Sunlight drives photosynthesis but can also cause photodamage. To protect themselves, photosynthetic organisms dissipate the excess absorbed energy as heat, in a process known as nonphotochemical quenching (NPQ). In green algae, diatoms, and mosses, NPQ depends on the light-harvesting complex stress-related (LHCSR) proteins. Here we investigated NPQ in *Chlamydomonas reinhardtii* using an approach that maintains the cells in a stable quenched state. We show that in the presence of LHCSR3, all of the photosystem (PS) II complexes are quenched and the LHCS are the site of quenching, which occurs at a rate of ~150 ps$^{-1}$ and is not induced by LHCCI aggregation. The effective light-harvesting capacity of PSII decreases upon NPQ, and the NPQ rate is independent of the redox state of the reaction center. Finally, we could measure the pH dependence of NPQ, showing that the luminal pH is always above 5.5 in vivo and highlighting the role of LHCSR3 as an ultrasensitive pH sensor.

**Significance**

Photosynthetic organisms utilize sunlight as a form of energy. Under low light, they maximize their capacity to harvest photons; however, under excess light, they dissipate part of the harvested energy to prevent photodamage, at the expense of light-use efficiency. Optimally balancing light harvesting and energy dissipation in natural (fluctuating light) conditions is considered a target for improving the productivity of both algae and plants. Here we have studied the energy dissipation process in the green alga *Chlamydomonas reinhardtii* in vivo. We found that it is remarkably different from that of higher plants, highlighting the need of developing tailor-made strategies to optimize the light harvesting–energy dissipation balance in different organisms.

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1To whom correspondence may be addressed. Email: l.tian@vu.nl or r.croce@vu.nl.

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Acid are indicated by red dots.

Acid-induced NPQ and Its pH Dependence. The most frequently used medium to grow *C. reinhardtii* is TAP (Tris-acetate-phosphate) (19), which contains 17.4 mM acetic acid that serves as a source of carbon and energy. However, acetic acid was also proven to induce quenching when added to the cell solution (9, 10, 20). This approach was employed in this work to lock the cells in their quenched state. A state transition-deficient mutant, stt7-9 (21), was used, which permits us to separate the effect of NPQ from that of state transitions. Indeed, upon addition of acetic acid, the chlorophyll (Chl) fluorescence of *C. reinhardtii* decreases in a few seconds and fully recovers by titrating the pH back to 7.0 (Fig. L4). The high sensitivity of Chl fluorescence to environmental pH allows us to explore the pH dependence of the NPQ process; note that the ionophore nigericin was added during the pH titration to ensure complete pH equilibrium within the cells (22). The pH titration curve (Fig. 1B) shows that the fluorescence quenching starts already at pH values around 6.7 and saturates at pH 5.5, well above the suggested cytoplasmic pH <5 (23). In higher plants, zeaxanthin (Zea) has been reported to significantly affect the pH dependence of LHII fluorescence quenching (24). This is not the case in *C. reinhardtii*, as the level of NPQ in high light (HL)-grown cells, which contain Zea [Zea/violaxanthin (Vio) ratio of 0.41 ± 0.07; Z++], and in dark-adapted cells, in which the amount of Zea is reduced by 75% (Zea/Vio ratio of 0.10 ± 0.01; Z−), is similar and shows an identical pH dependence (Fig. 1B).

In higher plants, it was shown that without PSB5, the LHC antennae can still be quenched by low luminal pH (25), and thus it is essential to verify whether the acetic acid-induced quenching is LHCSR-dependent. To do so, we compared the NPQ levels induced by either high light or acetic acid in cells with different amounts of LHCSRs. In wild-type *C. reinhardtii*, the expression level of LHCSR1 and LHCSR3 changes dramatically under different growth conditions, leading to different NPQ levels (26–28). The immunoblot results in Fig. 1C and D show that this is also the case in the stt7-9 mutant. We found that the NPQ levels caused by acetic acid positively correlate with the amount of LHCSRs (Fig. 1E), suggesting that it is indeed LHCSR-dependent. Note that PsbS is hardly expressed in the cells (SI Appendix, Fig. S1A) and does not trigger a significant amount of NPQ either, as tested by using a double mutant of npq4/stt7-9 (SI Appendix, Figs. S5 and S6 and more discussion in SI Appendix).

A question that follows is whether the acid-induced and the high light-induced fluorescence quenching mechanisms are the same. Fig. 1E also shows that the NPQ levels induced by HL and acetic acid are very similar, showing the same dependence on LHCSRs. The similarity is also observed in the low-temperature time- and spectrally resolved fluorescence kinetics (SI Appendix, Figs. S6 and S7 and Table S1). This indicates that the quenching induced by HL and by acetic acid proceeds via the same mechanism. Therefore, acetic acid can be used to study NPQ at a physiologically relevant temperature.

