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Non-photochemical quenching of chlorophyll *a* fluorescence: early history and characterization of two xanthophyll-cycle mutants of *Chlamydomonas reinhardtii*

Govindjee[B] *and Manfredo J. Seufferheld*[A]

Department of Biochemistry and Center for Biophysics and Computational Biology,
University of Illinois, Urbana, IL 61801, USA.
[A]Current address: Laboratory of Molecular Parasitology, Department of Pathobiology,
University of Illinois, Urbana, IL 61802, USA.
[B]Corresponding author; email: gov@uiuc.edu

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'Those who cannot manage to look from many viewpoints sometimes attribute to one entire object what actually belongs only to the little they are aware of. The neatness of their ideas hinders them from being suspicious.'
Marquis de Vauvenargues

Abstract. This paper deals first with the early, although incomplete, history of photoinhibition, of 'non-Q_A-related chlorophyll (Chl) *a* fluorescence changes', and the xanthophyll cycle that preceded the discovery of the correlation between non-photochemical quenching of Chl *a* fluorescence (NPQ) and conversion of violaxanthin to zeaxanthin. It includes the crucial observation that the fluorescence intensity quenching, when plants are exposed to excess light, is indeed due to a change in the quantum yield of fluorescence. The history ends with a novel turn in the direction of research — isolation and characterization of NPQ xanthophyll-cycle mutants of *Chlamydomonas reinhardtii* Dangeard and *Arabidopsis thaliana* (L.) Heynh., blocked in conversion of violaxanthin to zeaxanthin, and zeaxanthin to violaxanthin, respectively. In the second part of the paper, we extend the characterization of two of these mutants (*npq1*, which accumulates violaxanthin, and *npq2*, which accumulates zeaxanthin) through parallel measurements on growth, and several assays of PSII function: oxygen evolution, Chl *a* fluorescence transient (the Kautsky effect), the two-electron gate function of PSII, the back reactions around PSII, and measurements of NPQ by pulse-amplitude modulation (PAM 2000) fluorimeter. We show that, in the *npq2* mutant, Chl *a* fluorescence is quenched both in the absence and presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). However, no differences are observed in functioning of the electron-acceptor side of PSII — both the two-electron gate and the back reactions are unchanged. In addition, the role of protons in fluorescence quenching during the 'P-to-S' fluorescence transient was confirmed by the effect of nigericin in decreasing this quenching effect. Also, the absence of zeaxanthin in the *npq1* mutant leads to reduced oxygen evolution at high light intensity, suggesting another protective role of this carotenoid. The available data not only support the current model of NPQ that includes roles for both pH and the xanthophylls, but also are consistent with additional protective roles of zeaxanthin. However, this paper emphasizes that we still lack sufficient understanding of the different parts of NPQ, and that the precise mechanisms of photoprotection in the alga *Chlamydomonas* may not be the same as those in higher plants.

Keywords: chlorophyll *a* fluorescence, fluorescence transient (Kautsky effect), history of non-Q_A-related chlorophyll *a* fluorescence changes, *npq1* mutant, *npq2* mutant, violaxanthin, xanthophyll cycle, zeaxanthin.

Abbreviations used: Chl, chlorophyll; Chl**a*, excited state of Chl *a*; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; F_m, maximal Chl *a* fluorescence level in dark-adapted samples; F_m', maximal fluorescence level during continuous illumination; F_o, minimal fluorescence level at the beginning of illumination; HS, high salt; k_h, rate constant of heat loss; LED, light-emitting diode; NPQ, non-photochemical quenching of Chl *a* fluorescence; OD, optical density; O–P–S–M–T fluorescence transient, origin–peak–semi-steady-state–maximum–terminal-steady-state fluorescence transient; PEA, plant efficiency analyser; PMS, phenazine methosulfate; Q_A, first quinone acceptor of PSII (a one-electron acceptor); Q_B, second quinone acceptor of PSII (a two-electron acceptor); Q_A^-, singly-reduced Q_A; Q_B^-, semiquinone (singly reduced Q_B); QH, reduced and protonated quinone; *q*E, high-energy-related quenching of fluorescence; S_1, stable redox state of the manganese complex; S_2, S-state with one more positive charge than S_1; TAP, Tris–acetate phosphate; wt, wild type.

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Introduction

Photosynthesis is driven by light (see summary of the process in Whitmarsh and Govindjee 1999; Ke 2001; Blankenship 2002). However, in light that is in excess of the capacity of photosynthesis, the phenomena of photoinhibition and photoprotection are observed. There is a trade-off between photosynthetic efficiency and photoprotection mechanisms. Plants cope with the onslaught of excess light in various ways, including increased wax formation and/or hairs on leaves, reorientation of leaves, chloroplast movement away from the direction of light, quenching of excitation energy through formation of antheraxanthin and zeaxanthin from violaxanthin, repair of D1 protein, production of free-radical scavenging components including α-tocopherol, cycling of electrons around PSI and PSII, and increased photorespiration (see reviews by Robinson *et al.* 1993; Chow 1994; Long *et al.* 1994; Osmond and Grace 1995; Baker 1996; Demmig-Adams *et al.* 1996; Horton *et al.* 1996, 1999; Kramer and Crofts 1996; Gilmore 1997; Anderson *et al.* 1998; Andersson and Aro 1999; Frank *et al.* 1999; Gilmore and Govindjee 1999; Niyogi 1999). Our paper is divided into two parts: (i) early history of photoinhibition, and the relationship between photosynthesis, fluorescence, and 'heat loss' or NPQ; and (ii) characterization of two NPQ xanthophyll-cycle mutants of *C. reinhardtii* (Niyogi *et al.* 1997*a*).

Part 1. Early history

Light energy is converted into chemical energy by oxygenic photosynthetic organisms, producing food and oxygen. Light (in the form of photons) is a reactant in the following process:

$$CO_2 + 2H_2O + 8\text{–}12 \text{ photons} \rightarrow O_2 + \{CH_2O\},$$

where $\{CH_2O\}$ represents a carbohydrate moiety. For the history of the evolution of this equation, see Rabinowitch (1945).

Photons are absorbed by antenna Chl *a* molecules. These excited Chl *a* molecules (Chl**a*) have three major pathways via which absorbed energy is lost: (i) excitation energy transfer to reaction centre Chl *a* molecules leading to photochemistry (charge separation: production of oxidized reaction centre Chl *a* and a reduced electron acceptor); (ii) light emission (prompt fluorescence); and (iii) internal conversion (heat loss). Chl *a* fluorescence *in vivo* is low mainly because of photochemistry. This decrease in fluorescence is called 'photochemical quenching'. However, if a decrease in fluorescence occurs because of increase in heat loss, it is called 'non-photochemical quenching'. For a history of Chl fluorescence, see Govindjee (1995).

Discovery of 'photoinhibition': a 1940 story of the PhD thesis of Jack Myers

Myers (1996) has described his very first experiments on photosynthesis and the observation of 'photoinhibition' in the following words: 'Cranking up for my first real experiment was an exciting day. Carefully pipette a cell sample into the Warburg vessel and let it come to temperature in darkness. Then turn in the projection lamp to give a bright light spot already measured at 38000 foot-candles, almost 4 times as bright as sunlight.… That first experiment was a complete bust. There was only a short burst of the increasing pressure I expected. Thereafter, the pressure change became negative in evidence of oxygen uptake. Something was wrong. So I repeated the procedure with the same result. Only when the intensity was much reduced (1000 foot-candles, by wire screens) did I see the expected high and steady rate of oxygen evolution. Though it took a lot of confirming and polishing experiments, that was an exciting day in the life of an young photosynthetiker. I had made a discovery. I knew something unknown to anyone else in the world. That had been my romantic vision of the fruit of research. And it has not changed in the sixty years since.' This experiment was published by Myers and Burr (1940) — the discovery of quenching of photosynthesis by high light, the phenomenon of photoinhibition, but without its name. Only in 1956, did Kok characterize this phenomenon in an elegant manner (Kok 1956).

What did Eugene Rabinowitch know in 1951 about 'heat loss' (internal conversion)?

Rabinowitch (1951) has discussed the various factors that limit the yield of Chl *a* fluorescence *in vitro*. In discussing fluorescence results *in vivo*, he was aware of the role of 'heat loss' when he wrote 'In other words, fluorescence freed of one of its two competitors — the primary photoprocess — will face a stronger second competitor — internal dissipation — and will suffer a net loss.' Further, Rabinowitch (1951) pointed out that the actual competition was between the yield of photochemistry and the sum of the yields of fluorescence and heat loss. Thus, the antiparallel relationship between photosynthesis and fluorescence could be observed only when heat loss was constant. However, whenever parallel relationships were observed, the heat loss must be changing. Thus, if both photosynthesis and fluorescence were decreasing, heat loss may be increasing.

