activity. Dephosphorylated LHCCI moves back to PSII, leading to state 1.

In higher plants, qE is by far the most prominent photoprotective response (Horton et al., 1996). Conversely, quenching due to state transitions (qT) does not provide significant protection from light stress, as indicated by the comparative analysis of the fitness of *Arabidopsis thaliana* mutants lacking state transitions and/or qE under variable environmental conditions in the field (Frenkel et al., 2007). This is likely because state transitions are of limited amplitude in higher plants. They are thought to adjust the relative absorption capacity of the two photosystems to optimize light use (Allen, 1992). On the other hand, state transitions are a prominent phenomenon in *C. reinhardtii*, where up to 80% of the light-harvesting complexes can reversibly migrate between PSII and PSI following redox-driven phosphorylation (Delosme et al., 1996). This difference can be explained by the observation that only a few LHCCI are phosphorylated in plants, while both the minor (CHLOROPHYLL PROTEIN [CP]) and major (LIGHT HARVESTING COMPLEX II [LHCCI]) antennae are targeted in *C. reinhardtii* (Minagawa, 2011). Because of the large change in the PSII and PSI absorption capacity, state transitions in *C. reinhardtii* do not only serve the purpose of balancing the absorption of the two photosystems. It has been proposed instead that they may play another role in this alga (i.e., enhancing PSI activity in order to favor cyclic electron flow around PSI to increase ATP production) (Vallon et al., 1991; Cardol et al., 2009). Consistent with this hypothesis, a supercomplex containing PSI, the cytochrome 

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complex, FERREDOXIN NADP REDUCTASE, and PROTON GRADIENT REGULATORS LIKE1, a protein involved in cyclic flow (Dal Corso et al., 2008; Tolleter et al., 2011), was recently purified from state 2 acclimated cells (Iwai et al., 2010).

An additional role for state transitions can be envisaged in this alga. The psbS gene is present but not expressed under normal growth conditions (Bonente et al., 2008). In *C. reinhardtii*, the extent of qE is modulated by LHCSR3 (Peers et al., 2009; Bonente et al., 2011; Petrooutsos et al., 2011), a member of the light-harvesting complex stress-related (LHCSR) protein family, formerly known as LI818 (Savard et al., 1996). LHCSR genes are only found in algae and mosses (Elrad and Grossman, 2004; Nymark et al., 2009). In *C. reinhardtii*, LHCSR3 is induced after a prolonged exposure (several hours) to high light stress (Peers et al., 2009). During this period, photodamage can take place because of the lack of photoprotection. State transitions, which develop in a few minutes (Delepeaire and Wollman, 1985), could play an active role in counterbalancing high light stress in this alga by transferring a large part of the PSI antenna to PSI, which is a very efficient quencher of absorbed energy.

To investigate this possibility, and to assess the respective roles of state transitions and qE in high light acclimation in *C. reinhardtii*, we employed a genetic approach to generate a double mutant lacking both state transitions and qE. By comparing fluorescence quenching, photosynthetic activity, and reactive oxygen species (ROS) production in the wild type, single mutants deficient in state transitions (*sst7*; Depège et al., 2003) and qE (*npq4*; Peers et al., 2009), and in the double mutant (*npq4 sst7*), we show that both qE and qT act together during the development of NPQ in this alga. In particular, while qE could be the major photoprotective response in steady state (as in higher plants), state transitions seem to play a prominent role during the early phase of NPQ induction, probably by preventing hydrogen peroxide (H₂O₂) production, but also provide some protection in steady state conditions. We interpret this result in terms of the possible benefit provided by the reduced size of the PSI antenna in state 2, but also by the diminished reactivity of the PSI-generated electron with molecular oxygen due to the generation of the cyclic PSI supercomplex (Iwai et al., 2010).

**RESULTS**

**Generation of a Double Mutant Impaired in State Transitions and qE in *C. reinhardtii***

To address the relative role of NPQ-qE and NPQ-qT in light acclimation of *C. reinhardtii*, we produced a double mutant impaired in both qE and state transitions. This was done by crossing two mutants. One was the state transition–deficient strain *sst7-9*, a clone that bears the same mutation as the *sst7* mutant (Depège et al., 2003) but that has a higher mating capacity (Cardol et al., 2009). The second was *npq4*, a mutant in which the qE response is largely impaired, due to the disruption of the *LHCSR3.1* and *LHCSR3.2* genes (Peers et al., 2009). We isolated five clones (*npq4 sst7-9*, clones #2, #4, #7, #14, and #32) lacking both state transitions and qE, as detected by an in vivo fluorescence screen, and their characteristics are presented in Supplemental Figures 1 and 2 online. Two of them (clones #2 and #7) were further analyzed. To assess state transition capacity, maximum
fluorescence yield was recorded at room temperature upon a 2.5-s illumination on cells acclimated in state 1 and in state 2 (Figure 1; see Supplemental Figure 1 online). State 1 was induced by placing the cells in darkness under strong aeration and state 2 by adding the ionophore carbonylcyanide- p-trifluoromethoxyphenyl hydrazine (FCCP; 5 μM) to the incubation medium in the dark. This compound uncouples respiration, thereby lowering the cellular ATP content. This leads in turn to the activation of glycolysis and, therefore, to the accumulation of NADPH, which can reduce the PQ pool via the chlororespiratory chain (reviewed in Wellman, 2001). A reduced PQ pool activates the STT7 kinase, triggering the transfer of a large fraction of P7-LHCII to PSI, which acts as a strong energy quencher (Delosme et al., 1996). Ultimately, this results in a drop in the maximal fluorescence yield in state 2 conditions (Figure 1A; see Supplemental Figures 1A and 1C online), which was present in the two strains containing an active kinase (the wild type and npq4) but absent in stt7-9 (as expected because of the disruption of STT7) and in the five npq4 stt7-9 clones. In these strains, a small fluorescence increase was observed in state 2 conditions, a phenomenon already observed upon reduction of the PQ pool in strains impaired in state transitions (Hohmann-Marriott et al., 2010).

