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An *Arabidopsis thaliana* mutant, altered in the γ-subunit of ATP synthase, has a different pattern of intensity-dependent changes in non-photochemical quenching and kinetics of the *P*-to-*S* fluorescence decay

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Abstract. A major photoprotective mechanism that plants employ against excess light involves interplay between the xanthophyll cycle and the accumulation of protons. Using mutants in the xanthophyll cycle, the roles of violaxanthin, antheraxanthin and zeaxanthin have already been well established. In this paper, we present data on intact leaves of a mutant [coupling factor quick recovery mutant (cfq); atpC1:E244K] of *Arabidopsis thaliana* that we expected, based on 515-nm absorbance changes (Gabrys *et al*. 1994, *Plant Physiology* **104**, 769–776), to have differences in light-induced ΔpH. The significance of this paper is: (i) it is the first study of the photoprotective energy dissipation involving a mutant of the pH gradient; it establishes that protons play an important role in the pattern of non-photochemical quenching (NPQ) of chlorophyll (Chl) *a* fluorescence; and (ii) differences between the cfq and the wild type (wt) are observed only under subsaturating light intensities, and are strongest in the initial few minutes of the induction period. Our results on light-intensity dependent Chl* *a* fluorescence transients (the Kautsky effect), and on NPQ of Chl *a* fluorescence, at 50–250 μmol photons m^{-2} s^{-1} demonstrate: (i) the '*P*-to-*S*' (or '*T*') decay, known to be related to $[H^+]$ (Briantais *et al*. 1979, *Biochimica et Biophysica Acta* **548**, 128–138), is slowed in the mutant; and (ii) the pattern of NPQ kinetics is different in the initial 100 s — in the wt leaves, there is a marked rise and decline, and in the cfq mutant, there is a slowed rise. These differences are absent at 750 μmol photons m^{-2} s^{-1}. Pre-illumination and nigericin (an uncoupler that dissipates the proton gradient) treatment of the cfq mutant, which has lower ΔpH relative to wild type, confirm the conclusion that protons play an important role in the quenching of Chl *a* fluorescence.

Keywords: ATP synthase, chlorophyll *a* fluorescence induction, non-photochemical quenching, pH gradient, photoinhibition, 515–518-nm absorbance changes.

Introduction

Photoprotection is important to all plants for their survival in sunlight, as excess light can produce damaging chemical species (reviewed by Chow 1994). Among the many photoprotective mechanisms, is the release of excess energy as heat. It involves acidification of the thylakoid lumen, as well as conversion of violaxanthin to antheraxanthin and zeaxanthin. It is suggested that a lower energy state of either zeaxanthin or antheraxanthin accepts energy from the first singlet excited state of Chl* *a*, leading to a loss of energy as heat in the xanthophylls, or there are changes in the configuration of the Chl molecules so that the energy is lost as heat in the Chls themselves (for reviews on this topic, see Demmig-Adams *et al.* 1996; Gilmore 1997; Horton *et al.* 1999; Gilmore and Govindjee 1999; Niyogi 1999). Experiments with numerous mutants of *Arabidopsis* and *Chlamydomonas* have shown the importance of antheraxanthin and zeaxanthin, as well as of minor antenna proteins of

Abbreviations used: cfq, coupling factor quick recovery; Chl *a*, chlorophyll *a*; DA, dark re-adaptation; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; *F*, fluorescence intensity at a given time of measurement; F_m, maximal chlorophyll *a* fluorescence in dark-adapted leaf; F_m', maximal chlorophyll *a* fluorescence of light-adapted leaf; F_o, minimal chlorophyll *a* fluorescence in dark-adapted leaf; LA, light adapted; LED, light emitting diode; NPQ, non-photochemical quenching of Chl *a* fluorescence; *P*, peak fluorescence; PAM, pulse amplitude modulation; PEA, plant efficiency analyser; pmf, proton motive force; qN, non-photochemical quenching parameter of Chl *a* fluorescence $[(F_m - F_m')/(F_m - F_o)]$; qP, photochemical quenching parameter of Chl *a* fluorescence $[(F_m' - F)/(F_m' - F_o)]$; *S*, steady-state fluorescence; wt, wild type; *Y*, quantum yield of photosynthesis $[(F_m' - F)/F_m']$; ΔA 518, light-induced absorbance change at 518 nm, reflecting changes in membrane potential.

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PSII, in the photoprotective mechanism (Gilmore *et al.* 1996, 1998; Niyogi *et al.* 1998; Li *et al.* 2000).