What did Louis N. M. Duysens know in 1963 about Q_A and non-Q_A-related fluorescence changes?

Duysens and Sweers (1963) wrote 'the first direct experimental suggestion of a different effect on fluorescence of two light beams of different colors was obtained by Govindjee *et al.* (1960). The authors concluded from experiments with *Chlorella* that the total fluorescence caused by far-red and blue was smaller than the sum of the fluorescence intensities in each beam, and also that …'. Duysens and Sweers (1963) were the ones who explained these results. Based on their detailed two-light experiments, they provided the concept that PSII reduces a quencher Q

(now called Q_A), and PSI oxidizes the reduced quencher (they had called it QH). In addition, they did another experiment. They measured the Kautsky transient right after a similar transient in a dark-adapted sample — the rise in fluorescence from 'O' (the origin) to 'P' (the peak), followed by a decay to a steady state (S). They observed that the second exposure did not take the fluorescence to the P level (as observed in the first exposure), but it was very low (quenched). If P-to-S fluorescence decay was mainly due to reoxidation of QH to Q, the second exposure should have given the same curve as the first. Thus, they proposed the hypothesis that QH was converted into another quencher Q′, and Q was produced in the dark from Q′. A part of the P-to-S fluorescence quenching is thus 'non-Q_A-related' quenching.

Non-Q_A-related fluorescence studies in the late 1960s and the 1970s

During a 5-year period (1967–1973), there were several observations that could not be attributed to Q_A-related fluorescence changes: (i) a decrease of Chl *a* fluorescence by phenazine methosulfate (PMS) in chloroplasts that had been treated with DCMU (unpublished experiments of L. Yang and Govindjee, cited in Govindjee *et al.* 1967; Murata and Sugahara 1969); (ii) Chl *a* fluorescence changes due to addition of uncouplers of photophosphorylation and DCMU to intact cells of green algae and cyanobacteria (Papageorgiou and Govindjee 1967, 1968*a*, *b*); (iii) parallel rise in Chl *a* fluorescence transient (the so-called 'S-to-M rise') and rate of oxygen evolution (Papageorgiou and Govindjee 1968*a*, *b*; Mohanty *et al.* 1971).

In a seminal work, Wraight and Crofts (1970), using various uncouplers of photophosphorylation, provided the first detailed understanding that non-Q_A-related fluorescence changes are due to changes in proton concentration. The pH maximum of Q_A-related changes was shown to be 6.5, whereas that by 'high-energy state' to be 8.8. Mohanty *et al.* (1973) established that the PMS-induced changes in DCMU-treated chloroplasts must be due to changes in rates of heat loss (k_h). Finally, Briantais *et al.* (1979, 1980) showed that the P-to-S decay in fluorescence was indeed related to internal proton concentration in intact chloroplasts and algae.

Non-photochemical quenching of Chl a *fluorescence and its relationship to the xanthophyll cycle*

The current version of the xanthophyll cycle was discovered by Yamamoto *et al.* (1962), and manipulation of the xanthophyll cycle by dithiothreitol was discovered by Yamamoto and Kamite (1972) (Fig. 1). One of the earliest and major contributions to the relationship between decreased Chl *a* fluorescence in high light and increased heat loss in whole plants was that by Björkman (1987) and Demmig *et al.* (1987). It was Demmig *et al.* (1987, 1988) who first provided the connection between Chl *a* fluorescence quenching, heat loss and zeaxanthin. We note that J.-M. Briantais, C. Vernotte and G. H. Krause had already suggested a connection between fluorescence quenching and the ΔpH (see above, and Briantais *et al.* 1986). Demmig-Adams *et al.* (1989) proposed that all of the fluorescence quenching processes may be related to the xanthophyll cycle (see Demmig-Adams 2002). Bilger and Björkman (1990, 1994) showed the relationship between Chl *a* fluorescence quenching, xanthophyll cycle and membrane conformation in several plants (Fig. 2). It was followed by extensive quantitative work by Gilmore and Yamamoto (1993, 2001), Gilmore *et al.* (1994, 1995, 1996*a*, *b*, 1998) and Gilmore (2001) on the role of the xanthophyll cycle in NPQ. Gilmore *et al.* (1995) were the first to measure the lifetime of Chl *a* fluorescence and, thus, the true quantum yield of fluorescence. When the quantum yield of fluorescence decreased even in the presence of

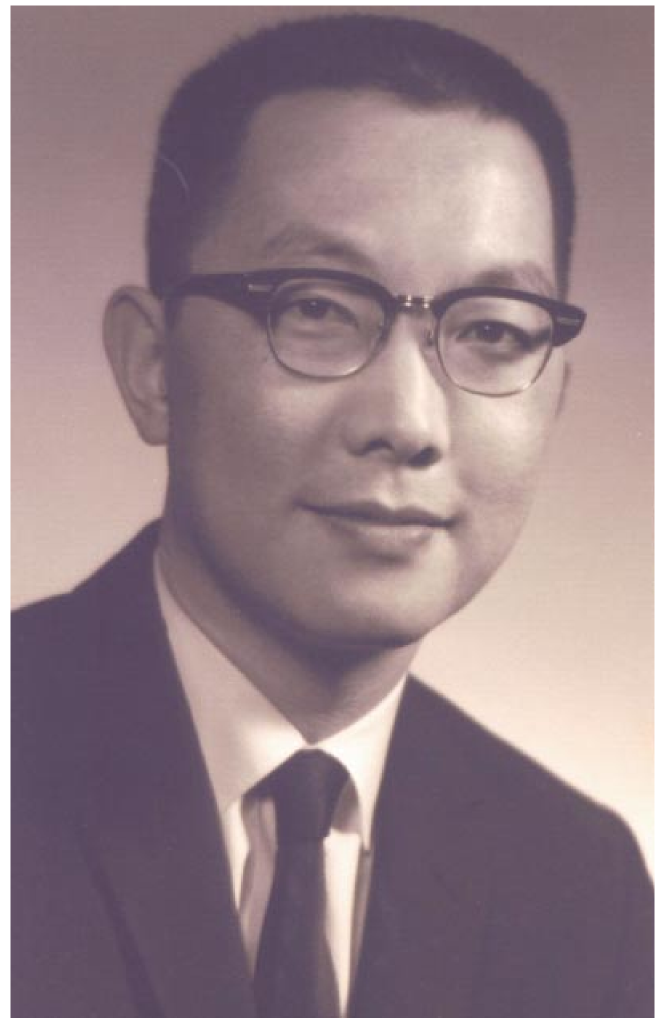

Fig. 1. Harry Yamamoto. Photo taken in 1969 by the Institute of Food Technologies.

DCMU, one could be sure that the only interpretation was an increase in heat loss. Horton and Ruban and co-workers have done extensive work since the early 1990s on NPQ (see reviews by Horton *et al.* 1996, 1999). Their thesis involves the role of aggregation of light-harvesting complexes of PSII, particularly of LHCIIb. This is supported by *in vitro* results. However, results of Gilmore *et al.* (1996*a*) and Briantais *et al.* (1996) for barley mutants do not apparently support this thesis. Moya *et al.* (2001) present detailed lifetime of fluorescence data on antenna proteins, including LHCIIb, in detergent micelles and liposomes, suggesting that intrasubunit conformational change and intersubunit interactions may also be important for photoprotection mechanisms *in vivo*. Further, data of Jahns *et al.* (2000) suggest a role of zeaxanthin in turnover of the D1 protein of PSII. In this paper, we do not enter into any debate over the detailed arguments on the topic of mechanisms of photoprotection *in vivo*, but instead consider the idea that nature may have evolved several alternate ways of reaching the same goal. The story of photoprotection may be analogous to different authors looking at the different parts of an 'elephant' and describing them as they see them. Experiments of Chow *et al.* (2000) on greening bean leaves, and those of Elrad *et al.* (2002) on the *npq5* mutant of *Chlamydomonas*, do raise the question of the involvement of LHCIIb in photoprotection in some systems. Future research is needed to answer this question (Govindjee 2002). On the other hand, Li *et al.* (2000) have established that the *psbS* gene product is essential to the process of photoprotection in higher plants.