Measurements of fluorescence emission at cryogenic temperatures confirmed the state transition phenotype of the selected clones (Figure 1B; see Supplemental Figures 1B and 1D online). Unlike in state 1, the fluorescence spectrum was dominated by PSI emission (λ = 715 nm) in the wild-type and npq4 strains under state 2 promoting conditions. This reflects the increased absorption capacity of this complex, induced by LHCII binding (Bonaventura and Myers, 1969). On the other hand, in the same conditions, the PSII emission bands (λ = 685 and 695 nm) were predominant in the stt7-9 strain, where LHCII remained associated with PSII, and in the selected clones, confirming their lack of state transition phenotypes.

In parallel, progeny from the stt7 × npq4 cross were analyzed for their capacity to develop qE in high light (Figure 2; see Supplemental Figure 2 online). Cells were grown mixotrophically in a Tris-acetate-phosphate (TAP) medium at low light (50 μmol photons m⁻² s⁻¹) to prevent the accumulation of LHCSR3 (Peers et al., 2009). They were harvested in the exponential phase, resuspended in a minimum high-salt (HS) medium (without CO₂ supply), and then transferred to high light (500 μmol photons m⁻² s⁻¹) to induce LHCSR3 accumulation (Peers et al., 2009). Fluorescence quenching was measured at different times during high-light exposure, along with the accumulation of LHCSR3, which was evaluated by immunoblotting with a specific antibody. Figure 2 shows that quenching was induced after a few hours of high-light exposure in the wild type and the stt7-9 strains, in parallel with the accumulation of LHCSR3. A small NPQ was seen in the npq4 as well as in the putative npq4 stt7-9 clones (Figure 2A; see Supplemental Figure 2A online), in which LHCSR3 did not accumulate in high light (Figure 2B; see Supplemental Figure 2B online). In these clones, the absence of qE was not due to impaired carotenoid deepoxidation (Figure 3; see also Peers et al., 2009 for npq4). In fact, a slightly higher deepoxidation was observed in the double mutant at the end of illumination (Figure 3A). In both the npq4 and npq4 stt7-9 mutants, a lower intensity band cross-reacting with the anti-LHCSR antibody was observed at the end of the illumination period (Figure 2B). Because no quenching increase was associated with the induction of this protein, and because of its apparent lower molecular weight (Peers et al., 2009; Bonente et al., 2011), we tentatively ascribed this band to LHCSR1, an LHCSR

Figure 1. State Transition Phenotype of the Different Strains.

Cells were harvested in the exponential phase and resuspended in minimum HS medium at a concentration of 2 × 10⁷ cells mL⁻¹, r.u., relative units. (A) Fluorescence induction curves. Traces were recorded in the presence of 10 μM DCMU, in the wild type (WT), stt7-9 (lacking the STT7 kinase), npq4 (lacking LHCSR3), and npq4 stt7-9 clones. Strains were placed either in state 1 (solid squares) or in state 2 (open circles) conditions by placing them in darkness under strong aeration (for state 1) or by incubating them with 5 μM FCCP in the dark for 20 min (for state 2). A decrease of the Fm level under state 2 conditions is indicative of a decrease in the size of the PSII antenna due to LHCII migration to PSI. Similar results were obtained when cells were placed in state 1 by illumination in the presence of 20 μM DCMU.

(B) Low-temperature (77 K) fluorescence emission spectra of cells under state 1 (closed squares) and state 2 (open circles) inducing conditions. A high ratio between the fluorescence emission band at 715 nm (PSI) and at 685 nm + 695 nm (PSII) is indicative of a transition to state 2 due to enhanced energy collection by PSI, following LHCII migration from PSII. PSII emission was normalized to 1 in all genotypes.
Cells were harvested in the exponential phase and resuspended in minimum HS medium at a concentration of 2 × 10^7 cells mL^-1. WT, the wild type.

(A) NPQ efficiency. Cells were exposed to high light (500 µmol photons m^-2 s^-1, white light) without external carbon dioxide addition for the indicated times and then briefly (10 min) dark adapted. NPQ capacity was evaluated from the (Fm - Fm');Fm' parameter (Bilger and Björkman, 1990) using a fast imaging setup. Values represent mean ± SE (n = 4 biological replicates).

(B) LHCSR accumulation. Cells were harvested at the indicated times, and samples were analyzed by immunoblotting with an anti-LHCSR antibody. ATPB (β-subunit of the CF0F1 ATPase) is shown as loading control. One microgram of chlorophyll was loaded in each lane.

**Figure 2.** NPQ induction in Wild-Type, stt7-9, npq4, and npq4 stt7-9 Strains.

Cells were harvested in the exponential phase and resuspended in minimum HS medium at a concentration of 2 × 10^7 cells mL^-1. WT, the wild type. NPQ efficiency. Cells were exposed to high light (500 µmol photons m^-2 s^-1, white light) without external carbon dioxide addition for the indicated times and then briefly (10 min) dark adapted. NPQ capacity was evaluated from the (Fm - Fm');Fm' parameter (Bilger and Björkman, 1990) using a fast imaging setup. Values represent mean ± SE (n = 4 biological replicates).

