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# Interplay between LHCSR proteins and state transitions governs the NPQ response in *Chlamydomonas* during light fluctuations

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## Abstract

Photosynthetic organisms use sunlight as the primary energy source to fix CO<sub>2</sub>. However, in nature, light energy is highly variable, reaching levels of saturation for periods ranging from milliseconds to hours. In the green microalga *Chlamydomonas reinhardtii*, safe dissipation of excess light energy by non-photochemical quenching (NPQ) is mediated by light-harvesting complex stress-related (LHCSR) proteins and redistribution of light-harvesting antennae between the photosystems (state transition). Although each component underlying NPQ has been documented, their relative contributions to NPQ under fluctuating light conditions remain unknown. Here, by monitoring NPQ in intact cells throughout high light/dark cycles of various illumination periods, we find that the dynamics of NPQ depend on the timescales of light fluctuations. We show that LHCSRs play a major role during the light phases of light fluctuations and describe their role in growth under rapid light fluctuations. We further reveal an activation of NPQ during the dark phases of all high light/dark cycles and show that this phenomenon arises from state transition. Finally, we show that LHCSRs and state transition synergistically cooperate to enable NPQ response during light fluctuations. These results highlight the dynamic functioning of photoprotection under light fluctuations and open a new way to systematically characterize the photosynthetic response to an ever-changing light environment.

## KEYWORDS

bioenergetics, chlorophyll fluorescence, light-harvesting complex stress related, microalgae, nonphotochemical quenching, photoprotection

**Abbreviations:** CCM, CO<sub>2</sub> concentration mechanism; C<sub>i</sub>, inorganic carbon (CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup>, CO<sub>3</sub><sup>2-</sup>); HL, high light; LHC, light-harvesting complex; LHCSR, light-harvesting stress related protein; NPQ, nonphotochemical quenching; PAM, pulse-amplitude modulation; PS, photosystem; qE, energy-dependent quenching; qI, photoinhibition; qT, state transitions; qZ, zeaxanthin-dependent quenching; STT7, serine/threonine-protein kinase; TCSPC, time-correlated single photon counting.

Collin J. Steen and Adrien Burlacot contributed equally to this study.

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## 1 | INTRODUCTION

Most life on Earth is sustained by photosynthetic organisms that capture sunlight energy to convert CO<sub>2</sub> and water into chemical energy. Light is captured by light-harvesting antenna complexes that contain a network of pigments absorbing photons and funneling the energy towards photosystems II and I that use it to perform photochemical reactions. Under light-limiting conditions, efficient light harvesting is crucial for maximizing the rate of CO<sub>2</sub> fixation (Björkman & Demmig, 1987). However, high light (HL) intensities can saturate reaction centers and lead to the build-up of excess excitation energy, which, if unchecked, can lead to the production of reactive oxygen species and damage to both reaction centers (Khorobrykh et al., 2020). In nature, light exposure rapidly fluctuates in intensity with periods of HL ranging from milliseconds to hours (Graham et al., 2017), requiring photosynthesis to acclimate to different timescales of HL exposure. For each period of HL acclimation, photosynthetic organisms exhibit photoprotective mechanisms that regulate light harvesting and safely remove excess energy (Erickson et al., 2015; Pinnola & Bassi, 2018; Roach, 2020).

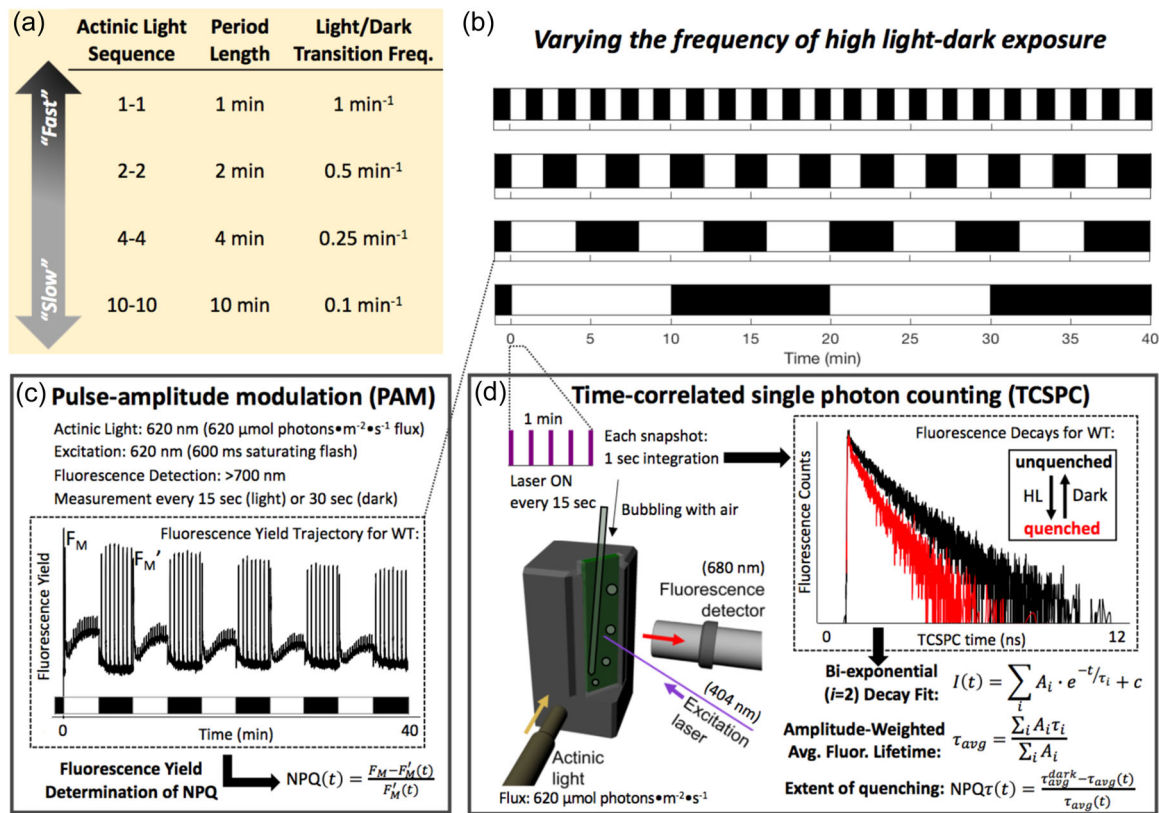
Upon light absorption, the energy can be dissipated as heat in a process called nonphotochemical quenching (NPQ). NPQ involves five components, each of which has been distinguished by its time of induction and relaxation during transition between dark and HL (Erickson et al., 2015). The fastest component, called energy-dependent quenching (qE), is triggered by luminal acidification (Briantais et al., 1979) and is induced and relaxed within seconds. State transition (qT) occurs within minutes and involves the phosphorylation of light-harvesting complexes (LHCs) (Allen, 1992) resulting in their detachment from Photosystem (PS) II and subsequent aggregation in a quenched state and/or association to PSI (Nagy et al., 2014; Nawrocki et al., 2016; Ünlü et al., 2014). Zeaxanthin-dependent quenching (qZ) requires the accumulation of zeaxanthin and probably involves quenching in the minor LHCs of PSII (Dall'Osto et al., 2005; Nilkens et al., 2010; Wehner et al., 2006). On longer time scales, two more sustained forms of NPQ occur: qH that takes place in the antennae of PSII (Malnoë et al., 2018) directly in the LHCII trimers (Bru et al., 2021) and photoinhibition (qI) that occurs when degradation of the PSII core protein D1 exceeds its capacity for repair due to excess excitation energy (Aro et al., 1993).

In the green microalga *Chlamydomonas reinhardtii*, qE is mediated by pigment-binding LHC stress-related (LHCSR) proteins (Peers et al., 2009; Rochaix & Bassi, 2019). LHCSRs contain protonatable residues, which sense the decreasing luminal pH generated under HL conditions (Ballottari et al., 2016; Tian et al., 2019); the protonation of LHCSRs triggers NPQ within the protein (Kondo et al., 2017; Liguori et al., 2013; Troiano et al., 2021), allowing fast activation of qE. While there are two types of LHCSR proteins (LHCSR1 and LHCSR3), both of which bind pigments (Bonente et al., 2011; Perozeni et al., 2020), LHCSR3 (for which two homologs are present in *Chlamydomonas*)

is thought to be the main actor in qE (Peers et al., 2009; Truong, 2011). On the other hand, qT is activated by the buildup of reducing equivalents in the thylakoid membrane, which activates a serine/threonine-protein kinase (STT7) (Depege et al., 2003; Lemeille et al., 2009) that phosphorylates LHCII, enabling it to detach from PSII and ultimately reattach to PSI (Iwai, Takizawa, et al., 2010; Minagawa, 2011). While qZ has been described in *Chlamydomonas* (Niyogi et al., 1997), it does not seem to play a significant role in overall NPQ (Girolomoni et al., 2019; Tian et al., 2019), and its potential mechanism of action remains to be determined. Finally, while qH has not been described in *Chlamydomonas*, qI occurs upon continued excess illumination (Aro et al., 1993; Erickson et al., 2015) at the level of the PSII center, where oxygen-mediated sensitization creates the irreversible formation of a quenching site (Nawrocki et al., 2021).