Fig. 2. Olli Björkman. Photo taken around 1962, courtesy of Joe Berry.

It has been established by many that both ΔpH (acidic lumen pH) and xanthophyll-cycle pigments are important for photoprotection (see for example Gilmore *et al.* 1998). The importance of ΔpH in NPQ was recently shown in a mutant of *Arabidopsis* (Govindjee and Spilotro 2002).

Xanthophyll-cycle mutants of C. reinhardtii

Niyogi *et al.* (1997*a*, *b*, 1998) have provided a novel tool to study the relationship of the xanthophyll cycle to the photoprotection process by isolating and characterizing mutants that are deficient in either violaxanthin de-epoxidase (*npq1*) or zeaxanthin epoxidase (*npq2*). Thus, *npq1* mutants accumulate violaxanthin, whereas *npq2* mutants accumulate zeaxanthin. Figure 3 [obtained in collaboration with O. Holub, C. Gohlke and R. Clegg (pers. comm.; see Holub *et al.* 2000 for methods used)] establishes that an *npq2* mutant cell of *C. reinhardtii* has a decreased lifetime of fluorescence and, thus, decreased quantum yield of fluorescence relative to a wild-type (wt) cell. However, the *npq1* mutant cell gives a similar, although not identical, picture to that of a wt cell (data not shown). The conclusion is consistent with *npq2* cells having more zeaxanthin quenching Chl *a* fluorescence — it is particularly compelling because we show that an *npq2* cell that has a higher fluorescence intensity than a wt cell has a decreased lifetime of fluorescence.

Part 2. Characterization of *npq1* and *npq2* mutants of *C. reinhardtii*

We have extended the characterization made by Niyogi *et al.* (1997*a*) of two mutants, *npq1* and *npq2*. A preliminary report of our experiments was presented at an international conference on *Chlamydomonas* (Seufferheld *et al.* 2000). Here, we provide parallel measurements of growth, oxygen evolution, fluorescence transients (with and without DCMU and nigericin), NPQ (with and without DCMU and nigericin), the 'two-electron' gate of PSII, and the back reaction around PSII.

Growth

Cultures of wt (cell wall^{-}), *npq1* mutant (which accumulates violaxanthin) and *npq2* mutant (which accumulates zeaxanthin), provided by Dr Krishna Niyogi of the University of California at Berkeley, were used in our studies. Figure 4 shows growth curves for cell suspensions of *C. reinhardtii* mutants (*npq1* and *npq2*) and wt, measured as optical density (OD) at 750 nm, as a function of hours of growth.

The measured OD values had maximum errors of 10% above 0.3 OD, but the error increased to 20% at (or less than) 0.1 OD. Figure 4*A* is for cells grown photoheterotrophically [17.4 mM acetate, Tris–acetate phosphate (TAP); Harris 1989] at 100 µmol photons m^{-2} s^{-1}. The experimental conditions for Fig. 4*B* are as for those in *A*, except that the cells were grown at 10 µmol photons m^{-2} s^{-1}. Conditions for Fig. 4*C* are also the same as those for *A*, except that cells were grown photoautotrophically in minimal high-salt (HS) medium (Harris 1989). For Fig. 4*D*, conditions were the same as in *C*, except that cells were grown at 10 µmol photons m^{-2} s^{-1}. The results in Fig. 4*A* confirm those obtained earlier by Niyogi *et al.* (1997*a*) for cells grown at 50 µmol photons m^{-2} s^{-1}. No significant difference is observed between the two mutants and wt. The three strains had higher cell density during photoheterotrophic growth, relative to autotrophic growth (Fig. 4*A cf. C*). Further, the time to reach half maximum was about 100 h at 100 µmol photons m^{-2} s^{-1} in both TAP and HS media. On the other hand, *npq2* grew to only about half the cell density of that of *npq1* and wt cells in TAP medium at 10 µmol photons m^{-2} s^{-1} (Fig. 4*B*), and about one-third the cell density of that of *npq1* and wt cells in HS medium at 10 µmol photons m^{-2} s^{-1} (Fig. 4*D*). Although the overall

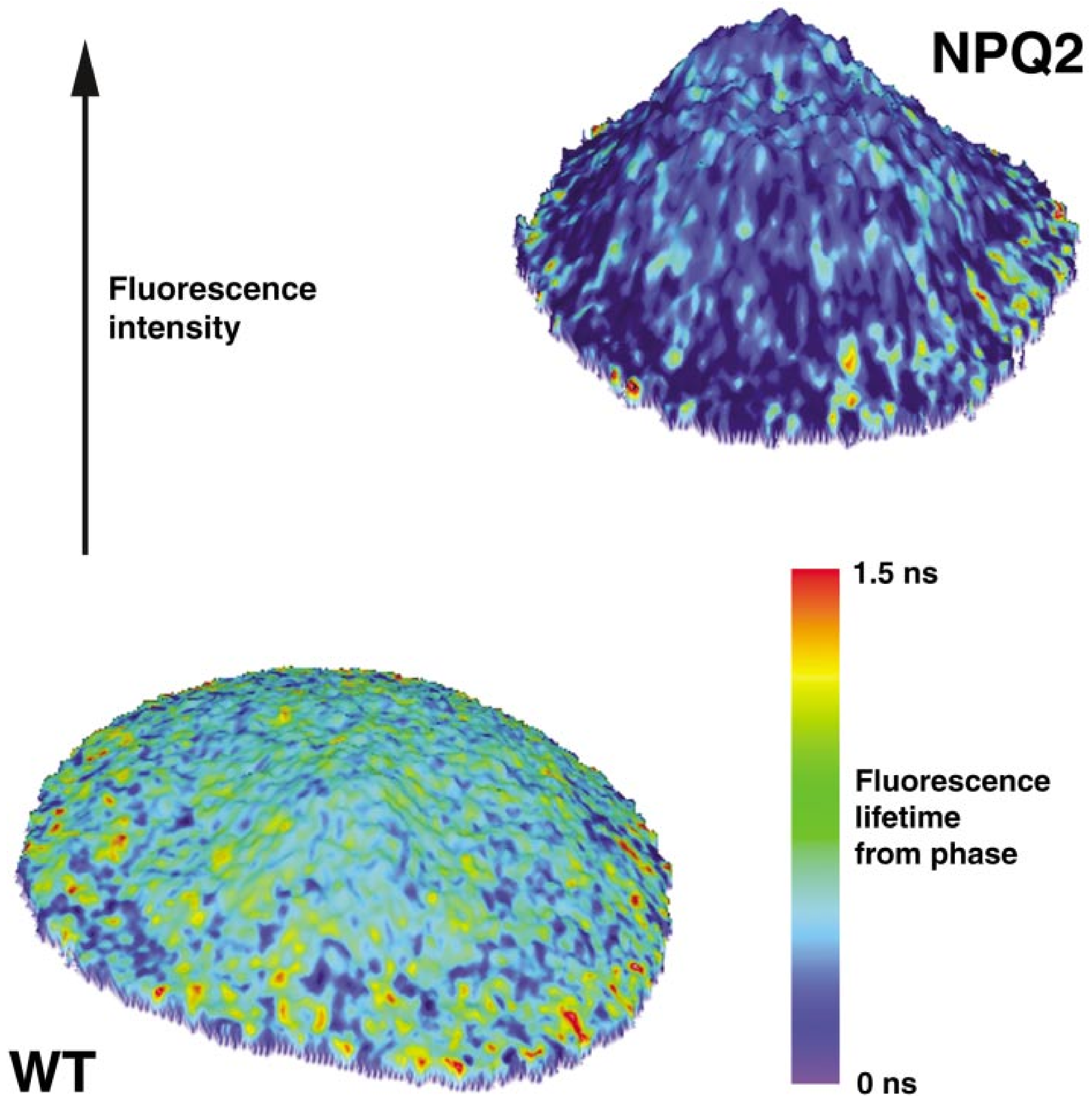

Fig. 3. Chlorophyll *a* fluorescence lifetime images of a single cell of wild type (WT) and *npq2* mutant of *Chlamydomonas reinhardtii* displayed as surface renderings. The apparent single lifetime calculated from the phase is displayed as colour mapped on the fluorescence intensity. The *npq2* mutant, which accumulates zeaxanthin, shows a shorter lifetime than WT. The 2-dimensional display of similar images with details can be found as Fig. 9 in Holub *et al*. (2000). Photo: Christoph Gohlke and Oliver Holub.

growth rate of the three strains was much lower in HS than TAP medium at 10 μmol photons m^{-2} s^{-1} (Fig. 4*B cf. D*), its pattern was different. Initially, the HS cells grew faster than TAP cells. Further experiments are needed to unravel the reasons for these differences. At 10 μmol photons m^{-2} s^{-1}, wt and *npq1* cells had, after 250 h of growth, about half the cell density of that at 100 μmol photons m^{-2} s^{-1} in TAP media. *npq2* had about one-fourth the cell density at the lower light intensity relative to the higher (Fig. 4*A cf. B*). However, after 150 h at the lower light intensity the growth rate of *npq2* was only one-thirteenth of that at the higher intensity.