Fluorescence quenching dynamics in C. reinhardtii Wild Type and Mutants Impaired in State Transitions and qE

To further evaluate the effect of this treatment on NPQ, fluorescence quenching dynamics were assayed in cells that were exposed to high light for 4 h and then vigorously aerated in the dark for 15 min. Normally, this treatment is sufficient to allow for a complete relaxation of NPQ and reversion of cells to state 1 (Figure 1). However, this was not the case for wild-type cells that had been subjected to a shift from mixotrophic conditions in low light to photoautotrophy in high light. We found that CP26, CP29, and LHCII type I (LhcbM3/-4/-6/-8/-9) were heavily phosphorylated in wild-type cells (as shown by immunoblotting using an antiphosphothreonine antibody; Figure 4A). In parallel, we observed changes in the 77K fluorescence emission spectra, which confirmed the occurrence of state 2 in the wild-type and npq4 strains (Figure 4B).

When the light was switched on, we observed NPQ (Figure 5) in agreement with earlier results (Peers et al., 2009; see also Figure 2). However, the fluorescence quenching pattern observed after high-light treatment turned out to be extremely complex (Figure 5) and different from the one normally seen in plants. In high light–treated wild-type cells, light exposure first leads to the development of a fast fluorescence quenching (phase I, Figure 5), which is completed in ~30 s. This phase is followed by a fluorescence recovery (phase II, Figure 5) that continues for around 3 min, and then by a second slower fluorescence quenching (phase III, Figure 5), until a steady state level is reached. When the light is switched off, quenching relaxation is observed (phase IV, Figure 5), and this is replaced by a slower decline (phase V, Figure 5), down to the initial dark level. In cells constitutively locked in state 1 (stt7-9), the fluorescence quenching profile is different: both the fluorescence rise (phase II) in high light and its decline in the dark (phase V) are abolished (Figure 5). Therefore, illumination led to the onset of a fast quenching (phase I), which rapidly reached a steady state level. The cells almost completely recover in the dark (phase IV). In the qE-deficient npq4 strain, phases II and V are maintained, while phases I, III, and IV are largely depressed (Figure 5). In the double mutant, all the phases observed in the wild type and in the single mutants are largely abolished. This indicates that the complex kinetics measured in wild-type cells is due to the superimposition of qE and state transitions (Figure 5). The comparison between the different genotypes suggests that phases I and IV, which are observed only in the strains capable of accumulating LHCSR3 (the wild type and stt7-9), should correspond to the development of qE in high light and to its relaxation in the dark, respectively. Phases II and V are inhibited by the stt7 mutation, suggesting a role for state transitions. Fluorescence changes are associated with the phosphorylation state of LHCII proteins, including the minor CPs (Allen, 1992; Takahashi et al., 2006; Rochaix, 2007). Thus, we tested the occurrence of phosphorylation-associated qT during the dark–high light–dark incubation by immunoblotting wild-type cells with an antiphosphothreonine antibody. We found that the phosphorylation level of LHCII slowly decreased upon illumination, confirming the occurrence of a state 2-to-1 transition during phase II (see Supplemental Figure 3A online). However, LHCII dephosphorylation also continued during phase III. When the light was switched off, LHCII phosphorylation recovered with a time course largely correlating with phase V.
We also employed chemical inhibitors to investigate the nature of NPQ in C. reinhardtii. qE is selectively suppressed by the dissipation of the ΔpH using the K+/H+ exchanger nigericin (Horton et al., 1996). Therefore, it is possible to exploit the different ΔpH sensitivity of these two processes to deconvolute the qE (i.e., of the nigericin sensitive) and qT (i.e., of the nigericin insensitive) components of NPQ starting from fluorescence traces measured in wild-type cells under control or nigericin poisoned conditions (see Supplemental Figure 3B online). We found a strong correlation between the kinetics of qT estimated by this approach (see Supplemental Figure 3C online) and the changes in the phosphorylation profile measured by immunoblotting. Therefore, we conclude that phases II and V correspond to a state 2-to-1 transition in the light and to the recovery of state 2 in the dark, respectively. In agreement with previous assignments based on the comparative analysis of fluorescence kinetics in the four genotypes (Figure 5), we also confirm that phase I represents the onset of qE-type quenching, while phase IV corresponds to its relaxation in the dark. To further investigate if phase II and IV could be due to state transitions, we employed the respiratory-deficient mutant dum22, which has been previously shown to be in state 2 in the dark (Cardol et al., 2003; see Supplemental Figure 4 online). Phase II is clearly observed in low light TAP-grown
to measurements of the dynamics of LHCII phosphorylation, this

Thus, this phase should correspond to a qE type of NPQ. How-

Phase III is abolished by the

...treated cells (Figure 5) but not

Cells were harvested in the exponential phase and resuspended in

Dunaliella tertiolecta to state 2 in the dark as previously observed in the green alga

Cells were placed either in state 1 (S1; 20 µM DCMU) or in state 2 (S2; 5 µM FCCP in the dark) conditions immediately after high-light incubation (500 µmol photons m⁻² s⁻¹, white light) for 4 h. PSI and PSII supercomplexes were isolated as described in Methods, and their protein composition was tested by immunoblotting with the specific antibodies. PSI-His and PSI-His supercomplex proteins (0.5 µg) were loaded in the respective lanes. Cyt, cytochrome.
indicates that LHCSR3 is also a part of the mobile fraction of the PSII antenna during state transitions. Its migration between the two photosystems could therefore modulate energy quenching in PSII, by changing the number of quenching effectors bound to this complex.