In this paper, we have investigated the patterns of Chl *a* fluorescence transients (Papageorgiou 1975) and the NPQ (Schreiber *et al.* 1986) of Chl *a* fluorescence, in leaves of wt and a cfq mutant (atpC1:E244K; based on Inohara *et al.* 1991) of *Arabidopsis*, since this mutant has the possibility of being different in the pH gradient across the thylakoid membrane. The mutant, cfq, was isolated by Gabrys *et al.* (1994) as follows (we describe some details of the isolation methods as they are necessary for appreciation of the reasons of our choice of this mutant). First generation (M1) plants were grown from ethane methane sulfonate-treated seeds. M2 generation plants were grown under 16-h photoperiod at 270 μmol photons m^{-2} s^{-1}. The stunted and abnormally pigmented plants were discarded. The normal looking plants were grown at 12 μmol photons m^{-2} s^{-1} in a photoperiod of 8 h. Then, all plants exhibiting normal properties were discarded, but those showing reduced growth were retained and returned to standard conditions. The plants that survived were dark-adapted for 12 h, and ΔA 518 (electrochromic shift) was measured. The cfq plants showed slowed ΔA 518 decay after 2-min exposure to 65 μmol photons m^{-2} s^{-1}, just as in dark-adapted plants. Wt plants had faster ΔA 518 decay after identical treatment. ΔA 518 is a measure of membrane potential, whereas the proton motive force (pmf) is a sum of $\Delta\psi$ and ΔpH (Junge and Jackson 1982). We expected ΔpH (and lumen pH) to be also affected by the mutation. Briantais *et al.* (1979) had established that the slow decay of fluorescence beyond 1 s of illumination [labelled as *P*-to-*S* (or *T*)] is a measure of the ΔpH, and the internal $[H^+]$ in thylakoid membranes. Our extensive experiments, presented here, show that indeed *P*-to-*S* decay in the Chl *a* fluorescence transient (Govindjee 1995; Lazar 1999) is slowed in the cfq mutant, confirming lowered ΔpH compared with the wt plants. Further, from the way the mutant was selected (see above), we expected it to show differences at low light intensities, not at higher light intensities. We show in this paper, differences in the kinetics of the *P*-to-*S* decline, as well as in the NPQ at 50 and 250, but not at 750, μmol photons m^{-2} s^{-1}. A preliminary summary of these observations was published in a conference proceedings (Spilotro *et al.* 1998). We present in this paper experiments characterizing these differences under several experimental conditions: (i) different light intensities; (ii) pre-illumination with light; (iii) reversal after removal of the actinic light; (iv) in the presence of an electron acceptor, methylviologen, and in the presence of an inhibitor of electron transport, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); and finally (v) in the presence of nigericin, which dissipates the proton gradient. Results establish the importance of the relationship between $[H^+]$, NPQ and the *P*-to-*S* (or *T*) decay during Chl *a* fluorescence transient, using a ΔpH mutant of *Arabidopsis*. Since nigericin slowed *P*-to-*S* decay, our results show that the cfq mutant has decreased ΔpH. Further, the differences between the mutant and the wt are observed mostly at low, not high light intensities.

Materials and methods

Plant material and growth conditions

Wt and a cfq mutant of *Arabidopsis thaliana* (L.) Heynh. (ecotype Columbia) were grown at light intensities of 120 μmol photons m^{-2} s^{-1} for 6 weeks in a day/night cycle of 16-h light/8-h dark in a growth chamber (model 1-60 LLVL; Percival, Boone, IA, USA). The day temperature was 22 and the night temperature was 20 ± 2°C. Twenty-watt fluorescent lamps (F20T12/CW; Philips, Eindhoven, The Netherlands), kept at a distance of approximately 10 cm, irradiated the plants. Three seeds per pot were sown in commercial potting soil (Sunshine LC1 mix; Sun Gro Horticulture, Bellevue, WA, USA). Plants were watered at regular intervals, including the day prior to fluorescence measurements. Miracle-Gro fertilizer (Scotts, Columbus, OH, USA), with N (nitrogen):P (available phosphorous):K (soluble potassium) ratio of 8:7:6, was added to water 2 weeks following seed placement. Jobe's plant 'food' spikes 13N-4P-5K_2O (purchased from a local nursery) were placed into the soil approximately 2.5 cm away from plants, once the root system was established. Plants (Fig. 1) were kept hydrated and illuminated before measurements were made.

Chlorophyll a *fluorescence measurements*

Similar looking pairs of 'large' leaves were selected, one leaf for the measurement of fluorescence induction (the Kautsky effect) and the other for NPQ of fluorescence. When the two measurements were needed for direct comparison (as, for example, in Fig. 3), errors due to variations in samples were avoided by using the same leaf (still attached to the plant) first for fluorescence induction, and then for NPQ measurement. This order of measurement was chosen to limit the possible influence of the saturating light pulses, used during NPQ measurements, on fluorescence induction measurements. Fluorescence induction was measured by a plant efficiency analyser (PEA; Hansatech, Kings Lynn, UK) Chl fluorometer, whereas NPQ was measured by pulse amplitude modulation (PAM)-2000 Chl fluorometer (Walz, Effeltrich, Germany). For experiments shown in Fig. 3, fluorescence was measured in a sequential fashion from low- to high-light: 50, then 250, and finally 750 μmol photons m^{-2} s^{-1}. Plants were dark-adapted for 10 min before fluorescence measurements began.