Decreased growth of *npq2*, compared with *npq1* and wt cells, at low light intensity is a new observation and may be explained as follows. Higher plants and algae posses an efficient light-harvesting mechanism to maximize light trapping. However, the larger pool of zeaxanthin present in *npq2* (Niyogi *et al.* 1997*a*), even at very low light intensity (Jahns *et al.* 2000), may dissipate more light energy in the form of heat rather than through the photosynthetic pathway. Therefore, *npq2* would reduce smaller amounts of carbon than *npq1* and wt, and grow less efficiently under very low light conditions. This predicts lowered oxygen evolution rates at these low light intensities. (However, due to large errors, we were unable to measure reliable rates of oxygen evolution at intensities in the 10 μmol photons m^{-2} s^{-1} intensity range.) These differences could not be ascribed to decreased antenna size in the *npq2* mutant, since *npq1*, *npq2* and wt seem to have comparable antenna sizes (Jahns *et al.* 2000). In addition, depression of photosynthetic efficiency in higher plants has been associated with maintenance of large pools of

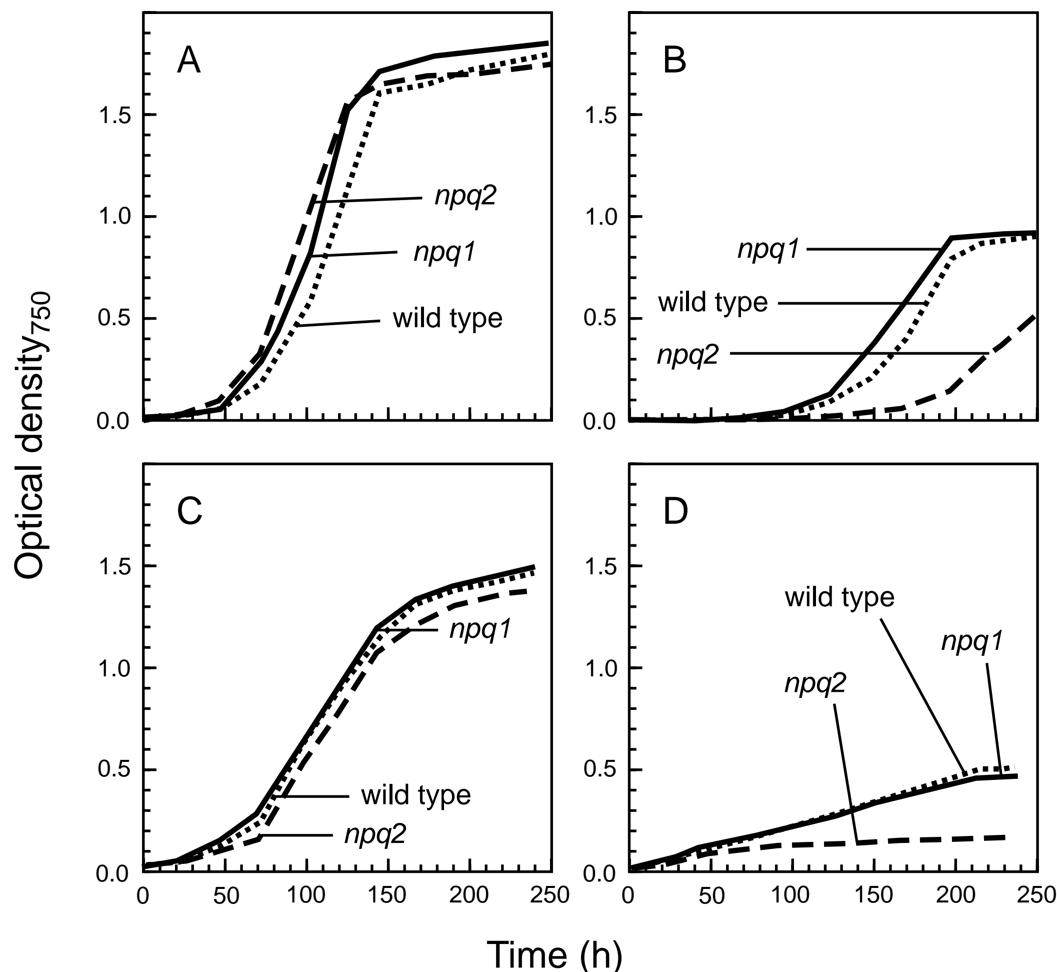

Fig. 4. Growth curves of *Chlamydomonas reinhardtii* mutants of the xanthophyll cycle (*npq1*, *npq2*) and wild type (cell wall^{-}). Cells were grown photoheterotrophically at 25°C in Tris–acetate phosphate medium (*A*, *B*) and photoautotrophically at 25°C in minimal high-salt (HS) medium (*C*, *D*). Cells were illuminated with ~100 (*A*, *C*) and ~10 μmol photons m^{-2} s^{-1} (*B*, *D*). UV 160 U spectrophotometer (Shimadzu, Kyoto, Japan) was used for optical density (OD) measurements. ODs of primary cultures were adjusted to be the same before inoculation of cultures for growth rate determination. This ensured similar cell densities in the initial cultures in all samples. To measure growth rates, cell suspensions were sampled during growth, and ODs measured at 750 nm. Original cultures were diluted before measurements, when necessary, for more accurate measurements.

zeaxanthin (Adams *et al.* 1999). In view of the lowered growth rates of cells grown at 10 μmol photons m^{-2} s^{-1}, these conditions were not used further in this research.

If we speculate that the decreased growth of the *npq2* mutant at low light intensities is due to significant heat dissipation, then how do we explain the similar growth rates of *npq2*, *npq1* and wt at higher light intensities? First of all, growth of cells is controlled by a complex set of factors. More importantly, rates of photosynthesis (a major determinant of growth) at high light intensities are limited by dark enzymatic reactions, particularly in the Calvin-Benson cycle, rather than by the quantum yields of the various de-excitation pathways.

Oxygen evolution

Rates of photosynthetic oxygen evolution (with carbon dioxide as an electron acceptor) for cells grown photoheterotrophically at 100 μmol photons m^{-2} s^{-1}, measured with an oxygen electrode (Yellow Springs, OH, USA) as a function of light intensity, are shown in Fig. 5. At light intensities between 1000 and ~3500 μmol photons m^{-2} s^{-1}, *npq1* cells showed significantly lower photosynthetic rates than *npq2* and wt cells. Decreased availability of zeaxanthin in *npq1* cells, and thus limited photoprotection, may be one of the reasons for this observation. However, there is no significant difference at lower light intensities. Results in Fig. 5 are consistent with the idea that zeaxanthin is involved in the process of photoprotection, especially at higher light intensities (600–3500 μmol photons m^{-2} s^{-1}). Preliminary measurements (data not shown) showed that illumination with 2500 μmol photons m^{-2} s^{-1} did not cause irreversible damage to the three cell types, as rates of oxygen evolution remained the same after 5 min of darkness. We cannot exclude the possibility of other 'downregulation' mechanisms playing additional roles in lowering rates of photosynthesis at high light intensities. As noted earlier, photosynthesis at high light intensities is limited by dark enzymatic reactions, particularly those of the Calvin-Benson cycle.