Role of State Transitions and qE in Photoprotection of C. reinhardtii

A major role of NPQ in photosynthetic organisms is to protect the components of the electron flow chain, and PSII in particular, from light damage. By showing that both LHCSR3-dependent quenching and state transitions are induced in response to high light, our data suggest that both processes could be involved in photoprotection in this alga. This possibility was investigated by measuring changes in PSII activity before and after the high-light treatment required to induce LHCSR3 accumulation. We observed that the variable fluorescence/maximum fluorescence parameter, which reflects the maximum quantum efficiency of PSII, was decreased to a greater extent in the single mutants than in the wild type after exposure to 500 μmol photons m⁻² s⁻¹ for 4 h (Figure 7A). This suggests that some photoinhibition was occurring in stt7-9 and npq4 during this treatment. The decline of the Fv/Fm was even more pronounced in the double mutant, indicating an increased light sensitivity in this strain. Assessment of PSI and PSII activities by measuring the electrochromic shift (ECS; Bailleul et al., 2010a) confirmed this observation. In the dark, a PSII:PSI ratio of ~1 was found in all strains, in agreement with previous estimates (Cardol et al., 2009). Conversely, a stronger decrease of the PSI photochemical activity was seen in npq4 stt7-9 when compared with the other genotypes after incubation in high light (Figure 7B). This indicates that PSII was more susceptible to high-light stress in the double than in the single mutants and the wild type. Consistent with this, besides changes due to state transitions, a lower PSII:PSI ratio was observed in npq4 compared with the wild type and npq4 stt7-9 compared with stt7-9 at cryogenic temperatures after high-light exposure (Figure 4B). The deleterious effects of the double mutation were confirmed when the analysis of the effects of high light on the photosynthetic properties of four strains was extended to 12 h (see Supplemental Figure 7 online). In this case, we observed some additional accumulation of LHCSR3, confirming the role of this protein in the long-term photoprotection of C. reinhardtii, as well as some increased photoinhibition, which was enhanced in the double mutant up to 8 h of high-light exposure.

Under conditions where the electron flow capacity is saturated, photoinhibition is triggered by the accumulation of ROS, which are produced at different levels in the photosynthetic electron flow chain (Krieger-Liszkay and Trebst, 2006). To understand the reasons for the increased photosensitivity of the npq4 stt7-9 mutant, we measured ROS levels in the wild type, the single mutants, and in the double mutant during the first hours of high-light exposure. We first evaluated the accumulation of H₂O₂ in the four genotypes and found that this species was produced in all strains during exposure to high light (Figure 8). However, differences were seen between the different strains in both the rate and the extent of H₂O₂ accumulation. The amount of H₂O₂ was lower in wild-type than in npq4 and stt7-9 cells. Conversely, H₂O₂ accumulation was increased in the double mutant, possibly explaining its higher photosensitivity. Moreover, we observed that stt7-9 and the double mutant accumulated more H₂O₂ than the npq4 and wild-type strains at the beginning of illumination. This suggests that state transitions may be of particular importance in preventing H₂O₂ generation during the first phase of high-light exposure (i.e., before the induction of qE). To provide a more complete scenario of ROS production in C. reinhardtii, we also tried to measure ¹⁰O₂ generation in the different genotypes analyzed in this work using the specific dye DanePyoxalate (Fischer et al., 2007). Unfortunately, the sensitivity of this dye was insufficient to detect ¹⁰O₂ in vivo unless enhancers (bromoxynil or methylene blue) were added to the cells (see Supplemental Figure 8 online). Nonetheless, we detected lower amounts of α-tocopherol in high light-treated npq4 stt7-9 cells when compared with the other genotypes (Figure 3A). α-Tocopherol acts as an efficient scavenger of intracellular ¹⁰O₂ (Krieger-Liszkay and Trebst, 2006; Li et al., 2012). It may be that its lower concentration in this strain is due to its rapid consumption to counteract the increased generation of this ROS. The observation of some chlorophyll bleaching after high-light exposure (Figure 3B) is also consistent with the idea that ROS production was increased in the double mutant.

**DISCUSSION**

In this work, we studied the development of state transitions and of qE during high-light acclimation of the green alga C. reinhardtii and their respective roles in protecting the photosynthetic machinery from photodamage. Using a combined biochemical, biophysical, and genetic approach, we first demonstrated that high-light treatment for several hours (without any additional
Cells were harvested in the exponential phase and resuspended in minimum HS medium at a concentration of $5 \times 10^6$ cells mL$^{-1}$. They were exposed to high light (500 µmol of photons m$^{-2}$ s$^{-1}$, white light), and samples were collected at different time points. H$_2$O$_2$ production was assessed as described in Methods. Values represent means ± se ($n = 4$ to 6 biological replicates). Statistical comparison was performed using one-way ANOVA followed by the Tukey multiple comparison test ($P < 0.05$). Symbols in the graph denote significant differences: #, from stt7-9 and npq4 stt7-9; §, from the wild type and npq4 stt7-9; @, from the wild type, npq4, and npq4 stt7-9. WT, the wild type.

**Figure 8.** H$_2$O$_2$ Production during High-Light Exposure of *C. reinhardtii* Cells.

The concomitant occurrence of qE and qT in high light suggests that both responses could be induced by the same metabolic signal(s) in the cell. Information concerning the nature of the photosynthetic step responsible for the induction of qE and qT in high light can be obtained from the kinetic analysis of the NPQ response presented in Figure 5. There, we show that high light–treated cells are in state 2 in the dark (Figure 5; see also Figure 4 and Supplemental Figure 3 online) and revert to state 1 upon illumination (phase II). This behavior can be rationalized based on previous knowledge of the mechanisms of state transition. The first step in the activation of state 2 transition is the reduction of the PQ pool (Wollman, 2001), either via PSII photochemistry or via a nonphotochemical (chlororespiratory) process. A net reduction of the PQ pool is expected in high light because the rate of PQQ oxidation by the cytochrome b$_6$f complex at this intensity. Thus, high-light exposure should promote the transition to state 2 instead of the transition to state 1 as observed in Figure 5. On the other hand, previous work has shown that the state 2 transition is inhibited by high light (Rintamäki et al., 2000; Vink et al., 2004), either due to the negative redox control exerted by the ferredoxin/thioredoxin system on STT7/STN7 kinase (Rintamäki et al., 2000) or to a conformational change within the PSII antenna that prevents its phosphorylation by the active kinase (Vink et al., 2004). Both mechanisms can equally account for the rapid recovery of state 1 observed upon illumination of the cells (phase II). No such inhibitory effect is expected upon dark adaptation, since both mechanisms are light dependent (Aro and Ohad, 2003). Therefore, both hypotheses are compatible with the high phosphorylation levels seen in wild-type and npq4 cells after high-light exposure (Figure 4; see Supplemental Figure 3 online). High-light treatments in the absence of any exogenous carbon source, as required to induce LHCSR3 (Peers et al., 2009), should lead to a limitation of photosynthetic activity by carbon assimilation at the level of the Calvin cycle, thereby promoting NADPH accumulation. After illumination, this NADPH can be consumed by the chlororespiratory pathway in reducing the PQ pool and thereby promoting a rapid state 2 transition. This could explain the origin of phase V. Consistent with this, exposure of *C. reinhardtii* cells to high light enhances the capacity for nonphotochemical reduction of the PQ pool at the expense of stromal reducing equivalents (Houyoux et al., 2011).