The photophysical and biochemical bases for NPQ have been studied for decades (Erickson et al., 2015), however, the in vivo operation has mostly been assessed under a single dark-to-HL transition (Nedbal & Lazár, 2021) leaving our understanding of photoprotection under more complex light patterns limited. While LHCSR and STT7 activity are both known to be important for steady-state NPQ based on measurements performed under nonrepeating HL/dark periods (Allorent et al., 2013), their relative contributions to NPQ have not been quantified, and their response to faster-timescale fluctuating light remains unstudied. Recent work has started looking at the response of NPQ to some specific light fluctuations in *Chlamydomonas* (Nawrocki et al., 2020; Roach, 2020) and in the moss *Physcomitrella* (Gao et al., 2022). However, the physiological role and the functioning of the NPQ components under the wide diversity of light patterns that are present in the natural environment is unexplored.

Here we utilized two distinct methods to monitor chlorophyll fluorescence in intact cells of *Chlamydomonas* that were exposed to various periodically fluctuating light sequences with HL/dark periods ranging from 1 to 10 min (Figure 1). The roles of qE and qT were investigated using single or double mutants of LHCSRs and STT7. Our analysis of LHCSR mutants (*npq4* [Peers et al., 2009], *lhcsr1* [Truong, 2011], and *npq4lhcsr1* [Truong, 2011]) revealed that LHCSR3 is the main contributor to the NPQ response during the HL phase of light fluctuations. Using mutants impaired in state transition (*stt7-9* [Depege et al., 2003] and *stt7npq4* [Allorent et al., 2013]), we showed that qT quenching occurs primarily during the dark portion of the fluctuating light sequence and represents a significant part of NPQ during repeated light fluctuations. Our results showed that while qE and qT sustain most of the NPQ throughout light fluctuations, their relative importance varies during different phases of the fluctuating light response, with qT playing a larger role during dark periods and after repeated HL-dark fluctuations. Surprisingly, the timescale of light fluctuations did not seem to have a major impact on the respective contributions of qE and qT although the contribution of qE during the dark phase was period-dependent. Nonetheless, the various components of NPQ are not completely independent, and we



**FIGURE 1** Experimental design for Chl *a* fluorescence measurements throughout exposure of *Chlamydomonas* cells to fluctuating light with various periods of high light (HL)-dark exposure. (a, b) Representation of the HL-dark cycles used for the 40 min of light fluctuation and their corresponding period, frequency, and name used throughout the main text. Nonphotochemical quenching (NPQ) was measured using pulsed-amplitude modulation (PAM, c) and time-correlated single-photon counting (TCSPC, d). (c) Characteristics of the PAM measurement and representative data of fluorescence yield in WT cells. (d) Characteristics of the TCSPC apparatus. Shown are two decays representative of two snapshots taken in a quenched and unquenched state. WT, wild type

reveal an interplay between LHCSR- and STT7-mediated NPQ that enables the wild-type photoprotective response. We further show that while *stt7* mutants are not impaired in growth under light fluctuations, short time scale light fluctuations highly impair LHCSR mutants. These findings represent an important first step in investigating the photosynthetic response to the diversity of HL periods that occur in nature.

## 2 | MATERIALS AND METHODS

### 2.1 | Strains and culture conditions

*Chlamydomonas* mutants and their respective wild-type 4A- were previously described (*npq4* [Peers et al., 2009], *lhcsr1* [Truong, 2011], *npq4lhcsr1* [Truong, 2011], *stt7-9* [Cardol et al., 2009], *stt7npq4* [Allorent et al., 2013]). Note here that because the *stt7-9* mutant was obtained by complementing an arginine-deficient strain (Depege et al., 2003) with a subsequent selection of a clone more prone to crossing (Cardol et al., 2009), it does not have a proper control strain. Accordingly, we used 4A- as the best

possible control strain for all mutants. All strains were grown photoautotrophically under moderate light (50 μmol photons m<sup>-2</sup> s<sup>-1</sup>) in minimal HS medium under air level of CO<sub>2</sub> (20°C). Except for the growth test, cell cultures (5–8 μg Chl mL<sup>-1</sup>) were incubated overnight at high light (400 μmol photons m<sup>-2</sup> s<sup>-1</sup>), for maximizing the expression of LHCSR proteins (Tibiletti et al., 2016). Before each measurement, cells were illuminated for at least 15 min with low intensity far-red light (3in1LED panel with far-red LED; 3LH series, NK system) to ensure a complete State 1 configuration (Bonaventura & Myers, 1969). All replicates shown are biological replicates from independent cultures.

### 2.2 | Chlorophyll fluorescence measurements

In this study, we employ two techniques to monitor the activation and deactivation of NPQ throughout 40 min of exposure to repeated periods of high light and dark on the basis of changes in Chl fluorescence emission (see Figure 1 for an illustration of the experimental design). Time-resolved Chl fluorescence was measured via time-correlated single photon counting (TCSPC) while

Chl fluorescence yield was measured in parallel experiments using pulse-amplitude modulation (PAM) fluorimetry. Although both methods can monitor NPQ, the fluorescence lifetime is not susceptible to a range of nonquenching processes that can impact the fluorescence yield (such as chromophore bleaching, changes in chlorophyll concentration, chloroplast movement or enhanced light scattering [Sylak-Glassman et al., 2016; Zaks et al., 2013]). Therefore, fluorescence lifetime measurements provide insight into processes that directly quench chlorophyll fluorescence.

### 2.2.1 | TCSPC measurement and fitting

The average chlorophyll fluorescence lifetime was measured by time-correlated single-photon counting (TCSPC), as previously described (Steen et al., 2020; Sylak-Glassman et al., 2016). A diode laser (Coherent Verdi G10, 532 nm) pumped a Ti:Sapphire oscillator (Coherent Mira 900 f, 808 nm, 76 MHz) and the output was subsequently frequency doubled using a  $\beta$ -barium borate crystal to obtain 404 nm light. These pulses were used for excitation of the sample with a power of 1.7 mW (20 pJ/pulse) and Chl fluorescence emission at 680 nm was detected via an MCP-PMT (Hamamatsu R3809U). A custom-built LABVIEW software was used to synchronize three shutters located in the laser path, actinic light path and the path between the sample and detector. Every 15 s, a fluorescence lifetime snapshot measurement was acquired by exposing the cells to the saturating laser (404 nm) for 1 s and detecting the emission. Fluorescence lifetime snapshots were measured by TCSPC using a Becker-Hickl SPC-850 data acquisition card and SPCM software. In between the snapshot measurements, high-light illumination of the cells was achieved by exposing the cuvette to white light set to an intensity of  $620 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Leica KL1500 LCD, peak 648 nm, FWHM 220 nm), chosen as the minimal light level necessary to saturate photosynthetic reactions and induce qE activity at the onset of illumination. It was also chosen such that PAM and TCSPC would use identical high light intensities. The sample concentration was adjusted to  $\sim 80 \mu\text{g Chl ml}^{-1}$  for TCSPC measurements. To control the gas composition of the culture and prevent cells from settling to the bottom of the cuvette, the sample was bubbled with air (ambient  $\text{CO}_2$  concentrations) at a rate of  $2\text{--}7 \text{ ml min}^{-1}$  throughout the entire 40 min experiment duration, although note that such bubbling increased the noise of the measurements.

For each fluorescence decay measurement, to ensure that PSII reaction centers were closed, we selected the 0.2 s step with the longest lifetime from the overall 1 s snapshot measurement duration (Sylak-Glassman et al., 2016). This longest step was then fit to a bi-exponential decay (Picoquant, Fluofit Pro-4.6) and the average amplitude-weighted fluorescence lifetime ( $\tau_{\text{avg}}$ ) was calculated for each snapshot measurement. The NPQ $\tau$  parameter is derived from the fluorescence lifetime snapshot measurements and is defined analogously to NPQ

(Sylak-Glassman et al., 2014, 2016):  $\text{NPQ}\tau(t) = \frac{\tau_{\text{avg}}(0) - \tau_{\text{avg}}(t)}{\tau_{\text{avg}}(t)}$ . The value of NPQ $\tau$  represents the magnitude of the quenching response based on the change in the average fluorescence lifetimes between time  $t=0$  (after far-red acclimation but before HL exposure) and any other time  $t$  during the 40 min snapshot trajectory. Therefore, using NPQ $\tau$  removes confounding effects arising from any differences in the average chlorophyll excited state lifetime of the different strains following far-red acclimation. For all TCSPC measurements, each biological replicate represents the average of three technical replicates measured on the same day.

### 2.2.2 | PAM measurements

Chlorophyll fluorescence yield was measured using a PAM fluorimeter (Dual-PAM 100, Walz GmbH) with the red measuring head. Red saturating flashes ( $8000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 600 ms, 620 nm) were delivered to measure  $F_M$  (maximal fluorescence yield in the dark-acclimated samples) and then every 15 or 30 s to measure  $F_M'$  under actinic light exposure or dark phase, respectively. Actinic light illumination (620 nm) was set to  $620 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Fluorescence emission was detected using a long-pass filter ( $>700 \text{ nm}$ ). NPQ was calculated as  $(F_M - F_M')/F_M'$ . The Chl concentration was  $\sim 5\text{--}8 \mu\text{g Chl ml}^{-1}$  and as for TCSPC, all PAM measurements and the sample was bubbled with air at a flux of  $2\text{--}7 \text{ ml min}^{-1}$  for proper control of the gas concentrations of the sample throughout the entire 40 min experiment duration, note that such bubbling increased the noise of the measurements (but to a lesser extent than for TCSPC).