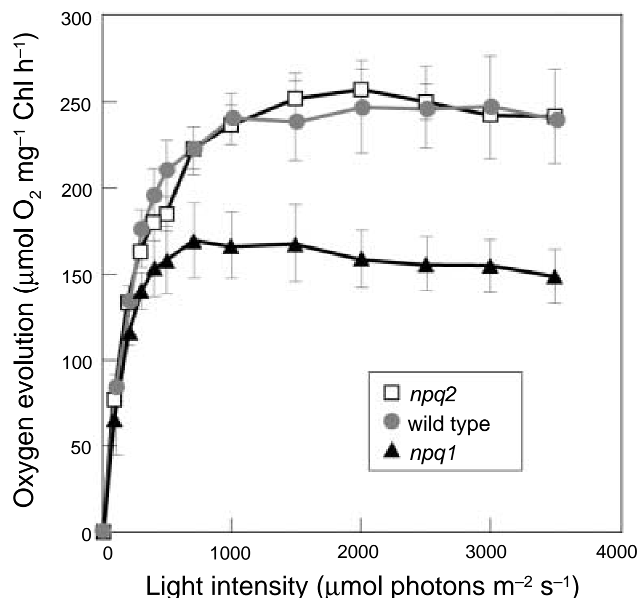

Fig. 5. Rates of oxygen evolution as a function of light intensity in *Chlamydomonas reinhardtii* mutants (*npq1*, *npq2*) and wild-type cells. Cultures were grown in Tris–acetate phosphate medium at 100 μmol photons m^{-2} s^{-1}. Cells used in these experiments were harvested at the late logarithmic phase of growth. Cell suspensions were dark adapted for 10 min, and then oxygen evolution was measured during illumination at different light intensities (provided by a Kodak Carousel 4200 projector using a 300 W lamp and neutral density filters). Sodium bicarbonate solution [45 μL of 0.5 M (pH 7.4)] was added to 1.98 mL of cell suspension prior to oxygen evolution measurements. Cells grown photoheterotrophically at 100 μmol photons m^{-2} s^{-1} were harvested at late log growth phase and adjusted to 15 μg Chl mL^{-1}. Each point represents the mean of six independent experiments. For experiments in this and all following figures, Chl concentration was measured spectrophotometrically based upon equations by Porra *et al.* (1989) after extraction from whole cells (for details see Harris 1989).

Fluorescence transient

Figure 6 shows Chl *a* fluorescence transients (Kautsky curves) for the two mutants and wt cells grown at 100 μmol photons m^{-2} s^{-1} in photoheterotrophic medium (see reviews on Chl *a* fluorescence by Govindjee 1995; Strasser *et al.* 1995, 2000; Lazar 1999), measured with a plant efficiency analyser (PEA) fluorimeter (Hansatech, Pentney, UK). To obtain uniformity and avoid interference from acetate, all cell suspensions were gently centrifuged and re-suspended in fresh HS medium, and adjusted to 15 μg Chl mL^{-1}. Further, cells were first dark adapted for 10 min before being exposed to strong red light (650 nm, 2200 μmol photons m^{-2} s^{-1}).

In all Chl *a* fluorescence measurements it is necessary to know the initial dark-level fluorescence (F_o), the level when all Q_A is in the oxidized state. This is easy to measure with the PEA in untreated control cells. F_o levels in untreated control *npq1* and wt cells were essentially the same within 5%. However in *npq2* cells, contrary to our expectations that F_o would be lower and quenched (due to the presence of higher [zeaxanthin]), values were apparently higher (20%). Since F_o has multiple origins (PSI, PSII, fluorescence due to decreases in excitation energy transfer from antenna to reaction centre core, among others), its interpretation could be complicated. We speculate that in *npq2* cells grown under our experimental conditions, some antenna may be dissociated from the reaction centre core, leading to apparently increased F_o. Thus, we normalized all our data at F_o and examined differences in variable fluorescence. Further, since in intact cells the ratio of Q_B to Q_B^- in darkness is usually 1:1 (see for example Rutherford *et al.* 1984; Xu *et al.* 1989), the true F_o in the presence of DCMU is difficult to measure. Even at very low light, measured F_o is higher than true F_o due to rapid equilibration between $Q_AQ_B^- \leftrightarrow Q_A^-Q_B$, since DCMU functions by displacing Q_B, driving the

reaction towards Q_A^- (Velthuys 1981; Wraight 1981). The higher the [Q_A^-], the higher the fluorescence (Duysens and Sweers 1963). Consistent with these ideas, the measured F_o with 10 μM DCMU added were about 1.7–1.8-fold higher in all three samples. However, nigericin (10 μM) alone did not significantly increase F_o.

Figure 6*A* shows fluorescence transients in untreated samples. Variable Chl *a* fluorescence, normalized at F_o, was much lower in *npq2* than in wt and *npq1*. There are differences between *npq1* and wt, with *npq1* having slower P-to-S decay (Fig. 6*A*) and higher fluorescence in the O-to-P phase after nigericin treatment (Fig. 6*B*) than wt. Similar observations were made for cells grown in HS medium (data not shown). Since *npq2* has an abundance of zeaxanthin, higher quenching of the P-level of fluorescence is consistent with the hypothesis that it may be involved in direct quenching of Chl**a*, especially because the lifetime of Chl *a* is decreased in the *npq2* mutant (Fig. 3). Addition of

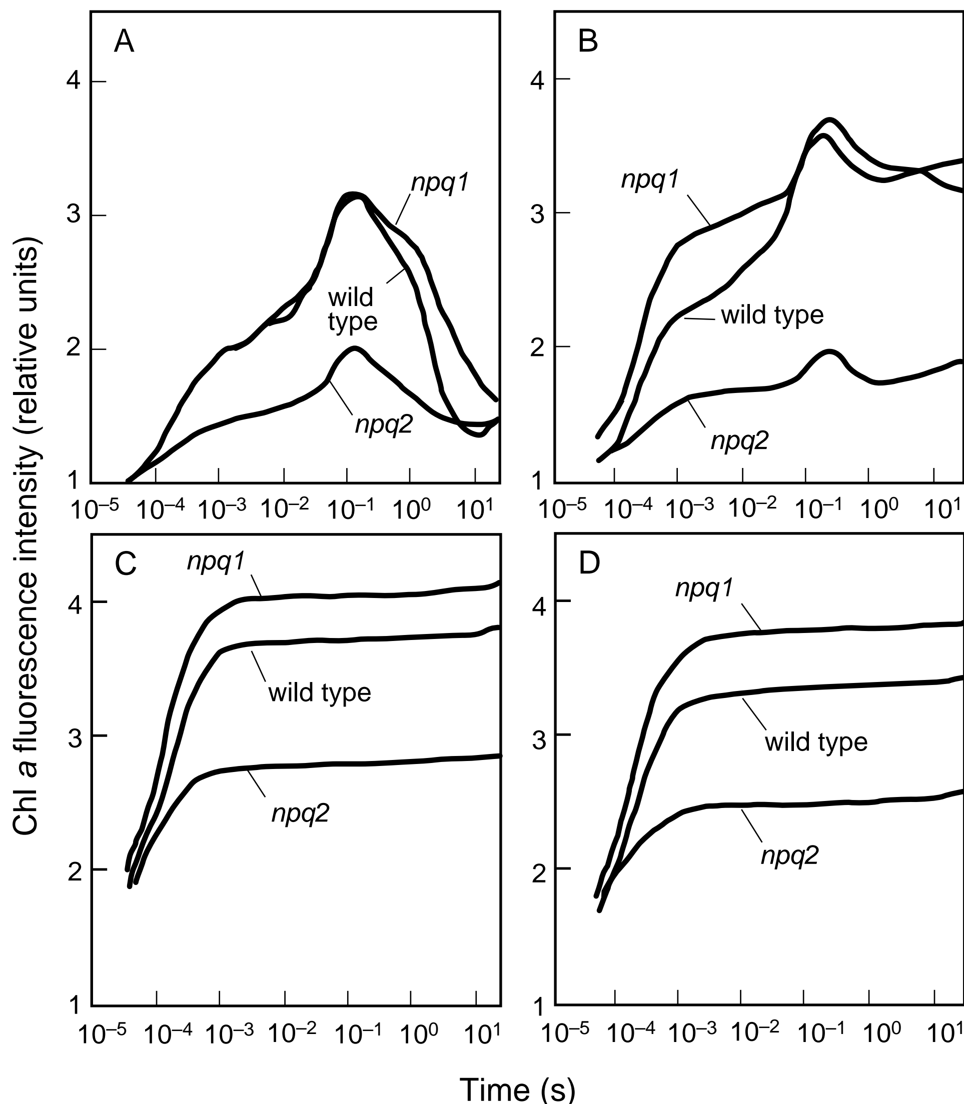

Fig. 6. Chlorophyll *a* fluorescence transients of cell suspensions of wild type, *npq1* and *npq2* mutants of *Chlamydomonas reinhardtii*, measured with a PEA fluorimeter. Cells were grown at 100 μmol photons m^{-2} s^{-1} in Tris–acetate phosphate medium. Cells harvested at late log growth phase were exposed to light (650 nm, 2200 μmol photons m^{-2} s^{-1}) after samples were dark adapted for 5 min. All traces were normalized by dividing measured values by the value for the data point at 40 μs, taken as F_o of untreated wild type. (*A*) Cells without treatment. (*B*) Cells incubated with 10 μM nigericin in the dark for 5 min. (*C*) Cells dark adapted for 5 min, then treated with 10 μM DCMU (final concentration) and incubated for a further 5 min in the dark. (*D*) Cells treated as in *C* except nigericin was added to a final concentration of 10 μM. In all cases, prior to measurements, cell suspensions were gently centrifuged and the pellet re-suspended in fresh high-salt medium. Cell suspensions were adjusted to 15 μg Chl mL^{-1}.