**Metabolic Links between qE and qT in *C. reinhardtii***

The tight relationship between state transitions and qE in high light suggests that both responses could be induced by the same metabolic signal(s) in the cell. Information concerning the nature of the photosynthetic step responsible for the induction of qE and qT in high light can be obtained from the kinetic analysis of the NPQ response presented in Figure 5. There, we show that high light–treated cells are in state 2 in the dark (Figure 5; see also Figure 4 and Supplemental Figure 3 online) and revert to state 1 upon illumination (phase II). This behavior can be rationalized based on previous knowledge of the mechanisms of state transition. The first step in the activation of state 2 transition is the reduction of the PQ pool (Wollman, 2001), either via PSII photochemistry or via a nonphotochemical (chlororespiratory) process. A net reduction of the PQ pool is expected in high light because the rate of PQQ oxidation by the cytochrome b$_6$f complex at this intensity. Thus, high-light exposure should promote the transition to state 2 instead of the transition to state 1 as observed in Figure 5. On the other hand, previous work has shown that the state 2 transition is inhibited by high light (Rintamäki et al., 2000; Vink et al., 2004), either due to the negative redox control exerted by the ferredoxin/thioredoxin system on STT7/STN7 kinase (Rintamäki et al., 2000) or to a conformational change within the PSII antenna that prevents its phosphorylation by the active kinase (Vink et al., 2004). Both mechanisms can equally account for the rapid recovery of state 1 observed upon illumination of the cells (phase II). No such inhibitory effect is expected upon dark adaptation, since both mechanisms are light dependent (Aro and Ohad, 2003). Therefore, both hypotheses are compatible with the high phosphorylation levels seen in wild-type and npq4 cells after high-light exposure (Figure 4; see Supplemental Figure 3 online). High-light treatments in the absence of any exogenous carbon source, as required to induce LHCSR3 (Peers et al., 2009), should lead to a limitation of photosynthetic activity by carbon assimilation at the level of the Calvin cycle, thereby promoting NADPH accumulation. After illumination, this NADPH can be consumed by the chlororespiratory pathway in reducing the PQ pool and thereby promoting a rapid state 2 transition. This could explain the origin of phase V. Consistent with this, exposure of *C. reinhardtii* cells to high light enhances the capacity for nonphotochemical reduction of the PQ pool at the expense of stromal reducing equivalents (Houyoux et al., 2011).

**qE and qT Dynamics during Induction of NPQ in *C. reinhardtii***

The occurrence of significant light stress in this strain is also confirmed by the slight increase in its deepoxidation state (Figure 3A), the reduced α-tocopherol content in high light (Figure 3A), possibly to counteract O$_2$ accumulation, and the bleaching of chlorophyll (Figure 3B). The observation of a tight link between qE and state transitions seems different from previous data reported in *C. reinhardtii* (Peers et al., 2009). However, this apparent contradiction can be explained by the fact that the cells were systematically exposed to 5 min of far-red light to ensure transitions into state 1 before the NPQ measurements (Peers et al., 2009). This may have prevented the observation of fluorescence changes related to state transitions during illumination, in contrast with the data shown in this work (Figure 5).
online). However, phase III also comprises a state 2-to-1 transition, as indicated by measurements of protein phosphorylation kinetics in the light (see Supplemental Figure 3 online). The observation that this phase is slower than phase I (i.e., qE onset in state 2) may suggest that qE development is slower in state 1 than in state 2. However, this possibility is not consistent with the observation that the rate of fluorescence quenching at the onset of illumination is similar in wild-type and stt7 cells, which are in states 2 and 1, respectively. Moreover, the different rates of phases I and III cannot be explained by a different accumulation of LHCSR3 and zeaxanthin, which accumulated to a maximum extent during high-light treatment that precedes the measurement of NPQ (Figures 2 and 3, respectively). Under these conditions, lumen acidification becomes the only time-limiting process for qE onset (Joliot and Finazzi, 2010), leading in principle to a very fast quenching response (Johnson et al., 2009). To explain the different qE kinetics measured in phases I and III, we propose instead that phase III represents the signature of the dynamics of LHCSR3 binding to PSII during the state 2-to-1 transition. LHCSR3 reversibly binds PSII in state 2, possibly because of its phosphorylation by STT7 (Bonente et al., 2011) (i.e., the same kinase involved in PSII antenna phosphorylation) (Depège et al., 2003). Therefore, the observation that qE quenching increases (phase III) at the end of the antenna movement to PSII (state 1 transition, phase II + phase III) suggests that the reassociation of LHCSR3 to PSII during a state 2-to-1 transition could be slower than the binding of the antenna complexes to the reaction center. This would explain why the fluorescence emission is first increased by the state 1 transition (phase II) due to the increased PSII absorption capacity, and then decreased (phase III) due to the reassociation of LHCSR3 with PSII. This latter process should amplify fluorescence quenching in PSII by increasing the number of quenching effectors bound to the PSII supercomplex, as already shown in the case of the plant qE effector PSBS (Li et al., 2002).