**Results**

**Acetic Acid-Induced NPQ and Its pH Dependence.**}
measurement, as it offers several advantages: (i) no distortion of the kinetics from any intermediate NPQ state; (ii) stable luminal pH; and (iii) absence of PSI heterogeneity, as all of the measurements can be done with closed PSII RCs. Indeed, independent of the state [unquenched (UQ) or quenched (Q)], all of the PSII RCs are closed by preilluminating the cells in the presence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethyleurea] and HA (hydroxylamine) (29). It should be emphasized that this is possible for cells in the quenched state, only thanks to the use of acid. In the case of HL-induced quenching, DCMU and HA would block the linear electron flow, inhibiting the lumen acidification and consequently NPQ.

A dramatic shortening of the fluorescence decay at pH 5.5 compared with 7.0 is directly visible from the streak-camera images (Fig. 2A and B and SI Appendix, Fig. S8), as well as from the fluorescence decay curves at 683 nm (Fig. 2C). Reconstructed steady-state spectra (Fig. 2D) show that the fluorescence quantum yield drops by nearly 80% upon quenching. It corresponds to an NPQ value of 4.0, slightly higher than the value of 3.5 obtained with pulse-amplitude modulation (PAM), which can be explained by the presence of the PSI contribution in the PAM signal (30).

**Target Analysis of Time-Resolved Fluorescence Data.** The data of UQ and Q cells upon 400- and 475-nm excitations were fitted simultaneously with a four-compartment model (see fitting qualities in SI Appendix, Fig. S9), in which all of the spectra, energy inputs at time 0, and rate constants are free parameters. In the model, one compartment represents PSI while three were used for PSII (Fig. 3A). The PSI compartment (black in Fig. 3A) has the typical PSI spectrum with a maximum at 690 nm [and emission above 700 nm (∼31% of the energy upon 400-nm excitation and ∼70% at 475-nm excitation. A second compartment (blue in Fig. 3A) receives 15.4 and 11.9% of the initial excitation at 400 and 475 nm, respectively. Note that the spectra of PSII-Ant and PSII-core were successfully separated at room temperature. These two compartments reach an energetic equilibrium within 15 ps after excitation (see calculated lifetimes and their decay-associated spectra in SI Appendix, Fig. S10), and then the PSII-core transfers an electron to a nonemitting radical pair (RP; white in Fig. 3A), from where the electron is further transferred down or recombines. For the description of the quenched sample, initially we had tried to associate quenching with each compartment except the RP, but we found that the quenching on PSI-Ant. dominates (SI Appendix, Fig. S11), meaning that it is taking place on the antenna. Indeed, the fitting quality of the model in which only PSI-Ant. is quenched remains the same. There is no need to include heterogeneity of PSII in the model to reach a satisfactory fit, meaning that all individual PSII supercomplexes are protected by NPQ. The quenching rate is estimated to be ∼150 ps−1, and the shape of the spectrum of PSI-Ant. remains unchanged (Fig. 3B, Inset) during the NPQ process. In particular, no new species with different spectra are required to fit the fluorescence decay curves of the quenched cells. This conclusion is further supported by the low-temperature time-resolved fluorescence data (see SI Appendix, Figs. S12–S14 and the fitted curves in SI Appendix, Figs. S15–S18), where the differences in spectral shape, if present, are often enhanced.

**How Does the NPQ Process Affect PSII Functional Antenna Size in Vivo?** The approach of acid-induced quenching is well-suited for estimating the functional effect of NPQ on the PSII electron transfer rate, as the acid keeps the cells in a constant quenched state without the necessity of sample illumination and thus light-induced RC closure. This permitted us to measure the functional antenna size of PSII upon NPQ in vivo by fluorescence induction in the presence of DCMU (33) (Methods and SI Appendix, Text 2): The comparison of the fluorescence value at the plateau in the presence and absence of acid represents the NPQ value (F_M/F_S; Fig. 4B), while the area above the normalized induction curve (Fig. 4A) represents the functional antenna size (see SI Appendix, Text 2 for more details). Since the fluorescence

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**Fig. 2.** Time-resolved fluorescence data at room temperature at 475-nm excitation. (A–C) Streak-cam images of intact cells in the unquenched state, UQ (A), and quenched state, Q (B); their fluorescence decay curves at 683 nm are shown (C). (D) Reconstructed steady-state spectra of UQ and Q cells.