nigericin, which dissipates the proton gradient, is known to decrease/eliminate the P-to-S fluorescence decline (see for example Briantais *et al.* 1979, 1980; Govindjee and Spilotro 2002). This effect on the fluorescence transient was confirmed in *npq1* and *npq2* as well as in wt cells (Fig. 6*B*) during the 0.2–10 s time range. The above results confirm the role of ΔpH in fluorescence quenching in intact cells, but the relationship of this quenching to the xanthophyll cycle is not yet known.

Treatment of cell suspensions with 10 μM DCMU (which blocks electron transport beyond Q_A) showed that *npq2* had much lower variable fluorescence yield than both *npq1* and wt cells (Fig. 6*C*). Furthermore, in the presence of DCMU, wt cells had lower fluorescence levels than *npq1*. This suggests that even in the absence of linear electron flow, zeaxanthin is responsible for quenching of fluorescence. Addition of nigericin to DCMU-treated cells (Fig. 6*D*) showed the absence of significant further effects. We note that the absence of linear electron flow when DCMU is present does not imply absence of a proton gradient, since the cytochrome b_6/f complex and PSI may be involved in a cyclic reaction that includes a proton gradient.

It is important to add a caveat in this paper. The conclusions from data in Fig. 6 are semi-quantitative, since they are affected by interpretation of measured F_o. If we assume that true F_o in *npq2* mutant cells is quenched, and measured F_o contains antenna fluorescence due to disconnected antenna, the true F_m of the *npq 2* mutant would be somewhere between its measured value and wt. On the other hand, NPQ (shown in Fig. 7) for the *npq2* mutant would be higher than measured values. Although further research is needed to obtain more quantitative information, this caveat does not affect the overall conclusions reached in this paper.

Our results suggest that increased zeaxanthin enhances development of NPQ in the *npq2* mutant. According to Gilmore *et al.* (1996*a, b*, 1998), Niyogi *et al.* (1997*b*) and Horton *et al.* (1996), ΔpH is required to induce NPQ of the *q*E (energy-dependent) type. Although the molecular mechanism of NPQ is still to be discovered, it has been shown, at least in higher plants, that a specific minor protein, PsbS, plays a crucial role in fluorescence quenching (Li *et al.* 2000).

There are significant chemical differences between xanthophyll cycle pigments (Yamamoto and Bassi 1996). For example, zeaxanthin is more hydrophobic than violaxanthin, and it is possible that in *npq2* part of the zeaxanthin pool is free in the lipid domain. This could affect membrane fluidity, thus affecting quenching of Chl *a* fluorescence by a mechanism differing from direct quenching of Chl**a* (see Frank *et al.* 2000). Further, stability of antenna complexes of PSII can be reduced, as reported by Tardy and Havaux (1996), in an *Arabidopsis* mutant that overexpresses zeaxanthin. One can envision antenna complexes that are not

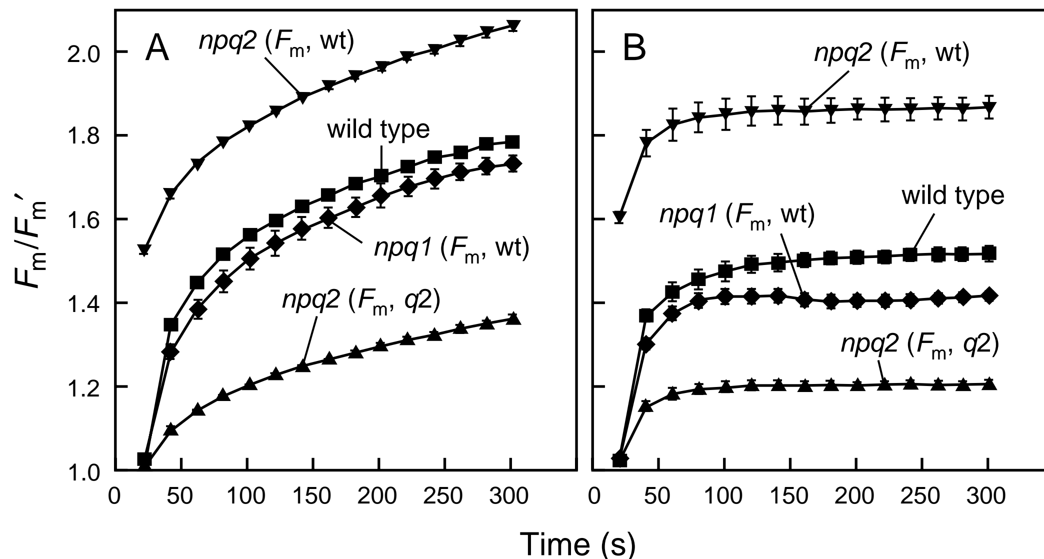

Fig. 7. Induction of NPQ measured by a pulse amplitude modulation device (PAM-2000). Cultures were grown at 100 μmol photons m^{-2} s^{-1}. Ordinates show values of F_m/F_m' for wild type and *npq* mutants (*npq1* and *npq2*) of *Chlamydomonas reinhardtii.* wt and *q2* (*npq2* mutant) in parentheses refer to the source of F_m, used for calculations of F_m/F_m'. Actinic illumination (880 μmol m^{-2} s^{-1}) was provided by a halogen lamp. In all cases, cells were harvested at late log growth phase. Cells were adjusted to 35 μg Chl mL^{-1} and deposited on a 25 mm diameter nitrocellulose filter disc by filtration. The filter disc was then placed in the leaf disc chamber of the PAM fluorimeter and dark adapted for 10 min prior to measurements. (*A*) Cells suspended in Tris–acetate phosphate medium. (*B*) Cells suspended in minimal high-salt medium. Each point represents the mean of three experiments. For points lacking error bars, the error was smaller than the symbol. (Note: another way to plot NPQ is as $F_m/F_m' - 1$. Both methods give the same conclusions; *cf*. Gilmore *et al*. 1998.)

attached to the reaction centre core, and thus explain the higher observed F_o in the *npq2* mutant noted earlier. Therefore, we should keep in mind that the presence of a large pool of zeaxanthin in *npq2* may affect, at least in part, protonation and/or specific binding sites of zeaxanthin to the xanthophyll-binding proteins (and consequently the energy dissipation patterns) by a mechanism other than that suggested earlier.

Non-photochemical quenching

Measurements of non-photochemical changes (as obtained by F_m/F_m') in 10 min dark-adapted *npq1* and *npq2* mutants and wt cells grown in TAP but suspended either in TAP or HS medium, using a pulse amplitude modulation device (PAM-2000; Walz GmbH, Effeltrich, Germany), are shown in Fig. 7. Chlorophyll concentration was 35 μg mL^{-1} of suspension deposited on a nitrocellulose filter disc. TAP or HS medium (50 μL) provided humidity. F_m was taken as the maximal fluorescence level during the saturating flash (1 s, 4000 μmol photons m^{-2} s^{-1}) given before any exposure to continuous illumination, and F_m' as the maximal fluorescence level in the saturating flash during continuous illumination (880 μmol photons m^{-2} s^{-1} for up to 5 min, provided by a halogen lamp). The kinetics of NPQ was biphasic (as already noted by Niyogi *et al.* 1997*a*) and somewhat different in the two culture media (Fig. 7*A*, TAP; Fig. 7*B*, HS). The curves initially show fast development of NPQ in mutant and wt cells in both TAP and HS media. However, in TAP medium the second phase continued to rise, even up to 300 s, whereas in HS medium it reached a plateau at 100 s. These differences between TAP and HS media may be related to acetate, since it can induce slow fluorescence changes (see Endo and Asada 1996).