### 2.2.3 | Quantifying the contributions of LHCSR1 and STT7 to NPQ

To assess the relative contributions of LHCSR1, LHCSR3, and STT7 to overall NPQ, we analyzed the NPQ (PAM) and NPQ $\tau$  (TCSPC) trajectories for WT and each mutant. The relative contribution of each protein was determined as the percent change in the integrated snapshot trajectory of NPQ or NPQ $\tau$  for each mutant relative to the control WT strain. As the contribution of each actor was found to be overall independent of HL-dark fluctuation frequencies in the range of  $1\text{--}0.1 \text{ min}^{-1}$ . (Supporting Information: Figure 5), the average contribution of each protein under the four light fluctuating sequences for both PAM and TCSPC was considered. Additionally, to characterize the involvement in activation or deactivation of NPQ, the quenching trajectories were integrated solely under HL or dark periods, respectively (Supporting Information: Figure 6). The contributions of each protein to the early versus late responses were further assessed by integrating from 0 to 20 min and 20–40 min, respectively (Supporting Information: Figure 7). These results are summarized in Figure 6 of the main text. Since it has been suggested

that the *stt7-9* mutant may be “leaky” (Bergner et al., 2015), our estimates for the STT7 contribution are lower bound estimates. The potential leakiness of the *stt7-9* mutant does not affect any of the conclusions presented in the manuscript. In addition to quantifying the relative contribution to NPQ of each protein relative to the WT strain, we also assessed the contribution of STT7 in the absence of LHCSR3 by integrating the PAM NPQ trajectory of *npq4stt7* relative to that of *npq4*. Likewise, the contribution of LHCSR3 was assessed independently of STT7 by integrating the PAM NPQ trajectory of *npq4stt7* relative to that of *stt7*.

### 2.3 | 77 K chlorophyll fluorescence emission

Chlorophyll fluorescence emission spectra of *Chlamydomonas* cells at 77 K were obtained by freezing whole cells (~5–8  $\mu\text{g Chl ml}^{-1}$  final concentration) in liquid nitrogen. The emission spectrum was then measured between 600 and 800 nm (435 nm excitation wavelength, RF-5300PC spectrophotometer, Shimadzu).

### 2.4 | Growth tests

The different *Chlamydomonas* strains were cultivated photoautotrophically under moderate light (50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in minimal medium under air level of  $\text{CO}_2$  (20°C). Cells were harvested during exponential growth and resuspended in fresh minimal medium to 0.1, 0.5, or 2  $\mu\text{g Chl ml}^{-1}$ . Eight-microliter drops were spotted on minimal medium plates at pH=7.2 and exposed to the various light conditions for 8 days. Homogeneous light was supplied by LED panels. Temperature was maintained at 25°C at the level of plates.

### 2.5 | Accession numbers

Genes studied in this article can be found on <https://phytozome.jgi.doe.gov/> under the loci Cre08.g365900.t1.2 (LHCSR1), Cre08.g367500.t1.1 (LHCSR3.1), Cre08.g367400.t1.1 (LHCSR3.2), Cre02.g120250.t1.1 (STT7).

## 3 | RESULTS

### 3.1 | Varying light fluctuation periods affect the dynamic NPQ response

While the photosynthetic response of *Chlamydomonas* to some light fluctuations has been reported (Cantrell & Peers, 2017; Roach, 2020), an analysis of NPQ for various timescales of light fluctuations is lacking. We therefore measured chlorophyll fluorescence during periodic light-dark cycles of 40 min with individual HL or dark periods ranging from 1 to 10 min (Figure 1).

Chlorophyll fluorescence yield was measured using pulse-amplitude modulated (PAM) fluorometry and used to calculate NPQ (Klughammer & Schreiber, 2008). In tandem experiments, time-correlated single-photon counting (TCSPC) was used to measure the chlorophyll fluorescence lifetime (Amarnath et al., 2012), which was used to calculate NPQ $\tau$  (Sylak-Glassman et al., 2014). For all timescales of light fluctuations in the wild-type strain, NPQ quickly turned on upon illumination but turned off more slowly (Figure 2). The same trend was observed in NPQ $\tau$  (Figure 2). The 1 min HL period light fluctuation led to a nearly square-like response of NPQ and NPQ $\tau$  (Figure 2).

For the longer HL periods ranging from 2 to 10 min, after an initial burst of NPQ in HL, the level of NPQ decreased with continued illumination, eventually reaching a steady state for the 10 min HL period (Figure 2). A similar trend was also seen in NPQ $\tau$  (Figure 2). Therefore, these kinetics are directly related to chlorophyll fluorescence quenching, rather than other non-quenching processes that affect chlorophyll fluorescence. This phenomenon of decreasing NPQ during the light has been previously attributed to the consumption of the proton gradient by the activity of the  $\text{CO}_2$  concentration mechanism (CCM) (Burlacot et al., 2022). In addition to the effect arising from CCM, state transition via the induction of State 1 could also play some role in the NPQ decrease observed during HL periods, though this would not contribute during the first HL period in which cells are already in State 1 (following far-red acclimation). For fluctuating light periods longer than 4 min, upon a transition from HL to dark, the NPQ turned off rapidly but was then followed by a gradual rise in NPQ during further darkness, a trend that was also observed in NPQ $\tau$  (Figure 2). However, compared with NPQ, NPQ $\tau$  showed a larger magnitude of increase during the long dark periods (Figure 2c,d). Differences between the NPQ and NPQ $\tau$  traces are considered in the discussion.

We conclude from these experiments that, when exposed to periodic light fluctuations with HL periods ranging from 1 to 10 min, at least three components of NPQ are present: (i) a rapidly responding component, (ii) a slowly inducible component induced throughout the light fluctuations and (iii) a component induced in the dark phases of light fluctuations.

### 3.2 | The majority of NPQ during light fluctuations is mediated by LHCSR proteins

It has been well established that LHCSR proteins are crucial for NPQ in *Chlamydomonas* during a single dark-to-light transition (Correa-Galvis et al., 2016; Peers et al., 2009; Truong, 2011). To examine the relative importance of each LHCSR protein for the functioning of NPQ during light fluctuations, we measured the chlorophyll fluorescence yield and lifetime during the same light-dark cycles on mutants impaired in the accumulation of LHCSR1 (*lhcsr1*) (Truong, 2011), LHCSR3-1 and LHCSR3-2 (*npq4*) (Peers et al., 2009), or all three LHCSRs (*npq4lhcsr1*) (Ballottari et al., 2016;

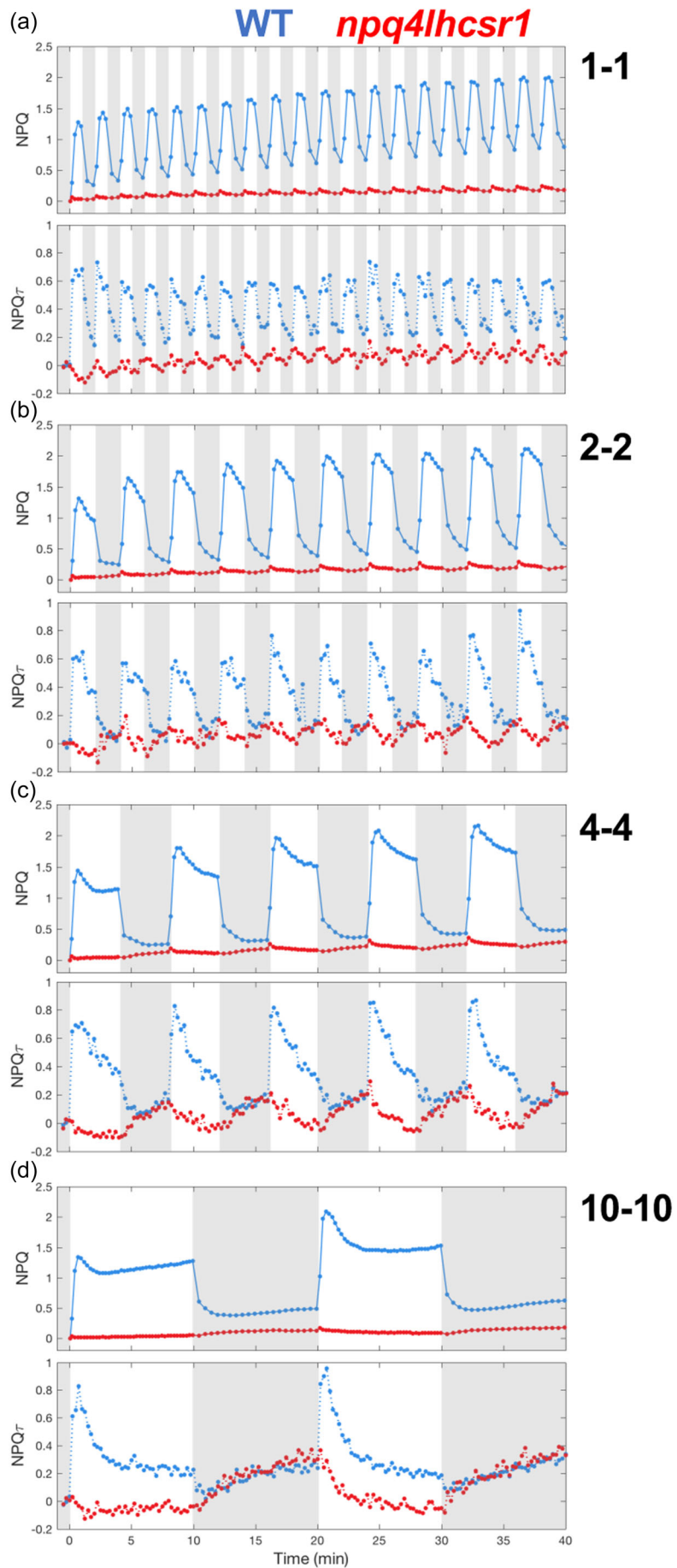


FIGURE 2 (See caption on next page)

Truong, 2011). While the *npq4lhcsr1* mutant was highly impaired in its NPQ capacity for all light fluctuations (Figure 2), single *npq4* and *lhcsr1* mutants showed some NPQ in response to light fluctuations (Figure 3). Noticeably, for HL periods longer than 4 min, the increasing NPQ observed during dark phases was more pronounced in the *npq4lhcsr1* mutant (Figure 2). We conclude from these experiments that although LHCSR3s are responsible for most of the NPQ during the light phase of all light fluctuations, a substantial portion of the NPQ in WT is nonetheless mediated by other biochemical processes, part of which is induced during the dark periods of light fluctuations.