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**Fig. 3.** Target analysis results. (A) Schematic model used to describe the time-resolved fluorescence data; rates are given in ns−1. k_q is the rate of quenching, and asterisks indicate the initial excitations at 400 nm (black) and 475 nm (gray). (B) Species-associated spectra (SASs) of the compartments with the same colors as used in A. (B, Inset) Normalized spectra of the UQ_Ant. and Q_Ant. are shown.
induction curve in the presence of DCMU provides the PSII rate before the RC closes ($F_{M}$), it gives access to information about the competition between NPQ and photochemistry. Our data show a linear decrease in functional antenna size concomitant with increasing NPQ (Fig. 4C), yet to a significantly lower extent than suggested by the NPQ value, demonstrating that NPQ competes for the excitation with the photochemical traps.

Finally, we investigated the slope of the dependence of the functional antenna size vs. NPQ to verify whether the rate of NPQ, $k_{NPQ}$, remains similar with open ($O_{L}$) and closed ($O_{L}^{-}$) RCs. Within the precision of our measurements, the data closely follow the theoretical scenario where $k_{NPQ}$ is identical with open and closed RCs (Fig. 4C), resulting in a different value of NPQ in the two situations. This indicates that in *Chlamydomonas*, NPQ decreases the functional antenna size of PSII, and that its rate does not depend on the redox state of the PSII RC.

**Discussion**

**The Dynamic Range of NPQ in *Chlamydomonas* is 1.2 pH Units.** Our NPQ titration curve shows that the quenching is activated at pH 6.7 and saturates at pH 5.5, meaning that it only covers ~1.2 pH units, whereas for higher plants it was reported to attain ~4 pH units (25). This difference is most likely related to the different properties of the pH sensors in plants and green algae, PsbS and LHCSR, respectively. The midpoint of the pH titration curve is at about 6.25, which indicates that the pKₐ of the pH-sensing residues must be close to neutral. For LHCSR3, it has been shown that the protonation of Asp and Glu residues at its C terminus triggers quenching (12, 34). Since these residues have a side chain with a pKₐ as low as ~4.0 in water, the present results suggest that the environment strongly modulates their pKₐ. In higher plants, it is zeaxanthin that modulates the pH sensitivity of NPQ (24, 25). This is not the case in *C. reinhardtii*, since we do not observe any influence of zeaxanthin on the NPQ response to pH. The lack of a zeaxanthin effect on NPQ agrees with the observation that a Zea-lacking mutant of *C. reinhardtii* has a similar NPQ level as wild-type *C. reinhardtii* (8).

**NPQ as pH Probe in Vivo: The pH of the Lumen Does Not Go Below 5.5.** The value of the luminal pH is a matter of debate, as its determination is technically challenging (23). Several approaches have been developed that either use pH-sensitive dyes (25) or pH-sensitive GFps (35). However, these approaches require labeling, which is often complicated for in vivo systems. Here, we show that the LHCSR-dependent NPQ can be used as a natural sensor to determine the pH of the lumen. Using the pH calibration curve and the value of NPQ obtained on the same cells after high light exposure, we can conclude that the pH of the lumen does not go below 5.5, even in very high light conditions (1,000 μmol photons m⁻² s⁻¹). Indeed, the NPQ level observed upon HL exposure is always a bit lower than the value obtained with acetic acid at pH 5.5. This result is in agreement with a previous proposal that the luminal pH does not go below 5, as lower values would damage the Mn cluster in PSII (36).

**Quenching Site, Rate, and Mechanism.** Electron microscopy on isolated PSII–LHCII–LHCSR3 supercomplexes has shown that LHCSR3 binds to the LHCs at different positions (37). In agreement with this, fluorescence upconversion experiments on isolated supercomplexes have suggested that both LHCSR (38) and LHCSR3 quenches LHCs and consequently the excitation energy within the PSII supercomplex (38). Our data indicate that this is also happening in vivo. Based on our target model (Fig. 3), LHCSR directly binds and quenches the PSII-Ant. and concomitantly the PSII-core but not PSI. The quenching rate at physiological temperature is estimated to be ~150 ps⁻¹, which is surprisingly fast considering that there are almost 200 Chls in a supercomplex (~4.6 LHCCI trimers per RC according to our estimations; see SI Appendix for details). With this rate, the quenching is very evident when the PSI reaction center is running at high-capacity, in agreement with our observation that $F_{M}$ was severely quenched (Fig. 4A) and that the light-limited charge separation rate of PSII decreases upon quenching (Fig. 4). A much faster quenching rate of 18 ps⁻¹ has been reported by Kim et al. (38) for isolated supercomplexes. However, since there is no energy equilibrium between the two compartments implemented in their model, this quenching rate is dramatically overestimated. Moreover, in their measurements, the open/closed state of PSII, which severely influences the kinetics, is not defined. In contrast, PSII in our measurements are fully closed in both UQ and Q states. This maximizes the time the excitation spends on the antenna, so that we could precisely fit both the energy equilibrium within PSII and the quenching rate.