A quick look at the data in Fig. 7 shows that *npq1* cells have somewhat lower NPQ than wt, whereas the *npq2* mutant gives an apparently paradoxical result. Results on *npq1* are consistent with the data and conclusions of Niyogi *et al.* (1997*a*), which show that *npq1* has somewhat lower NPQ than wt. However, *npq2* has the most zeaxanthin, but apparently shows the lowest value of NPQ. This is because in *npq2*, even F_m is quenched (see Fig. 6). We can correct for this phenomenon if we assume that in the absence of extra zeaxanthin, the *intrinsic* F_m value is the same in *npq2* and wt cells. With this assumption, NPQ in *npq2* is shown to be almost 1.5-times higher than in wt.

Our expectation was for the *npq1* mutant (which is blocked in violaxanthin) to have much smaller NPQ than wt cells. Gilmore (2001) has proposed that zeaxanthin or antheraxanthin can replace lutein in the *lut2 Arabidopsis* mutant. By analogy, one may propose that in the *npq1* mutant, lutein or a similar carotenoid can replace zeaxanthin and antheraxanthin. Other carotenoids may mimic zeaxanthin by fulfilling a structural or binding role in inducing NPQ. In agreement with Niyogi *et al.* (1997*b*) few lutein molecules bound to specific sites in the antenna complex may produce a direct effect on quenching of Chl**a*.

Quenching of Chl *a* fluorescence intensity could be the result of either a decrease in the quantum yield of fluorescence or in the absorption cross section of the pigment bed of fluorescing PSII. It is likely that both processes occur. Results of Holub *et al.* (2000) on the lifetime of fluorescence of single cells of wt and *npq1* and *npq2* mutants show that *npq2* cells have decreased quantum yield of fluorescence (Fig. 3), making the former explanation plausible.

Since PSII itself may act as a photoprotective device, we measured both the two-electron gate and back reaction functions of PSII.

Two-electron gate measurements

A series of fluorescence kinetic measurements were performed with a home-built fluorimeter (Kramer *et al.* 1990) to measure binary oscillations (Velthuys and Amesz 1974) due to faster electron transfer from Q_A^- to Q_B and slower electron transfer from Q_A^- to Q_B^- (Bowes and Crofts 1980). To evaluate the results, it is necessary to first know the details of experimental conditions. Cells were dark adapted for 10 min, and treated in the dark with 100 μM *para*-benzoquinone to convert all Q_B^- to Q_B. Cells were centrifuged and the pellet re-suspended in fresh TAP medium (or in HS medium as indicated). Cell suspensions were adjusted to 7 μg Chl mL^{-1}. A brief (7 μs) actinic flash (greater than 90% saturating) was provided by a Xenon discharge flash lamp (EG & G, Gaithersburg, MD, USA) doped with hydrogen. Excitation was with blue light given at 1 Hz, and a series of low intensity short red LED pulses were used to resolve sub-ms kinetic traces of fluorescence yield changes after each actinic flash. Each pulse was typically less than 1% of actinic light. Binary oscillations of the level of normalized variable Chl *a* fluorescence $[(F_t - F_o)/F_o]$ were measured from 75 μs–20 ms after the actinic flashes. Figure 8 shows plots of variable Chl *a* fluorescence at different times $(F_t - F_o)$, normalized to F_o, as a function of flash number in wt, *npq1* and *npq2* mutant cells. Binary oscillation is observed in all three cases. Thus, the two-electron gate on the electron-acceptor side of PSII remains unaffected by the mutation. On the other hand, the decreased levels of Chl *a* fluorescence in the *npq2* mutant confirm results for the fluorescence transients, shown in Fig. 6.

Measurements of back reactions of PSII

$S_2Q_B^-$ recombination

To test if the rate of the back reaction from $S_2Q_AQ_B^-$ to $S_1Q_AQ_B$ (where S_1 and S_2 are the redox states of the Mn cluster in the oxygen-evolving system) was altered by the mutation, the two-electron gate function was measured as a function of dark time (1, 5, 10, 20 and 30 s) between the first and second actinic flashes (Robinson and Crofts 1983). At

variable times after the actinic flash, the electron on Q_B^- will back react with the Mn cluster. If an actinic flash is delivered before the back reaction has occurred, then fluorescence will be high. If the back reaction has occurred, then fluorescence will be low. Figure 9 shows Chl *a* fluorescence 200 μs after flash 2, following different dark times after flash 1. The decay pattern is almost identical in the two *npq* mutants and wt cells, when corrected for the different levels of fluorescence at 1 s after flash 1. This observation shows that there is no significant effect of mutation on the back reaction of PSII electron donors and acceptors. Here again, the data for the *npq2* mutant confirm that its fluorescence is quenched throughout the experiment.

$S_2Q_A^-$ recombination

To measure $S_2Q_A^-$ recombination kinetics, Chl *a* fluorescence decay was followed after the first actinic flash in dark-adapted cells treated with 10 μM DCMU. Cells were adjusted to 7 μg Chl mL^{-1}. Fluorescence decay after the first

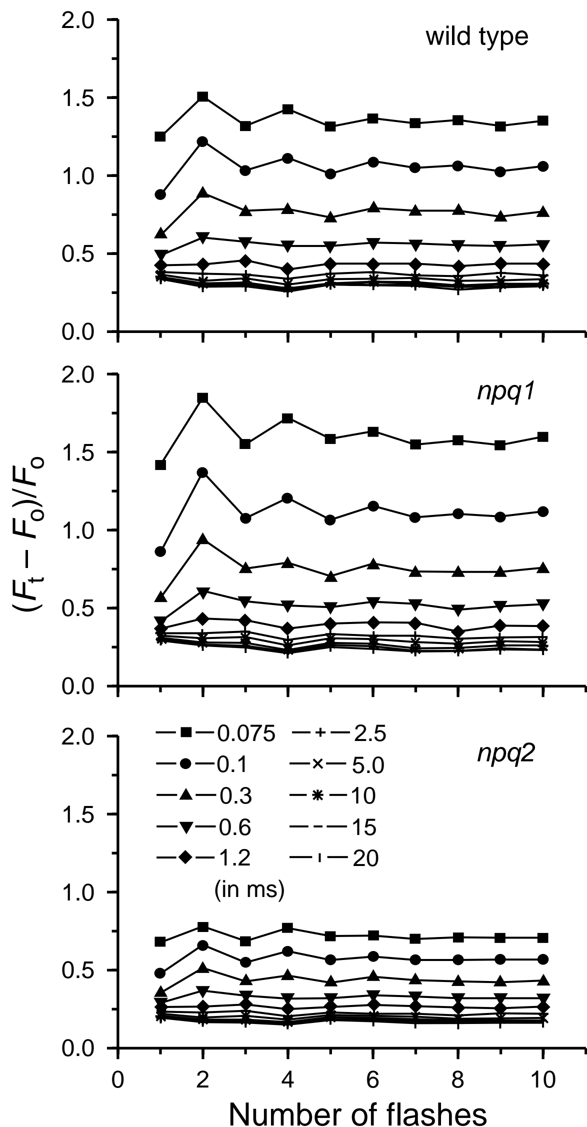

Fig. 8. Binary oscillations in the level of normalized variable Chl *a* fluorescence $[(F_t - F_o)/F_o]$ from *Chlamydomonas reinhardtii npq1* and *npq2* mutants and wild type, measured 0.075, 0.1, 0.3, 0.6, 1.2, 2.5, 5, 10, 15 and 20 ms after the flash. Flashes were given at 1 Hz. Cells were grown in Tris–acetate phosphate (TAP) medium at 100 μmol photons m^{-2} s^{-1}. Cells were harvested at late log growth phase, dark adapted for 10 min, and treated with 100 μM *para*-benzoquinone. Cells were centrifuged and the pellet re-suspended in fresh TAP medium. Cell suspensions were adjusted to 7 μg Chl mL^{-1}.