### 3.3 | The increasing quenching in the dark periods arises from state transition

Induction of NPQ during darkness has been previously reported in chlorophytes (Allorent et al., 2013; Casper-Lindley & Björkman, 1996), and qT has been proposed to be involved (Allorent et al., 2013). Reorganization of light-harvesting antennae between PSII and PSI was thus followed throughout a light fluctuation by measuring 77K fluorescence emission spectra for cells at three time points: after acclimation to far-red light (cells in State 1 [Zhang et al., 2021]), after 10 min HL, and after 10 additional min dark (see vertical lines in Figure 4a). In WT and *npq4lhcsr1*, an increase in the emission at 710 nm specific to PSI-bound LHCI was observed between the 10 (after HL) and 20 min (after dark) time points, suggesting that some reassociation of LHCI from PSII to PSI occurs during the dark portion of our measurements (Figure 4, Supporting Information: Figure 1). In contrast, mutants lacking the STT7 kinase responsible for qT (*stt7* and *stt7npq4*) showed negligible changes in the 77K fluorescence emission spectra (Supporting Information: Figure 2) and only showed a minimal increase in NPQ or NPQ<sub>T</sub> during the dark periods of light fluctuations (Figure 5) and with increasing duration of exposure to light fluctuations (Supporting Information: Figure 3).

To characterize the kinetics of qT occurring during the light-to-dark transition, we analyzed the response of the chlorophyll fluorescence lifetime for WT and *npq4lhcsr1* mutant cells that were exposed to 10 min of HL followed by 30 min of dark. Interestingly, upon light-to-dark transition, both strains showed steadily decreasing lifetimes for the first 10 min of darkness, after which, the fluorescence lifetimes began to reverse, eventually reaching the starting lifetime after 30 min of darkness (Supporting Information: Figure 4). In contrast, *stt7* showed no such quenching of fluorescence lifetime during the 10 min dark period. Therefore, we conclude that the quenching observed during the dark phases of light fluctuations in

*Chlamydomonas* arises from qT, which has an induction timescale of ~10 min and is reversible upon continued exposure to darkness.

### 3.4 | Contributions of qE and qT to NPQ and growth under fluctuating light

It has been previously proposed that, while LHCSR3s play an important role during short timescale illumination, state transitions are important for the cell's longer timescale response to HL acclimation (Erickson et al., 2015). To test this hypothesis and assess the scenario under fluctuating light conditions, we quantified the amount of NPQ that was mediated by each protein by comparing the remaining NPQ (or NPQ<sub>T</sub>) in each mutant relative to the NPQ (or NPQ<sub>T</sub>) in the WT reference strain (Figure 6a, Table 1). Surprisingly, the contribution of each protein to overall NPQ did not seem to depend on the period of the light fluctuation (Supporting Information: Figure 5). Therefore, as a first estimate for the contribution of LHCSR3s and STT7, we calculated the average contribution under all four light fluctuation sequences. The donut charts in Figure 6b,c,d depict the contribution of each protein as a percentage of the total NPQ observed in the WT strain (represented as the complete donut). While LHCSR3 is responsible for the majority of overall NPQ (72%, Figure 6b), STT7 had a substantial contribution mediating 42% of the NPQ, with LHCSR1 having a smaller contribution at 22% of NPQ. LHCSR3s were found to have a substantially larger contribution during light phases, where they are responsible for 94% of WT NPQ, while in the dark phases their contribution declined to 57% (Figure 6c, Supporting Information: Figure 6). On the other hand, STT7 played a significantly larger role in the NPQ during darkness (60%) than it does during illumination (36%) this latter result being likely due to inhibition of STT7 by high light (Allorent et al., 2013; Rintamäki et al., 2000; Vink et al., 2004). Interestingly, the amount of NPQ mediated by LHCSR3s was more important during the beginning of light fluctuations while state transitions contributed more after 20 min of light fluctuation (Figure 6d, Supporting Information: Figure 7), revealing increased relative contribution of qT and decreasing contribution of qE with increasing time of exposure to light fluctuations.

Since LHCSR3 is part of the mobile fraction of photosynthetic antenna and has been proposed to be affected by STT7 activity (Allorent et al., 2013), giving a potential mechanistic connection between qE and qT (Roach & Na, 2017), we aimed to interrogate the involvement of qT both together and separately from qE under fluctuating light. We compared the integrated PAM NPQ trajectories of *npq4stt7* relative to that of *npq4*, both of which lack LHCSR3, or

**FIGURE 2** Quenching trajectories during light fluctuations in *npq4lhcsr1* and its control strain. The response of nonphotochemical quenching (NPQ) and NPQ<sub>T</sub> (upper and lower panel, respectively) were measured in *npq4lhcsr1* mutant and its control strain (red and blue curves, respectively) during 40 min of light fluctuations with periods of 1, 2, 4 and 10 min (a, b, c and d, respectively) as described in Figure 1. Shown are the average of three biological replicates. For TCSPC data, each biological replicate was averaged from three technical replicates. The fluorescence lifetime values used to calculate NPQ<sub>T</sub> are shown in Supporting Information: Figure 13.