Photoprotective Role of State Transitions in C. reinhardtii

As discussed above, the phenotypic analysis of the npq4 stt7-9 double mutant suggests that both qE and qT counteract photodamage in high light. In particular, measurements of ROS production suggest that state transitions play a photoprotective role during the early phase of light stress (i.e., when qE has not yet developed). This role is illustrated by the enhanced \( \text{H}_2\text{O}_2 \) production (Figure 8) observed in the two strains lacking STT7 (stt7-9 and npq4 stt7-9) during the first hours of illumination. This observation is consistent with the kinetic analysis of NPQ (Figure 5), which shows that both wild-type and npq4 cells rapidly reach state 1 upon high-light exposure (phase II). Accordingly, no differences are expected between the four genotypes, which should all be in state 1 after a few minutes of illumination (either because of the inactivation of STT7 or because of the inhibition of LHCII phosphorylation by the mechanisms discussed above). However, this incoherence can be tackled by considering the fact that recovery of state 1 is not complete in high light. In steady state photosynthesis, a fraction of the photosynthetic chains is always in state 2 (Forti et al., 2003), possibly to ensure the proper balance between NADPH and ATP production. The existence of this fraction, which is only expected in the STT7-containing strains (the wild type and npq4), could explain why only a lower amount of \( \text{H}_2\text{O}_2 \) is produced by these strains during the early phase of high-light exposure (i.e., before the induction of qE).

The observation that the impairment of state transitions enhances \( \text{H}_2\text{O}_2 \) production is intriguing. The opposite would be expected because PSI turnover is increased in state 2, owing to the largely enhanced absorption capacity. This higher turnover capacity should translate into an increased \( \text{H}_2\text{O}_2 \) production under the conditions employed here, where the availability of \( \text{CO}_2 \) should be limiting to photosynthesis. This apparent contradiction can be explained considering (1) that a part of the \( \text{H}_2\text{O}_2 \) generated in the chloroplast is produced by PSII. This is consistent with recent data showing that this ROS can be produced directly by PSII, although to a very small extent (Pospíšil, 2012). Therefore, transition to state 2 may reduce the generation of \( \text{H}_2\text{O}_2 \) by reducing the excitation pressure on this complex. On the other hand, (2) recent data have shown that the state 2 transition in C. reinhardtii leads to the formation of supercomplexes containing PSI, FERREDOXIN NADP REDUCTASE, PROTON GRADIENT REGULATOR5 LIKE1, and the cytochrome b_{6,f} (Iwai et al., 2010). These supercomplexes should be able to sequester the soluble electron carriers (plastocyanin and ferredoxin) within a thermodynamically confined space, to enhance the efficiency of cyclic electron flow around PSI (Iwai et al., 2010). By doing so, it could also lower the probability of side reactions between PSI acceptors and molecular oxygen, thereby directly reducing the yield of \( \text{H}_2\text{O}_2 \) generation. Removal of both mechanisms in strains locked in state 1 could enhance the production of \( \text{H}_2\text{O}_2 \), thereby increasing the probability of damaging the photosynthetic apparatus. This negative effect could be amplified in a genetic background where production of other ROS species is also enhanced, such as npq4. Although the detection limits of DanePy in the absence of bromoxynil are too low to reliably measure \( \text{O}_2 \), other cellular responses (pigment bleaching, \( \alpha \)-tocopherol consumption, and photoinhibition) suggest that this is the case at least in the npq4 stt7-9 strain. This is in agreement with the notion that by accumulating a large amount of excited state in the antenna, decreased qE could increase the concentration of the dangerous triplet state of chlorophylls, which is the main source of \( \text{O}_2 \) (Krieger-Liszkay and Trebst, 2006). Consistent with this hypothesis, data obtained in intact chloroplasts from Arabidopsis indicate that \( \text{O}_2 \) production is higher in a mutant lacking PSBS (Roach and Krieger-Liszkay, 2012).

Physiology of State Transitions in C. reinhardtii Revisited

Previous data in higher plants have documented that state transitions do not play any relevant role in the high-light stress response (Frenkel et al., 2007) but are only involved in the short-term acclimation to a changing low light environment (Bellaire et al., 2005). The different results observed in higher plants and in C. reinhardtii likely reflect the observation that state transitions are by far larger in this alga than in plants. The reasons for the different extent of qT between the two types of organism have been largely debated (reviewed in Wollman, 2001; Finazzi, 2005; Rochaix, 2007; Minagawa, 2011). In the frame of the observations presented here, it is tempting to speculate that one possible reason for the larger capacity of state transitions in C. reinhardtii is its involvement in photoprotection. As discussed above, induction
of the qE response during a low-light to high-light transition is a much slower process in this alga than in plants, owing to the longer time required to accumulate LHCSR3 during high-light exposure (Peers et al., 2009, Figure 2; this work). The occurrence of a state 2 transition during this period may alleviate high-light pressure on PSII by reducing H$_2$O$_2$ production (likely via the establishment of the PSII-cytochrome $b$_6$-c$_1$ supercomplex) but also by reducing its light-harvesting capacity (via the transfer of a large fraction of the PSII antennas to the rather efficient energy quencher reaction center of PSI). Consistent with this possibility, the LHCSR proteins that modulate the qE response are constitutively expressed in other microalgae, such as the diatom Phaeodactylum tricornutum (Nymark et al., 2009; Bailleul et al., 2010b; Zhu and Green, 2010), while state transitions are absent (Ting and Owens, 1993). In the moss Physcomitrella patens, both PSBS and LHCSR3 contribute to qE (Alboresi et al., 2010), but the LHCSR isoform that modulates quenching is also constitutively expressed.