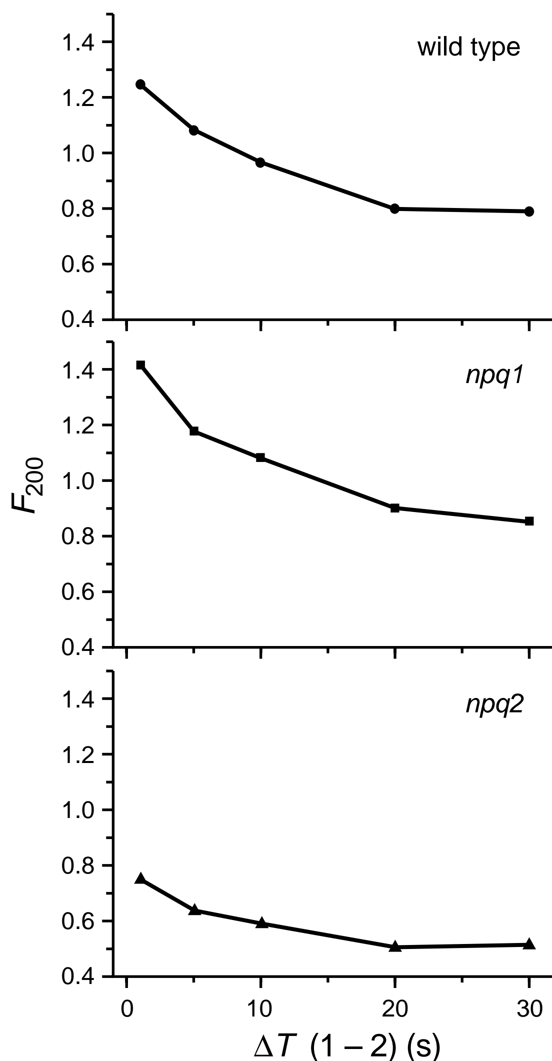

Fig. 9. Kinetics of the back reaction of Q_B^- with S_2 represented by the level of normalized variable Chl *a* fluorescence from *Chlamydomonas reinhardtii npq1* and *npq2* mutants and wild type, measured as a function of waiting time (in s) between the first and second actinic flashes [ΔT (1–2)]. Observation points are at 200 μs (F_{200}). Cells were grown in Tris–acetate phosphate (TAP) medium at 100 μmol photons m^{-2} s^{-1}. Cells were harvested at late log growth phase, dark adapted for 10 min, and treated with 100 μM *para*-benzoquinone. Cells were centrifuged and the pellet re-suspended in fresh TAP medium.

actinic flash in samples treated with 10 μM DCMU (recombination of S_2 with Q_A^- to form S_1Q_A) was essentially unaffected in both the *npq1* and *npq2* mutants and wt (Fig. 10*A*). A normalized plot of the data shows insignificant differences between the two *npq* mutants and wt (Fig. 10*B*).

Concluding remarks

Hints of NPQ have been around for a very long time (Rabinowitch 1945, 1951). The first clear abnormality was observed in the experiments of Duysens and Sweers (1963) on cells of red algae, when the fluorescence transient could not be repeated right after the P-to-S fluorescence decay. Wraight and Crofts (1970) provided the first detailed correlation between fluorescence quenching and pH in isolated chloroplasts. Mohanty *et al.* (1973) related the PMS-induced quenching of fluorescence observed by Murata and Sugahara (1969) to changes in the rate constant of heat loss. In our opinion, the discovery of the xanthophyll cycle by Yamamoto *et al.* (1962), the relationship of NPQ to the rate constant of heat loss and the xanthophyll cycle by Demmig *et al.* (1987), measurements on the lifetime of fluorescence proving that the observed changes are indeed due to changes in quantum yield of fluorescence (Gilmore *et al.* 1995, 1998), research using NPQ mutants by Niyogi *et al.* (1997*a*, *b*), and the discovery of the importance of the *psbS* gene product in photoprotection (Li *et al.* 2000) are the highlights of research in the area of photoprotection. Finally, the extensive work of Horton *et al.* (1996, 1999), Wentworth *et al.* (2000) and Moya *et al.* (2001) has provided great impetus and challenge to this area of research, and continues to raise questions that must be fully examined before a final picture can emerge.

Several conclusions can be made from the experimental results of Niyogi *et al.* (1997*a*) and those presented here on mutants of *C. reinhardtii*: (i) the violaxanthin-accumulating *npq1* mutant may be poorly protected against high light, since it has lower rates of oxygen evolution. The excess zeaxanthin in the *npq2* mutant may protect against photo-oxidative damage. These findings seem consistent with the concept that associates zeaxanthin with antioxidant action, in addition to its suggested role as a direct quencher; (ii) Although zeaxanthin and antheraxanthin are known to be involved in non-radiative dissipation of energy, the xanthophyll-cycle interconversions may not always be essential for NPQ. The zeaxanthin-accumulating *npq2* mutant shows strong fluorescence quenching even in the presence of DCMU. The *npq1* mutant that lacks the de-epoxidase has a significant steady-state level of NPQ, but its magnitude is lower than that in wt cells. Other carotenoids may also be involved in the mechanism of energy dissipation (see for example Gilmore 2001); (iii) The xanthophyll-cycle mutations do not affect either the two-electron gate (i.e. the acceptor side of PSII) or the back reactions from both Q_B^- and Q_A^- to the S_2 state of

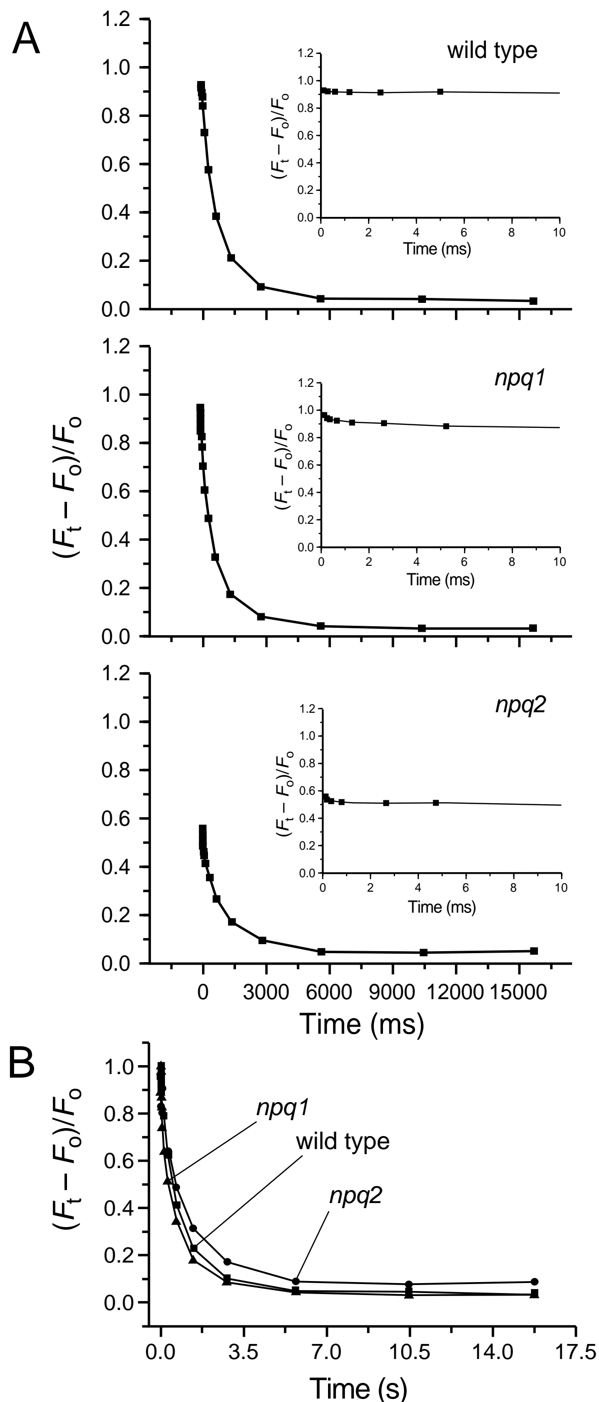

Fig. 10. (*A*) Kinetics of Chl *a* fluorescence decay following the first actinic flash in dark-adapted, 10 μM DCMU-treated *Chlamydomonas reinhardtii npq1* and *npq2* mutant and wild-type cells. Insets: data for fluorescence up to 10 ms after the flash, shown on an expanded scale. Cells were harvested at late log growth phase, and grown in Tris–acetate phosphate medium at ~100 μmol photons m^{-2} s^{-1}. Cells were adjusted to 7 μg Chl mL^{-1}. (*B*) Kinetics of Chl *a* fluorescence decay following the first actinic flash in dark-adapted, 10 μM DCMU-treated *Chlamydomonas reinhardtii npq1* and *npq2* mutant and wild-type cells. Data was normalized at F_m to compare and display the kinetics of $S_2Q_A^-$ recombination.

the oxygen-evolving complex. Thus, photoprotection may not involve cyclic reactions in PSII.

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