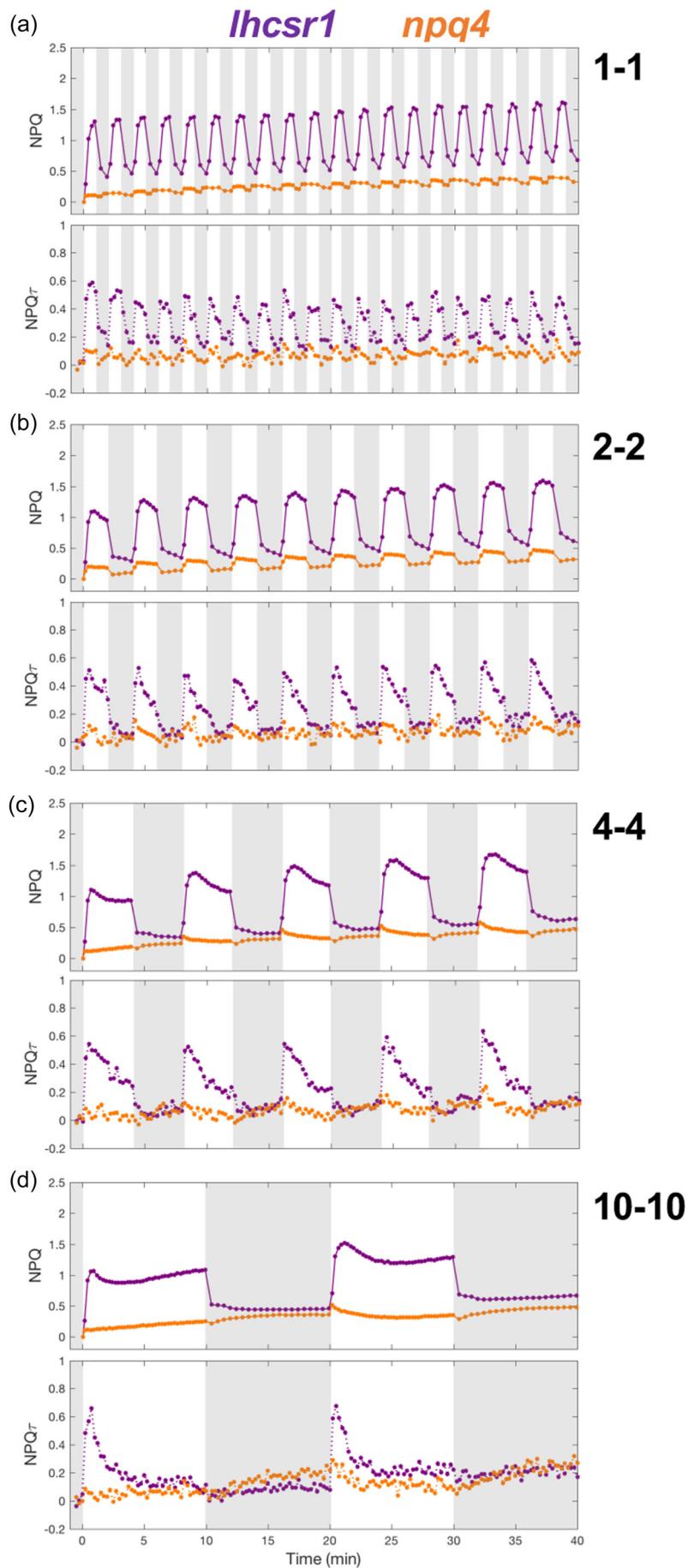
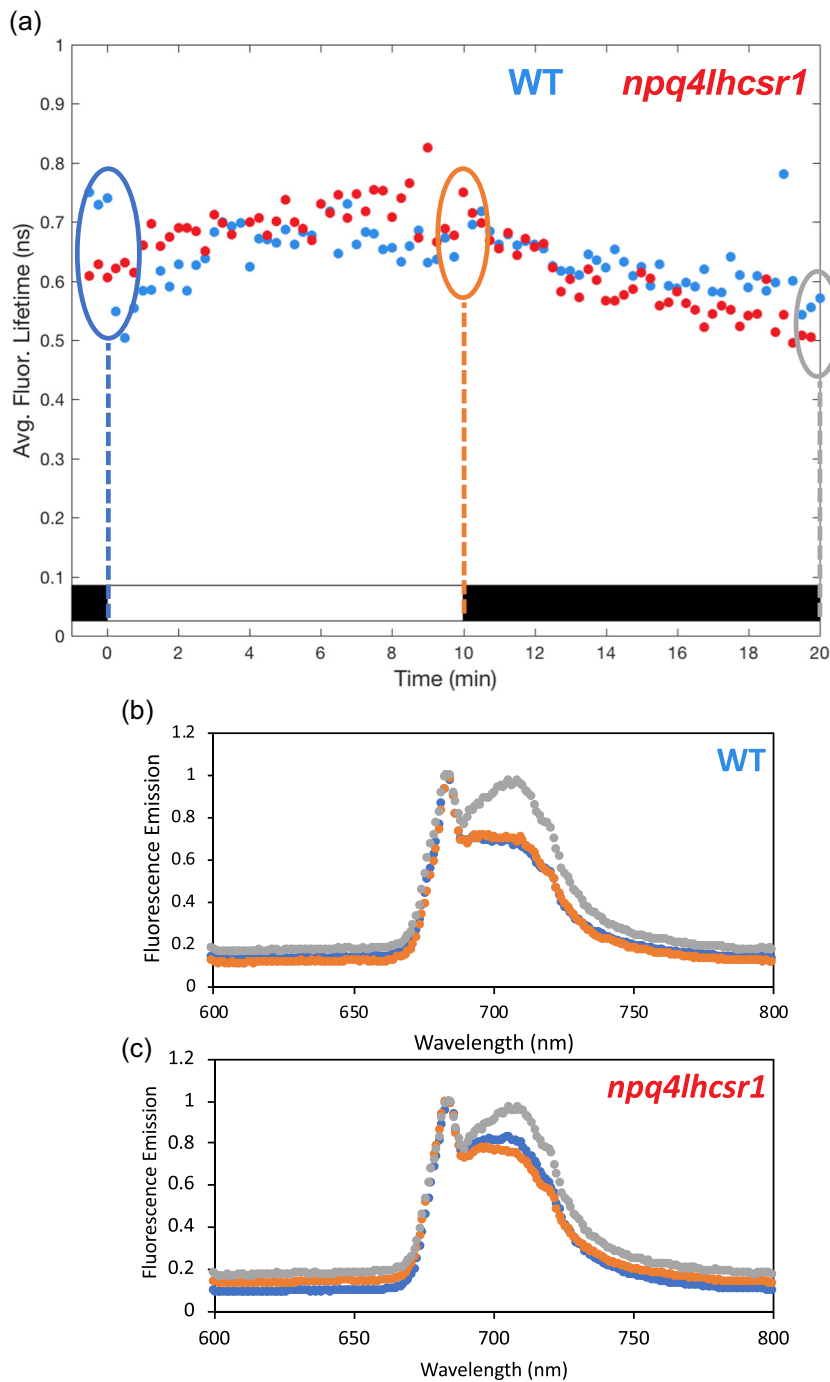


FIGURE 3 (See caption on next page)

*npq4stt7* relative to that of *stt7*, both of which lack STT7. The relative contribution of STT7 to NPQ was found to be different in the presence versus absence of LHCSR3, with STT7 playing a larger role when LHCSR3 is present (Figure 7 and Table 2). Likewise, the

contribution of LHCSR3 was larger when STT7 was present. These data suggest that STT7 and LHCSR3 synergistically cooperate to induce quenching during dark periods of light fluctuations. We propose that the activity of STT7 resulting in LHCSR phosphorylation



**FIGURE 3** Quenching trajectories during light fluctuations in *lhcsr1* and *npq4*. The response of nonphotochemical quenching (NPQ) and NPQ<sub>T</sub> (upper and lower panel, respectively) were measured in *lhcsr1* and *npq4* (purple and orange curves, respectively) during 40 min of light fluctuations with periods of 1, 2, 4 and 10 min (a, b, c and d, respectively) as described in Figure 1. Shown are average of three biological replicates. For TCSPC data, each biological replicate was averaged from three technical replicates. The fluorescence lifetime values used to calculate NPQ<sub>T</sub> are shown in Supporting Information: Figure 14.

**FIGURE 4** 77 K chlorophyll fluorescence emission spectra during the first high light-dark cycle of light fluctuations. Cells were placed in a time-correlated single photon counting (TCSPC) cuvette as described in Figure 1 and both fluorescence lifetime snapshots and 77 K chlorophyll fluorescence emission spectra were taken through 10 min of high light and 10 min darkness. (a) Fluorescence lifetime trajectory of *npq4lhcsr1* mutant (red dots) and its control strain (WT, blue dots). On the graph, dashed vertical lines depict the timepoints at which samples were taken for 77 K fluorescence spectra measurement. (b, c) 77 K fluorescence emission spectra of samples taken in (a) on the control strain (WT, b) and *npq4lhcsr1* mutant (c). Spectra were taken at 0, 10 and 20 min timepoints (blue, orange and grey spectra, respectively). Shown are representative spectra. Three independent biological replicate spectra for WT and *npq4lhcsr1* are shown in Supporting Information: Figure 1. 77 K spectra for the *stt7* and *stt7npq4* strains are shown in Supporting Information: Figure 2.

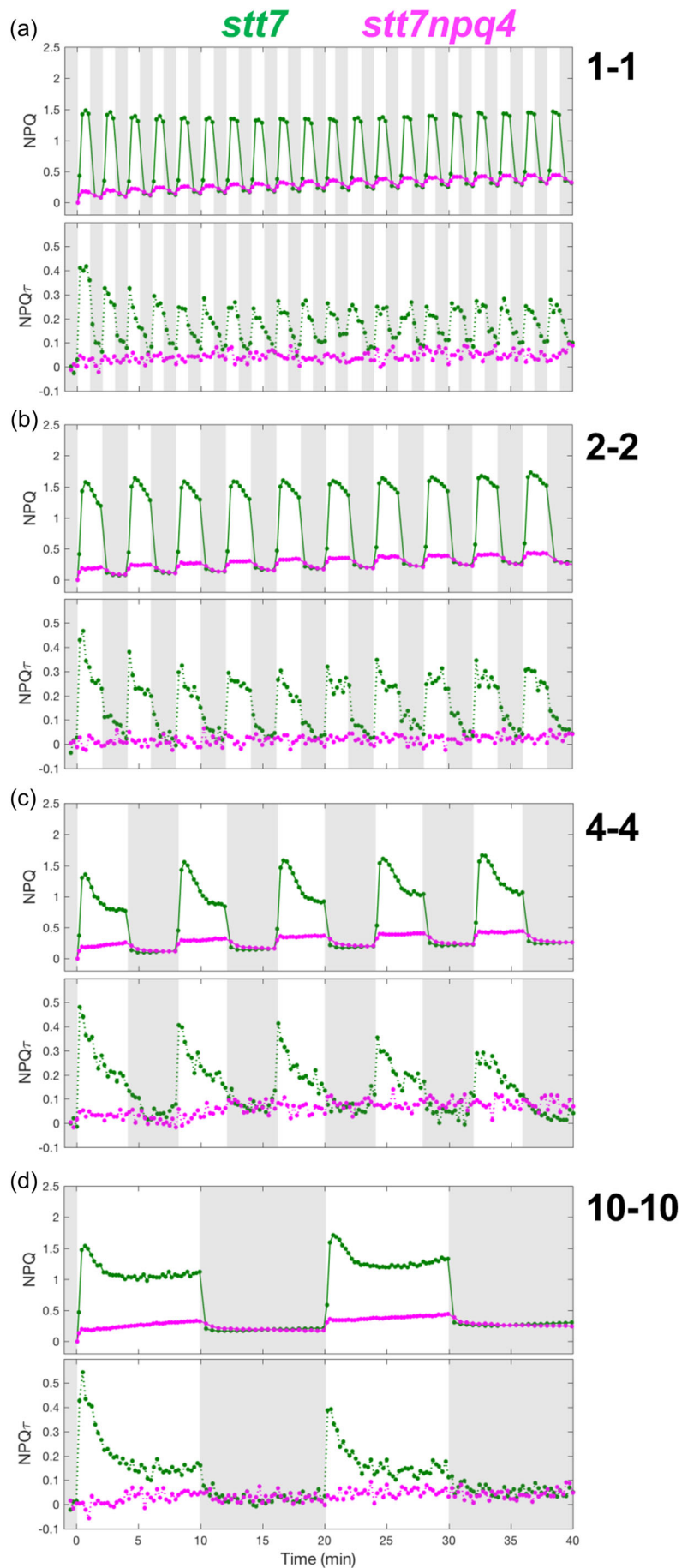


FIGURE 5 (See caption on next page)

and antenna mobility is responsible for this crosstalk between qE and qT during light fluctuations.