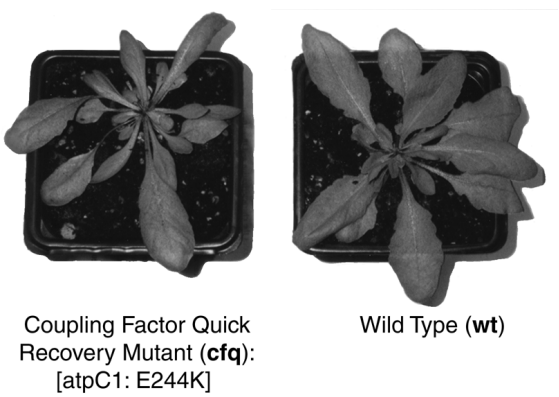

Fig. 1. Wt and cfq (coupling factor quick recovery; atpC1:E244K) mutant plants of *Arabidopsis*. Plants were grown in a 16-h photoperiod. Cfq mutant leaves exhibited slightly stunted growth, particularly in the early weeks of development. Refer cover for color image.

Plant efficiency analyser measurements

Chl *a* fluorescence induction (the Kautsky effect), that is the *OJIPS* transient (*O* for the initial or minimal level; *P* for the peak or the maximum level; *J* and *I* for intermediate inflections; and *S* for the semi-steady state fluorescence; Strasser *et al.* 1995), was measured using a shutter-less fluorometer (PEA, Hansatech). Data accumulation began at 70 μs and lasted for 60 s, with the fluorescence signal at 70 μs considered as F_o. Chl *a* fluorescence transient was measured using a 650-nm excitation light at irradiance of 50, 250, and 750 μmol photons m^{-2} s^{-1}. A leaf area of 12.5 mm^2 was illuminated by an array of six high intensity light emitting diodes (LEDs). A high performance pin photodiode, associated with an amplifier circuit, was used to collect the fluorescence signal. This signal was digitized in the control box using an analogue/digital converter. The initial fast rise in fluorescence was digitized at a rate of 100 000 readings s^{-1} to ensure accurate determination of F_o. After 2 ms, a data acquisition rate of 1000 readings s^{-1} was used until 1 s had elapsed. Finally, only ten readings s^{-1} were taken for the slow decline of fluorescence from the peak (*P*) to the semi-steady state (*S*) or the terminal level (*T*).

Pulse amplitude modulation measurements

Both the Chl *a* fluorescence induction and the NPQ of fluorescence were measured at room temperature by a PAM-2000 (Walz) during exposure to light intensities of 50, 250, and 750 μmol photons m^{-2} s^{-1}. Red actinic illumination (wavelength, 655 nm) was provided by five LEDs (H3000; Stanley, Irvine, CA, USA) focused onto the leaf surface (79 mm^2). Two other H-3000 LEDs, that emit 650-nm pulses, were used as measuring light. Leaf clip holder 2030-B, equipped with a microquantum sensor, monitored photosynthetically active radiation. An additional spacer was added to the clip in order to avoid pinching of the leaf. Chl *a* fluorescence was detected by a photodiode (BPY 12; Siemens, Munich, Germany) that was shielded by a long-pass far-red filter (RG9; Schott, Southbridge, MA, USA) and a heat filter. All the PAM-2000 fluorescence data were recorded in a time span of 5 min 20 s. The rate of data acquisition by the DA-2000 software in the preset program used (listed as run 3 in the PAM-2000 handbook) was 10 ms $pulse^{-1}$. Run 3 measures Chl *a* fluorescence while calculating values of qN (a measure of NPQ $[(F_m - F_m')/(F_m - F_o)]$), qP (photochemical quenching of fluorescence $[(F_m' - F)/(F_m' - F_o)]$), and *Y* (the photochemical yield $[(F_m' - F)/F_m']$; see van Kooten and Snel (1990) for the nomenclature of terms used here.) Recording of data began 15 s following the onset of run 3, and the determination of F_o (minimal fluorescence yield of dark-adapted sample) and F_m (maximal fluorescence of dark adapted sample).

Modifications of standard runs in data acquisition software

Experiments were conducted by modification of standard run 3 (see PAM-2000 handbook) within data acquisition software of PAM-2000 Chl fluorometer. The experiments that eliminated the saturation pulse (a 800-ms pulse of high-intensity white light) used for F_m determination prior to Chl induction recordings were accomplished by altering the standard 'report file', as described in the PAM-2000 handbook.