Importantly, because of the high spectral resolution of our data, we could show that there is not a spectral signature associated with NPQ at both room temperature (Fig. 3) and 77 K (SI Appendix, Figs. S12–S14). This conclusion is further supported by the indistinguishability of the spectral shape of UQ and Q in the original data after 100 ps, when the PSI contribution is minimized (see also the time-gated spectra in SI Appendix, Fig. S19).}

This is an important observation since LHCCI aggregates, which have a specific spectral feature even at room temperature (39, 40), serve as a well-received model for NPQ in higher plants. In contrast, PSIIs in isolated supercomplexes have a broad spectrum. This is an important observation since LHCCI aggregates, which have a specific spectral feature even at room temperature (39, 40), serve as a well-received model for NPQ in higher plants. In contrast, PSIIs in isolated supercomplexes have a broad spectrum. This is an important observation since LHCCI aggregates, which have a specific spectral feature even at room temperature (39, 40), serve as a well-received model for NPQ in higher plants. In contrast, PSIIs in isolated supercomplexes have a broad spectrum. This is an important observation since LHCCI aggregates, which have a specific spectral feature even at room temperature (39, 40), serve as a well-received model for NPQ in higher plants. In contrast, PSIIs in isolated supercomplexes have a broad spectrum.
The Effects of NPQ on PSII Light Harvesting. It was recently concluded that upon NPQ in plant thylakoids, the functional antenna size of PSII increases (44). Here we demonstrate that this is not the case in C. reinhardtii cells, for which we observed that the NPO induces a decrease of the PSII functional antenna size (Fig. 4). Moreover, we show that this decrease follows the expected value in the scenario where NPO competes with photochemical quenching without any change in the physical antenna size. Interestingly, our results also indicate that the rate of quenching in C. reinhardtii is identical at F₀ and at F₆₉₀, although small changes of kNPO on the order of ~10% could not be excluded. This is again different from what was recently observed in higher plants, where the quenching rate was found to be lower in the case of open RCs (45). The lack of the Qₐ redox state-dependent NPO regulation in C. reinhardtii results in a larger loss of energy in the presence of the open reaction center than in plants, meaning that NPO lowers the quantum yield of photochemistry.

Conclusions: Plants vs. Green Algae. Our results show that substantial differences in the mechanism of NPO exist between C. reinhardtii and vascular plants. It is likely that the mode of quenching, LHCSR- vs. PSBS-dependent, is the reason for these differences. Indeed, while LHCSR is a pigment-binding protein (8), this does not seem to be the case for PSBS (46), implying that the former can in principle directly act as a quencher while the latter cannot. This also means that while in C. reinhardtii the quenching rate depending on the redox state of the reaction center, is lacking. Taken together, our results suggest that the protective mechanism which does not require the aggregation of LHCSR-dependent quenching is an on-site and solely pH-driven process is far from identical. In green algae, LHCII aggregation is absent and the additional mechanism, which regulates the presence of open reaction centers under NPQ conditions. Interestingly, our results also indicate that the rate of quenching in accordance with it, as observed in plants (45). It is again different from what was recently observed in higher plants, meaning that NPO lowers the quantum yield of photochemistry.

Methods

Cell Growth Conditions. To eliminate the effects of antenna redistribution and focus exclusively on NPO, we have used the C. reinhardtii stt7-9 mutant, which is impaired in state transitions (21). Cells were grown under three different light/carbon supply conditions: high-light photosynthetic growth in high-salt medium (HSM) (51) (~500 μmol photons m⁻² s⁻¹) or in the presence of 5% CO₂ and normal-light mixotrophic growth in TAP (~50 μmol photons m⁻² s⁻¹) as previously described (26). The light source was made of fluorescence tubes (Philips; MASTER PLL-55W/840), the spectrum of which is shown in SI Appendix, Fig. S2.

Pigment Analysis. Pigments were extracted from the cells with 80% acetone. The relative amount of carotenoids was determined by HPLC as described (48).