Although mutants impaired in the accumulation of LHCSRs and STT7 have strongly impaired NPQ capacities, this does not seem to impair the growth of those strains under continuous high light conditions (Cantrell & Peers, 2017; Depege et al., 2003; Peers et al., 2009). Recent data have shown that the growth of *npq4* and *npq4lhcsr1* mutants is impaired under certain light fluctuation conditions (Cantrell & Peers, 2017; Roach, 2020). We therefore investigated whether the growth impairment of those strains could be dependent on the timescale of light fluctuations. While all the mutants grew as well as the control strain under continuous illumination (Supporting Information: Figure 8), *npq4*, *lhcsr1*, *npq4lhcsr1* and *stt7npq4* mutants exhibited impaired growth under fast light fluctuations with a 1-min HL period (Figure 8). In contrast, only *npq4lhcsr1* and *stt7npq4* mutants showed an impaired growth under slower light fluctuations with an HL period of 10 min, and the growth of all mutants was similar when the HL period was increased to 30 min (Figure 8). We conclude from this experiment that qE mediated by LHCSR proteins is critical for growth under light fluctuations and that this role is more important for rapid light fluctuations.

## 4 | DISCUSSION

The involvement of pH-sensing LHCSR proteins and state transitions in the photoprotective response of *Chlamydomonas* has been previously described (Allorent et al., 2013; Peers et al., 2009). While mutants impaired in accumulation of LHCSRs were shown to have limited growth when light is provided in a day/night cycle (Cantrell & Peers, 2017) or fluctuating with a 10-min timescale (Roach, 2020), our understanding of the contribution of LHCSRs and state transitions to photoprotection during fluctuating light remains limited. Here, by measuring the NPQ levels during light fluctuations in a range of mutants impaired in the accumulation of LHCSR3, LHCSR1 and/or STT7 we have unraveled their relative contributions to NPQ. Varying the timescale of fluctuating light periods from 1 to 10 min allowed us to assess the dynamics of rapid qE- and slower qT-type processes. Interestingly, we observe that qT builds up during the dark periods of the light fluctuations and continues to play a role in the NPQ response during subsequent light phases. It occurs even in the absence of LHCSR3 (see *npq4* and *npq4lhcsr1* mutant in Figures 2 and 3), has a timescale of 10 min, and is reversible (Supporting Information: Figure 4), which is consistent with recent literature (Allorent et al., 2013; Zhang et al., 2021). During a transition between

low-light and high-light stress, qE proteins take a few hours to be fully induced (Peers et al., 2009), and it was hypothesized that qT may substitute for qE during this delay (Allorent et al., 2013). Our results show that even when LHCSRs are fully activated (i.e., in high light-acclimated cells), the occurrence of qT remains substantial during light fluctuations (Figure 2). State transition or qE mutants were previously shown to have high reactive oxygen species (ROS) production (Allorent et al., 2013; Barera et al., 2021). Thus, the substantial amount of qT induced during the dark periods of light fluctuations may enhance photoprotection and limit ROS production by “anticipating” the next exposure to high light. The combination of fast qE (turns on rapidly upon HL exposure due to  $\Delta$ pH) and residual qT (from previous dark periods) could therefore provide effective photoprotection in an unpredictable fluctuating-light environment.

Since qE has long been ascribed as the fastest component of NPQ, directly responding to the thylakoid lumen proton concentration (Briantais et al., 1979), and qT as a slower component (Allorent et al., 2013), the contribution of qE to NPQ was proposed to be stronger for short periods of HL, with the contribution of qT becoming increasingly important during longer periods of high-light stress (Erickson et al., 2015). Our approach of systematically assessing the response of photosynthesis to various timescales of light fluctuations has revealed nuances in this interpretation. Surprisingly, we found that the overall contributions of qE and qT are different in the presence versus absence of the other component (Figure 7) revealing that interactions between qE and qT contribute to the NPQ response during all timescales of light fluctuation. Additionally, the *npq4* mutant showed significantly reduced NPQ capacity compared with WT in the dark periods (by 74%) under fast light fluctuations (1-1 sequence), but only a 17% impairment under slower fluctuations (10-10) (see Supporting Information: Figure 6b), showing that relaxation of qE (around 1 min) is mediated by LHCSR3 and contributes substantially to the response of NPQ to rapid light fluctuations. Such relaxation kinetics may contribute to a faster response of NPQ to the next illumination if the period of light fluctuations is shorter than 2 min. It is also worth noting that the relative importance of qE in NPQ decreased after 20 min of light fluctuations, while the opposite occurred for qT (Figure 6), reflecting a build-up in qT throughout the 40 min of light fluctuations. Modeling the response of photosynthesis to complex light fluctuations has been done (Tanaka et al., 2019; Zaks et al., 2012, 2013; Steen et al. 2020; Nedbal & Lazár,) and would allow targeting specific mechanisms for increasing plant yields in the field. Our results show that such efforts should consider both the period of light and dark as well as the total time exposed to fluctuating light. The light intensities used during light fluctuations may also affect the relative

**FIGURE 5** Quenching trajectories during light fluctuations in *stt7* and *stt7npq4*. The response of nonphotochemical quenching (NPQ) and NPQ<sub>T</sub> (upper and lower panel, respectively) were measured in *stt7* and *stt7npq4* (green and magenta curves, respectively) during 40 min of light fluctuations with periods of 1, 2, 4 and 10 min (a, b, c and d, respectively) as described in Figure 1. Shown are the average of three biological replicates. For TCSPC data, each biological replicate was averaged from three technical replicates. The fluorescence lifetime values used to calculate NPQ<sub>T</sub> are shown in the Supporting Information: Figure 15.

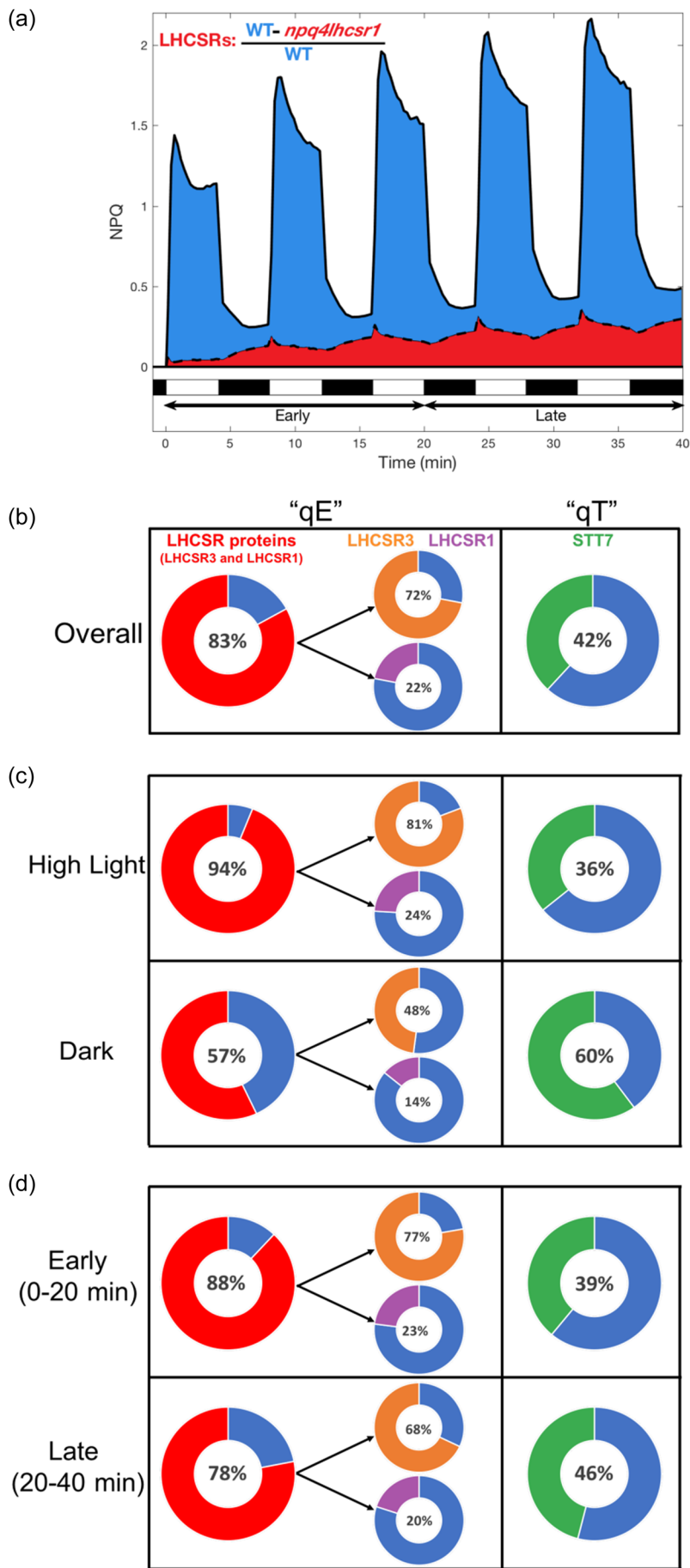


FIGURE 6 (See caption on next page)