Altogether, it appears that a large state transition capacity may have evolved to provide photosynthetic organisms with a high degree of flexibility in coping with abiotic stresses. Indeed, the possibility of modulating the PSI and PSII absorption to a large extent (by displacing most of the LHCII) and to regulate the ATP/NADPH synthesis capacity via changes in the linear versus cyclic electron flow ratio (Cardol et al., 2009; Iwa et al., 2010) could provide a clear benefit in high-light conditions when the electron flow chain is overreduced. The same system could also be useful under nutrient deprivation. Indeed, a systematic transition to state 2 is observed upon nutrient starvation in C. reinhardtii (i.e., a condition where the electron flow capacity is restricted and light is absorbed in excess), paving the way to photoinhibition (reviewed in Finazzi, 2005). By providing benefits in terms of ROS production (Figure 8), the large displacement of LHCII between PSI and PSII could mediate the qE response during a low-light to high-light transition is a much slower process in this alga than in plants, owing to the longer time required to accumulate LHCSR3 during high-light exposure (Peers et al., 2009, Figure 2; this work). The occurrence of a state 2 transition during this period may alleviate high-light pressure on PSII by reducing H$_2$O$_2$ production (likely via the establishment of the PSII-cytochrome $b$_6$-c$_1$ supercomplex) but also by reducing its light-harvesting capacity (via the transfer of a large fraction of the PSII antennas to the rather efficient energy quencher reaction center of PSI). Consistent with this possibility, the LHCSR proteins that modulate the qE response are constitutively expressed in other microalgae, such as the diatom Phaeodactylum tricornutum (Nymark et al., 2009; Bailleul et al., 2010b; Zhu and Green, 2010), while state transitions are absent (Ting and Owens, 1993). In the moss Physcomitrella patens, both PSBS and LHCSR3 contribute to qE (Alboresi et al., 2010), but the LHCSR isoform that modulates quenching is also constitutively expressed.

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METHODS

Cells and Growth Conditions

Several Chlamydomonas reinhardtii wild-type strains were studied in this work. The 137c and 222+ strains are derived from strain 137C. 4A+ is also derived from 137C (gift from J.-D. Rochaix, University of Geneva). The npq4 strain is a mutant lacking a functional copy of LHCSR3.1 and LHCSR3.2 (Peers et al., 2009). stt7-9 is a clone allelic to stt7 (Depège et al., 2003), which can be easily crossed, unlike the original strain (gift from J.-D. Rochaix; Cardol et al., 2009). The double mutant npq4 stt7-9 clones were obtained by crossing the npq4 mt$^-$ mutant with a stt7-9 mt$^-$ mutant using standard procedures. Five npq4 stt7-9 clones were isolated (see Supplemental Figures 1 and 2 online). The dun22 strain is a mitochondrial DNA deletion mutant lacking the left telomere, cob, and part of nd4 (Cardol and Remacle, 2008). Genetically modified C. reinhardtii strains PSII-His (Cullen et al., 2007) and PSI-His (Gulis et al., 2008), carrying a 6x His-tag at the C terminus of psbH and the N terminus of psaA, respectively, were used to isolate thylakoid membranes. C. reinhardtii cells were routinely cultivated at 50 µmol photons m$^{-2}$ s$^{-1}$ in mixotrophic (in a TAP medium) conditions.

Fluorescence Measurements

To evaluate state transitions (Figure 1; see Supplemental Figure 1 online), algae were grown mixotrophically, harvested in the exponential phase, and resuspended to a cell density of 2 x 10$^7$ cells mL$^{-1}$ in HS medium (Sueoka, 1960). Two milliliters was loaded onto a multiwell plate (24 wells) and subjected to continuous shaking (by putting a glass bead into each well and depositing the plate on a shaker) in the dark or illuminated in the presence of the PSI inhibitor DCMU (20 µM) to obtain state 1. State 2 was achieved by incubating the cells with FCCP (5 µM) for 20 min (Bulte et al., 1990). Fluorescence emissions were measured at 77K using a Speedzenn MX fluorescence imaging setup (JBeamBio) or a charge-coupled device spectrophotometer (JBeamBio).

Fluorescence Quenching Measurements

Algae in minimum HS medium were exposed to high-light intensity (500 µmol of photons m$^{-2}$ s$^{-1}$) while light provided by a WH 100 light-emitting diode panel (JBeamBio) to induce the accumulation of LHCSR3. The NPQ response was measured using a Speedzenn MX fluorescence imaging setup (JBeamBio). Excitation was done in the blue range ($\lambda = 450$ nm) using short pulses (10 µs). Emission was measured in the near far red. Saturation pulses (duration 250 ms) were provided by a green ($\lambda = 520$ nm) light-emitting diode array. Measurements were done 15 min after high-light exposure to ensure relaxation of the NPQ induced by the preillumination. The actinic light ($\lambda = 520$ nm) intensity was 700 µmol of photons m$^{-2}$ s$^{-1}$. NPQ was calculated as (Fm – Fm’)/Fm’ (Bilger and Björkman, 1990), where Fm and Fm’ are the maximum fluorescence emission level in the dark and light, respectively, measured after exposure to a saturating pulse of light, the intensity of which was 2700 µmol of photons m$^{-2}$ s$^{-1}$.

ROS Production

For H$_2$O$_2$ measurements, cells were exposed to 500 µmol photons m$^{-2}$ s$^{-1}$ provided by a 125-W cool fluorescent lamp. One milliliter of culture was diluted onefold and combined with 10 units of horseradish peroxidase and 0.5 µM Amplex Red (Invitrogen) forming the fluorescent resorufin product (excitation, 518 nm; emission, 583 nm). C. reinhardtii cells were removed by brief centrifugation before measurement. H$_2$O$_2$ was quantified against a standard curve. Measurements at time 0 were subtracted from all other time points. DanePy fluorescence was measured in the same conditions as those of Fischer et al. (2007).