Fluorescence Measurements. High light-adapted cells naturally express zeaxanthin; they were used throughout this work for all fluorescence measurements unless stated otherwise. To check the role of zeaxanthin in NPO, HL-grown cells were kept in the dark in the growing medium for ~3 h. Cells with/without zeaxanthin were pelleted down and resuspended in HSM before further experiments. Saturating pulse (2,000 μmol photons m⁻² s⁻¹, 250 ms) and actinic light (1,000 μmol photons m⁻² s⁻¹), both peaking at 630 nm, and a blue measuring beam peaking at 430 nm were used for all of the measurements, and were provided by the Dual-PAM-100 (Wals) flurometer.

Acetic Acid-Induced Chlorophyll Fluorescence Quenching. Cells were washed with fresh HSM (pH 6.9). For Fig. 1A, 4.5 mM acetic acid (1 M stock) was added to decrease the pH to 5.5, and KOH was used to adjust the pH back to 7.0. For the pH titrations in Fig. 1, different amounts of acetic acid were used together with 100 μM nigericin. pH values were recorded directly in the PAM cuvette by a microelectrode (pH-500; Unisense), while NPO values were measured by the Dual-PAM.

Immunoblots and Total Protein Extract Preparation. Total protein extract (TPE) was performed as described (26). Immunoblot analyses were performed as described (10); 10 and 2.5 μg of TPEs were loaded for the determination of LHCSR1 and LHCSR3, respectively. CP43 was used as loading control, and the CP43 antibody was used together with the other antibodies to evaluate the amount of LHCSR per sample. We used LHCSR3 and LHCSR1 apoprotein overexpressed in Escherichia coli as in refs. 10, 12, and 52. Both LHCSR1- and LHCSR3-overexpressed apoproteins have a His tag at the C terminus, which explains their different mobility in SDS/PAGE compared with the WT proteins. All antibodies used were from Agrisera, but the anti-PsbS was a gift from Stefano Caffarri, Université Aix-Marseille, St. Paul Les Durance, France (53).

Isolation of PSI Complexes. Photosystem I was purified as in ref. 54, and the isolated complex was used to estimate the PSII spectral shapes in a simultaneous target analysis of the 77 K data.

Time-Resolved Fluorescence. Measurements were performed with a sub-picosecond streak-camera setup (details in ref. 55) which combines a femtosecond laser source (Coherent Vitesse Duo + regenerative amplifier Coherent RegA 9000 + optical parametric amplifier Coherent QPA 9400) with a streak-camera detecting system (Hamamatsu; CS680). Fluorescence emission was collected at a right angle to the incident beam by a spectrograph (Chromex; 2505S; 50 grooves per mm ruling, blaze wavelength 600 nm, spectral width 260 nm), with the central wavelength set at 720 nm. Scattered excitation light was removed with an optical long-pass filter.

Two excitation wavelengths were used, 400 and 475 nm; the former more or less equally excites Chl a and b, and the latter selectively excites Chl b so that more antennae are initially excited. The laser beam was focused to a small spot of ~50-μm diameter, and laser power was set to 15 μW with a repetition rate of 250 kHz. Cells were pelleted down, resuspended in fresh HSM, and directly measured in a 10- by 10-mm quartz cuvette at room temperature. Magnetic stirring (~25 Hz) was used to prevent cell sedimentation. Care was taken to minimize the optical path length (<1 mm) to allow measurements on highly concentrated samples (OD₅₇₀ nm 3) without significant self-absorption. Note that, to keep the cells in a fully quenched state, 4.5 mM acetic acid was added and, to ensure the PSI RQs were in a homogeneous closed state, 20 μM DCMU and 1 mM HA were added for both UQ and Q states. For each fluorescence measurement, a high signal/noise ratio was achieved by averaging a sequence of 200 single images with a CCD exposure time of 10 s for each. All images were background- and shading-corrected before global and target analysis. The laser did not cause damage or actinic effects, as no change in fluorescence decay curves was observed after continuous laser illumination for 30 min; see typical examples in SI Appendix, Fig. S20.

Simultaneous Target Analysis. Data obtained with the streak-camera setup were globally analyzed with the R package TIMP-based Glotaran (56). The data were fitted with a kinetic model (so-called target analysis). For details of the methodology of target analysis, see ref. 57. With this approach, the spectrum of each resolved species and the energy transfer and quenching rates were estimated.

Determination of the Effect of Quenching on the Functional Antenna Size of PSI in Vivo. The measurements of the light-limited rate of PSI electron transfer (indicated below as the functional antenna size) were conducted measuring the...
fluorescence rise in the presence of DCMU. The JTS-10 spectrophotometer (Bio-
Logic) was used for all of the measurements in “fluorescence” mode, as described in ref. 58. A detailed description of the experiments and data analysis is provided in SI Appendix, Figs. S21 and S22.

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