**TABLE 1** Average contribution of each protein to overall wild-type NPQ for each light fluctuation sequence

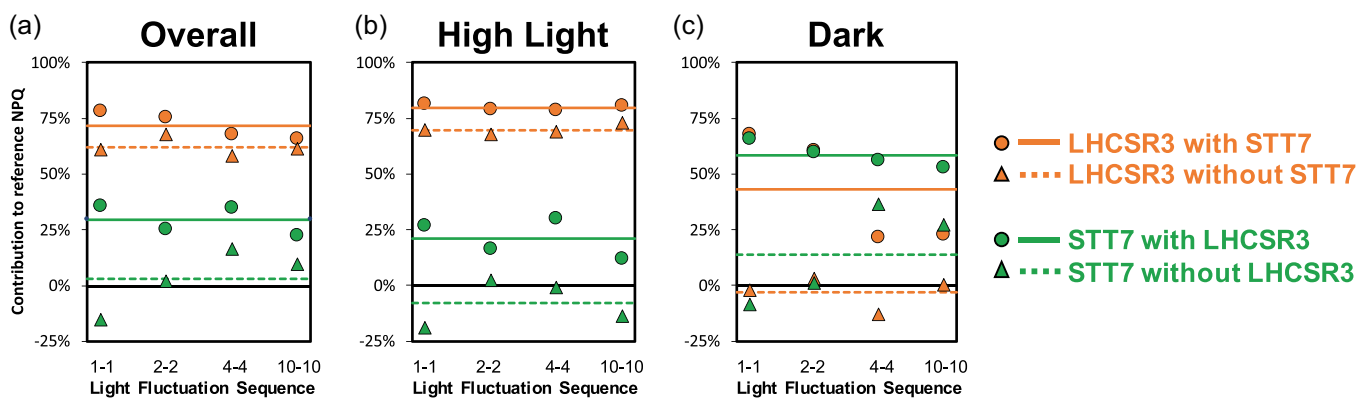
Overall contributions	1-1	2-2	4-4	10-10	Average
LHCSR3	89 ± 7%	84 ± 7%	83 ± 13%	77 ± 24%	83 ± 5%
LHCSR1	22 ± 12%	22 ± 10%	24 ± 16%	19 ± 30%	22 ± 2%
LHCSR3	81 ± 5%	78 ± 5%	73 ± 8%	58 ± 15%	72 ± 10%
STT7	46 ± 12%	38 ± 16%	46 ± 13%	39 ± 22%	42 ± 4%

Note: Shown is the average value ( $n = 6$ , evaluated from three TCSPC and three PAM replicates) and standard deviation of all individual replicates. The contributions of LHCSR3 (orange) and LHCSR1 (purple) were determined from the single mutants *npq4* and *lhcsr1*. The contribution of LHCSR3 overall (red) was evaluated from the *npq4lhcsr1* mutant. The contribution of qT was assessed from the *stt7* mutant. Each error in the right column represents the standard deviation of each protein's contribution across the four light fluctuation sequences. For simplicity, only the average values (shown in the right column) were used to generate Figure 6 in the main text. Full distributions of the individual TCSPC and PAM data points are shown in Supporting Information: Figure 5.

contributions of each mechanism. In green microalgae, it is tempting to speculate that in nature, where exposure to HL and dark occur repeatedly, qE may play a more important role in the beginning of light fluctuations while qT may provide a photoprotective response on a longer timescale.

Although qI is known to contribute to NPQ during prolonged illumination, it is likely negligible in our experimental conditions since both *npq4lhcsr1* and *npq4stt7* mutants did not show a strong buildup of NPQ or NPQ<sub>T</sub> during the 20 min of HL exposure during the light fluctuations (Figures 2 and 5). Additionally, Fv/Fm were not significantly different between strains before light fluctuations, with the exception of *npq4lhcsr1* which showed a lower Fv/Fm (Supporting Information: Figure 9). Likewise, the *npq4lhcsr1* double mutant showed a shorter starting fluorescence lifetime (Figure 4a), which likely reflects some constitutive qI following overnight HL acclimation in the complete absence of LHCSR proteins.

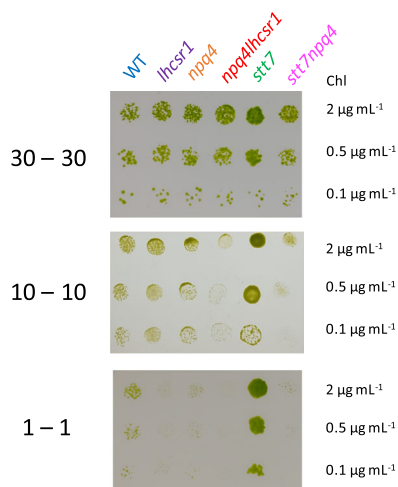
Interestingly, when comparing NPQ and NPQ<sub>T</sub>, the magnitude of the quenching decrease during HL was larger for NPQ<sub>T</sub> than NPQ

**FIGURE 7** Untangling qE and qT. The quantification of the contributions of LHCSR3 (orange) and STT7 (green) to the nonphotochemical quenching (NPQ) response both in the presence (solid lines, circle markers) and absence (dashed lines, triangle markers) of the other protein is plotted as a function of the light fluctuation period. Horizontal lines show the average contribution for each protein, considering all light fluctuation frequencies. For reference, the black lines represent zero contribution. The contribution of each protein was evaluated (a) over all 40min of the experiment, (b) only during HL periods, or (c) only during dark periods. All of these were evaluated from PAM trajectories using various combinations of WT along with the *npq4*, *stt7* and *stt7npq4* mutants as discussed in Section 2.**FIGURE 6** Quantification of the contribution of light-harvesting complex stress-related (LHCSRs) and STT7 to wild-type nonphotochemical quenching (NPQ) under fluctuating light. (a) Example of quantification of the relative NPQ mediated by LHCSRs. The area under the NPQ curve of *npq4lhcsr1* mutant (red) was subtracted from that of the control strain (blue) and expressed relative to the area of NPQ of the control strain. (b, c) Overall contribution of LHCSR3 (orange), LHCSR1 (purple) and STT7 (green) averaged over all 40 min (b) or specific periods of the light fluctuations (c). For each protein, the part of the donut shown in red, orange, purple or green is the percentage of wild-type NPQ lost in each mutant impaired in the accumulation of LHCSRs, LHCSR3, LHCSR1 and STT7, respectively. Given that the contribution of each protein was largely independent of HL/dark period (Supporting Information: Figure 6), shown are the average of all four light fluctuation sequences. Distribution of individual replicates and estimates of error (i) overall, (ii) during HL or dark and (iii) during early and late portion of light fluctuations are presented in Supporting Information: Figures 5, 6, and 7, respectively.

**TABLE 2** Average contribution of LHCSR3 and STT7 in the presence and absence of the other protein for each light fluctuation sequence

Overall contributions	1-1	2-2	4-4	10-10	Average
LHCSR3 w/STT7	78 ± 5%	75 ± 5%	67 ± 6%	66 ± 14%	72 ± 6%
LHCSR3 w/out STT7	61 ± 5%	67 ± 4%	58 ± 3%	61 ± 3%	62 ± 4%
STT7 w/LHCSR3	36 ± 4%	25 ± 11%	35 ± 8%	22 ± 9%	29 ± 7%
STT7 w/out LHCSR3	-16 ± 15%	2 ± 12%	16 ± 5%	9 ± 6%	2 ± 14%

Note: Shown is the average value (evaluated from three PAM replicates) and standard deviation of individual replicates. Each error in the right column represents the standard deviation of the four light fluctuation sequences. For simplicity, only the average values (shown in the right column) were used to generate Figure 7a in the main text.



**FIGURE 8** Growth of mutants impaired in qE and/or qT under various periods of dark/light cycles. *lhcsr1*, *npq4*, *npq4lhcsr1*, *stt7* and *stt7npq4* mutants and their control strain (WT) were diluted and spotted at different chlorophyll concentration and grown on plates under dark/light cycles with a period of 30 (30-30, upper panel), 10 (10-10, middle panel) or 1 min (1-1, lower panel). Each row represents a different chlorophyll concentration. Shown are representative spots of three biological replicates. Cells were grown for eight days. Growth under constant low light or high light are shown in Supporting Information: Figure 8. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

(Figure 2). The energetic requirement (and thus its proton gradient consumption) of the CCM depends on the inorganic carbon ( $C_i$ ) availability (Fei et al., 2022). Since the high cell concentration in the TCSPC sample leads to strong  $C_i$  consumption, this could deplete the  $C_i$  concentration even in the presence of bubbling, leading to a  $C_i$  level sensed by cells in the TCSPC sample being lower than what is experienced by cells in the PAM sample. This would lead to higher activity of CCM, and hence a larger decrease in quenching, under TCSPC sample conditions compared with PAM sample conditions. The decrease in both NPQ and NPQ<sub>T</sub> was also stronger for longer HL periods as well as later in the fluctuating light sequence (Figure 2). The slope of the initial decrease in NPQ<sub>T</sub> or NPQ during HL was similar for all four sequences (Supporting Information: Figure 10) and is likely dictated by  $C_i$  availability and its influence on the CCM kinetics. The magnitude of the decrease in NPQ and NPQ<sub>T</sub> was larger for longer light

periods (Supporting Information: Figure 10d,h), likely due to simultaneous activation of the CCM that dissipates the proton gradient and the onset of slower forms of NPQ such as state transitions. Conversely, the differences in the magnitude of the increase in NPQ and NPQ<sub>T</sub> during the dark periods could be due to differences in  $O_2$  concentrations sensed by the cells in both conditions, which is known to affect the extent and rate of state transition (Forti & Caldiroli, 2005).