Spectroscopy Analysis

Spectroscopy analysis was performed in vivo using a JTS-10 spectro-photometer (BioLogic). Changes in the amount of functional photosynthetic complexes were evaluated measuring the ECS spectral change, a shift in the pigment absorption bands that is linearly correlated to the number of light-induced charge separations within the reaction centers (Witt, 1979). Functional PSI and PSII content was estimated from changes in the pigment absorption bands that is linearly correlated to the number of light-induced charge separations within the reaction centers (Witt, 1979). Functional PSI and PSII content was estimated from changes in the amplitude of the fast phase of the ECS signal (at 520 to 546 nm) upon excitation with a saturating laser flash (520 nm, 5-ns duration). PSII contribution was calculated from the decrease in the signal amplitude upon the addition of DCMU (20 µM) and hydroxyamine (2 mM) to irreversibly block PSII charge separation. Conversely, PSI was estimated as the fraction of the signal that was insensitive to these inhibitors (Bailleul et al., 2010a).
Biochemical Analysis

Whole-Cell Analysis

C. reinhardtii cells were shock frozen at the time of sampling and pelleted later by centrifugation at 16,000g for 2 min. Proteins were extracted by resuspending the pellet in 100 μL of extraction buffer (60 mM DTT, 60 mM Na2CO3, 2% [v/v] SDS, and 12% [w/v] Suc) and collected in the supernatant after centrifugation at 16,000g for 10 min. Proteins were separated on 12.5% denaturing polyacrylamide gels (Laemmli, 1970), transferred onto nitrocellulose membrane (0.45 μm; Whatman), and revealed by immunodetection with the ECL+ detection kit (GE Healthcare).

Isolation of Thylakoid Membranes and Purification of PSII and PSI Supercomplexes

C. reinhardtii cells were harvested after an illumination of 500 μmol photons m−2 s−1 in HS medium for 4 h and disrupted twice by BioNeb (Glasm-col) at 7.5 kilogram force cm−2. Thylakoid membranes were isolated as described previously (Tokutsu et al., 2009) with a slight modification: The buffer used for preparing thylakoid membranes contained 25 mM MES, 0.33 M Suc, 5 mM EDTA, and 1.5 mM NaCl, pH 6.5. Purification of PSII supercomplex and PSI supercomplex was done using the respective His-tagged strains as described previously by Iwai et al. (2008, 2010), except that n-dodecyl-α-c-maltoside (α-DM; Anatrace) was used to solubilize the thylakoid membranes. Thylakoid membranes were adjusted to 0.4 mg chlorophyll mL−1 in thylakoid preparation buffer and solubilized with 1.0% (w/v) α-DM on ice for 5 min in the dark (Tokutsu et al., 2012). The solubilized membranes were applied to a column with ProBond resin (Invitrogen), which was equilibrated with the same buffer supplemented with 0.02% (w/v) α-DM. The column was washed with the same buffer supplemented with 15 mM imidazole and 0.02% (w/v) α-DM until the flow-through solution became clear. The photosystem supercomplexes were eluted with the same buffer supplemented with 25 mM imidazole and 0.02% (w/v) α-DM. SDS-PAGE and immunoblotting were conducted as described previously (Tokutsu et al., 2009).

Antibodies

Antibodies used to detect the polypeptides in the supercomplexes were as described previously (Iwai et al., 2010). Anti-LHCSR antibody was a kind gift of M. Guérin (Université Laval, Canada) to K.K.N., antiphosphotheonine antibodies were from Zymed Lab, and anti-ATPB was obtained from Agrisera.

Pigment Analysis

The method used to analyze and quantify carotenoids is detailed by Fraser et al. (2000). C. reinhardtii cells were frozen and resuspended in methanol. After three consecutive extractions, the supernatant was used to estimate pigment content by HPLC analysis using a C30 reverse-phase column (250 × 4.6 mm) manufactured by YMC Co. and purchased from Interchim. The following mobile phases used were methanol (A), water/ methanol (20/80 by volume) containing 0.2% (w/v) ammonium acetate (B), and tert-butyl methyl ether (C). The gradient used was 95% A/5% B isocratically for 12 min, a step to 80% A/5% B/15% C by 30 min (Fraser et al., 2000). Identification of pigments was achieved by comparing retention times and spectral properties of authentic standards. Quantification was achieved by evaluating the area below each peak using software provided by the manufacturer. Tocopherols were measured using the same method as for pigments, using a fluorescence detector instead of a diode array detector.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: STN7, AAO63768.1; STT7, AAO63768.1; and LCCSR3.2, EDP01087.1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. State Transition Phenotype of the Different Strains.

Supplemental Figure 2. High-Energy Quenching Phenotype of the Different Strains.

Supplemental Figure 3. Dynamics of Protein Phosphorylation and Fluorescence Quenching in High-Light-Treated C. reinhardtii Wild-Type Cells.

Supplemental Figure 4. Fluorescence Quenching Dynamics in durn22 Cells.

Supplemental Figure 5. Fluorescence Dynamics in C. reinhardtii Cells in Low Light.

Supplemental Figure 6. Sensitivity of Fluorescence Quenching to the Presence of a Proton Gradient in C. reinhardtii.

Supplemental Figure 7. Long-Term High-Light Acclimation in C. reinhardtii.

Supplemental Figure 8. In Vivo Detection of 1O2 Production by DanePy in C. reinhardtii.

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AUTHOR CONTRIBUTIONS

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A Dual Strategy to Cope with High Light in *Chlamydomonas reinhardtii*

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