Although LHCSR3 plays the dominant role in photoprotection under constant and fluctuating light conditions, we also observe a role for LHCSR1 in our measurements. While the chlorophyll fluorescence dynamics of *lhcsr1* are similar to those of WT (Figure 3), we observe a ~20% reduction in overall NPQ in this mutant under fluctuating light conditions (Figure 6). This small amount of photoprotection afforded by LHCSR1 in vivo is consistent with previous in vitro investigations (Dinc et al., 2016; Nawrocki et al., 2020) in which LHCSR1 has been suggested to mediate energy transfer between LHCII and PSI (Kosuge et al., 2018) and to compensate for the absence of LHCSR3 (Girolomoni et al., 2019). Interestingly, our results suggest that different from the case for LHCSR3, the qE that is mediated by LHCSR1 is largely independent of the timescale of light fluctuations (Supporting Information: Figure 5). Both LHCSR1 and LHCSR3 are thought to generate NPQ in response to (i) the proton gradient and (ii) carotenoid composition (Kondo et al., 2017; Troiano et al., 2021), thus the frequency-dependent LHCSR3 and frequency-independent LHCSR1 could differ in their dependence on the pH or carotenoid.

The relationship between NPQ capacities and growth have remained puzzling in *Chlamydomonas*, because only some light fluctuation regimes have consistently been shown to impair growth (Cantrell & Peers, 2017; Peers et al., 2009; Roach, 2020; Truong, 2011). Here we show that all mutants lacking LHCSRs showed impaired growth under rapid light fluctuations (1-1 sequence), and that this impairment was lower under slower fluctuations (10-10 sequence) and absent under an even slower 30-30 sequence or constant illumination (Figure 8 and Supporting Information: Figure 8). There seems to be a good relationship between defect of NPQ and growth deficiency under rapid fluctuations when considering *npq4lhcsr1* and *stt7npq4* mutants (Figure 8), clearly showing that LHCSR-dependent qE is critical for growth when light fluctuates within a short period of time. However, surprisingly, the growth defect of *lhcsr1* seemed larger than *npq4* under the 1-1 sequence. This suggests that the growth capacity of LHCSR mutants under light fluctuations may not depend only on the level of NPQ. The growth defects observed for

qE mutants under short timescale light fluctuations may be affected by the deactivation of the Calvin–Benson–Basham (CBB) cycle during the dark phases, as previously proposed by (Roach, 2020). Such a frequency dependence for the growth may thus additionally reveal the specific time scales of CBB cycle activation and deactivation during extended periods of fluctuating light. Other factors may include the activation of compensatory mechanisms that enable photoprotection at the expense of growth or the increased production of reactive oxygen species in *npq4* mutants (Barera et al., 2021; Roach et al., 2020), which could be greater in the *lhscr1* mutant. However, the NPQ dynamics may be different for cells grown on plates than in liquid medium due to differences in nutrient availability such as nitrogen or CO<sub>2</sub>. These differences would limit the validity of our conclusions regarding the growth outcomes under fluctuating light. Note here that in our conditions, due to slightly different genetic background between *stt7* and the WT control, we cannot make a conclusion on the mechanism by which *stt7* grew better under rapid timescale fluctuations (Figure 8). The WT background may be particularly sensitive to fast 1–1 and 10–10 fluctuations; another possibility could be that qT is detrimental for growth under medium to rapid timescale fluctuations.

Through 77 K fluorescence emission spectra analysis, we have shown that the increasing dissipation observed in the dark requires the STT7 kinase responsible for state transition (Figure 4 and Supporting Information: Figure 2). This effect, already described in *Chlamydomonas* (Allorent et al., 2013) and *Dunaliella salina* (Casper-Lindley & Björkman, 1996), greatly contributes to NPQ during light fluctuations. In plants, the occurrence of state transitions and its involvement in NPQ is thought to be minor (Allen, 1992; Minagawa, 2011) even if mutants of *Arabidopsis thaliana* impaired in state transition (*stn7*) exhibit impaired growth under light fluctuations (Bellafiore et al., 2005). Interestingly, an increase of NPQ during the dark periods of fluctuating light was recently reported in *npq4* leaves of *Arabidopsis thaliana* (Steen et al., 2020) for which about 53% of the WT NPQ<sub>T</sub> remained in the mutant after 40 min of exposure to light fluctuations despite the absence of the pH-sensing protein PsbS (Supporting Information: Figure 11). However, it remains unclear as to how much of the dark quenching in plants originates from qT as opposed to effects related to de-epoxidized xanthophyll pigments (Steen et al., 2020) or LHC protein conformation and/or aggregation (Goral et al., 2012). In the future, periodic illumination experiments performed on *A. thaliana* mutants impaired in qE and/or qT could clarify the relative importance of each mechanism and allow a comparison of the in vivo functioning of NPQ in higher plants and green algae under light fluctuations.

Tuning the relaxation kinetics of NPQ in higher plants has been shown to improve crop plant productivity (Kromdijk et al., 2016), and recent modelling of the response of plant canopies to natural light fluctuations has shown that there remains ample room for improving photosynthetic efficiency under nonsteady-state conditions (Wang et al., 2020). Similar opportunities for improvement also exist for increasing biofuel production from microalgae (Benedetti et al., 2018; Perin & Jones, 2019; Vecchi et al., 2020). In both cases, such optimization will require detailed understanding of the dynamic activity of NPQ mechanisms. The sum of the NPQ contributions of each protein is greater

than 100% (Figure 6) which cannot be explained by a difference in the amount of LHCSR3 between WT and *stt7* (Supporting Information: Figure 12). Additionally, our quantifications of qE and qT reveal that each of them depends on the presence of the other process (Figure 7), showing that there is an interaction between qE and qT in *Chlamydomonas* under fluctuating light. The possibility of an interaction between qE and qT has been previously suggested on the basis of a kinetic analysis of qT in the presence and absence of LHCSR3 protein (*npq4* mutant) (Roach & Na, 2017). Our findings are consistent with a partial overlap of the functions of LHCSR3 and STT7 in both qE and qT. This partial overlap highlights the need for further investigations of the interactions occurring between proteins that underlie the in vivo NPQ response, not only in HL but also in the dark, and more generally during light fluctuations. For example, in microalgae, the quenching mediated by LHCSR3 both at PSII and PSI levels (Girolomoni et al., 2019) could be tuned by the movement of LHCSR3 from PSI to PSII during state transitions (Allorent et al., 2013). Thus, the described LHCSR3 phosphorylation by STT7 (Bergner et al., 2015) could explain the crosstalk between qE and qT. LHCSR3 is also known to associate with LHCII trimers in the PSII supercomplex (Semchonok et al., 2017); therefore, a similar LHCSR3–LHCII interaction may also generate quenching in the trimers following the detachment of LHCII from PSII. It should be noted that in the thylakoid membrane, LHCII can exist in a range of different conformations and/or quenching states (Kawakami et al., 2019; Tian et al., 2015). At this point, it is not possible to distinguish the relative contributions of different forms of LHCII in individual snapshot measurements. The ensemble fluorescence lifetime likely originates from some combination of at least three LHCII subpopulations: unphosphorylated and bound to PSII (State 1) (Drop et al., 2014) with an intermediate fluorescence decay component, phosphorylated and unbound (free LHCII) (Iwai, Yokono, et al., 2010) which has been previously assigned to a long fluorescence decay component (Ünlü et al., 2014), and phosphorylated and bound to PSI (State 2) (Huang et al., 2021) with a short fluorescence decay component. In intact algal cells, the relative abundance of each form of LHCII likely dynamically evolves throughout exposure to the fluctuating HL–dark sequences. Further development of in vivo spectroscopic tools will be required to disentangle the dynamics of LHCII conformations and correlate them with photoprotection. Overall, a deeper understanding of the protein interactions underlying NPQ dynamics will be highly valuable in finding new ways to improve plant and microalgal productivity.

## 5 | CONCLUSIONS

LHCSR- and STT7-mediated NPQ processes (qE and qT) are known to underly the photoprotective response of the microalga *Chlamydomonas*. Here, we have applied a new method to disentangle the involvement of qE and qT in real time by exposing intact algal cells to repetitive cycles of high light and darkness alternating at different timescales. While both qE- and qT-type responses are present during all light fluctuations, LHCSR-dependent qE plays a larger role in the beginning of light fluctuations and during the HL periods. The contribution of STT7-dependent qT became



more pronounced upon longer exposure to fluctuating light and especially during the dark periods of light fluctuations. Over the long term, rapid light fluctuations reduced the growth of mutants impaired in LHCSR3, demonstrating the importance of LHCSR proteins during abrupt changes in light intensity. Overall, our work shows that a cooperativity between LHCSR proteins and STT7 constitutes an important regulatory feature of the photoprotective response in *Chlamydomonas*, likely mediated by phosphorylation of LHCSR3 by STT7. These findings provide a foundation for disentangling and modelling how the diverse molecular mechanisms involved in plant and microalgal acclimation to light fluctuations interact and enable robust photosynthesis in nature. We envision that further knowledge of the response of photosynthetic mechanisms to various timescales of light fluctuations will open new avenues for building a strong understanding of how photosynthetic organisms respond to complex light fluctuations.

#### AUTHOR CONTRIBUTIONS

Collin J. Steen, Adrien Burlacot and Graham R. Fleming designed the research; Collin J. Steen, Adrien Burlacot and Audrey H. Short performed research; Collin J. Steen, Adrien Burlacot, Audrey H. Short and Graham R. Fleming analyzed data; Collin J. Steen and Adrien Burlacot wrote the paper with inputs from Audrey H. Short, Krishna K. Niyogi and Graham R. Fleming.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supporting Information Materials.

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## SUPPORTING INFORMATION

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