Experiments were thus conducted to confirm whether the measurements themselves were affecting the fluorescent properties of the wt and cfq mutant plants. Figure 2 shows the effects of the removal of the saturation pulse prior to measurement. This experiment demonstrates that the F_m determination prior to measurement (not shown in figures) as determined by the PAM-2000, does not have significant influence on the quenching patterns of the wt and cfq mutant plants. In order to examine if the measurement techniques affect the outcome of the experiments, plants were also exposed to three consecutive runs (data not shown). No significant changes in quenching patterns were seen. Figure 2 shows the results for the fluorescence transient (labelled as 'F'), and the calculated qP, qN and *Y*, both with (curves *A* and *C*) and without (curves *B* and *D*) the F_m determination in the preceding dark phase for both the samples. No significant differences were observed, showing that the 'dark' F_m determination had no effect on the differences between wt (curves *A* and *B*) and cfq (curves *C* and *D*) reported in this paper. Additional modifications were made to standard run 3 by removing actinic light 1 min following onset of actinic, continuous light, thus allowing the calculation of qN recovery after darkness (as, for example, in Fig. 5). See 'Results and discussion' for details.

Fluorescence analysis

In most experiments, Chl *a* fluorescence data from the PEA fluorometer were plotted, on a logarithmic time scale, after all the data points were divided by the measured F_o, and the F_m value normalized to 100. Usually, this gave a F_o value of 20, and a ratio of 5 for F_m/F_o, consistent with published values for most unstressed normal plants. In some cases, the measured F_o values were simply subtracted from all the data points, and the F_m normalized to 100. When necessary, directly measured fluorescence data were also presented.

The PAM fluorometer data were retrieved from the files within the DA-2000 data acquisition software. The program automatically calculates the NPQ, qN, of Chl *a* fluorescence, as $(F_m - F_m')/(F_m - F_o)$. An alternative equation for the NPQ is $(F_m - F_o)/(F_m' - F_o)$. Both equations provide the same conclusions (data not shown).

Light treatment

High intensity white light

Wt and cfq mutant leaves were dark-adapted for 10 min before being exposed to white light at an intensity of 1400 μmol photons m^{-2} s^{-1}. The light source was a Kodak 4200 slide projector; the light was focused on to the leaf after it passed through a 12-cm wide round-bottom flask containing 1% $CuSO_4$ solution that filtered out the heat. Intact attached leaves were exposed to high light for 10 min. Measurements were made on the same leaves prior to, and following, the high light-intensity treatment.

Low intensity red light

A 3-s pre-illumination with red light of 65 μmol photons m^{-2} s^{-1} provided the conditions used by Gabrys *et al.* (1994). This allowed comparison of our data with theirs on the measurements of ΔA 518.

Treatment of intact leaves with the electron acceptor, methyl viologen, electron transfer inhibitor, DCMU, and nigericin, which dissipates proton gradients

To introduce methyl viologen, DCMU, and nigericin into intact, attached leaves, the upper and lower portions of the leaf were lightly abraded with silicon carbide 400 grit sandpaper. Following abrasion, small drops of the solution containing 0.1% Tween 80 and methyl viologen, DCMU, or nigericin, at a concentration of 3, 0.1, and 2–100 mM, respectively, were spotted on the leaf. Next, a tissue was folded and drenched with the respective solutions, placed over the leaf, and lightly fastened. The time used for soaking with chemicals was 30 min for methyl viologen and DCMU, and 10 min for nigericin.

Results and discussion

As mentioned in the 'Introduction', Gabrys *et al.* (1994) had shown that the relaxation kinetics of the flash induced electrochromic absorbance change at 518 nm, ΔA 518 (Witt 1979) was different in the cfq mutant (known later to be altered in the γ-subunit of ATPase, atpC1:E244K). The cfq plants were those that showed slowed ΔA 518 decay after

2 min of exposure to 65 μmol photons m^{-2} s^{-1}, just as in dark-adapted plants (for details, see 'Introduction'). Wt plants had, however, much faster ΔA 518 decay after identical treatment. A change in pmf was obvious, as Δψ must have changed. However, we also expected changes in the ΔpH of the membranes. Changes in the redox potential of sulfhydryl groups of the γ-subunit have been proposed by Gabrys *et al.* (1994). Further, Evron and Pick (1997) have shown that modification of different sulfhydryl groups of the γ-subunit affects the rate of proton conductance into the chloroplast stroma. Since Chl *a* fluorescence transient changes [the *P*-to-*S* (or *T*) decay] and NPQ of Chl *a* fluorescence have been shown to be related to the lumenal [H^+], and thus to ΔpH in thylakoid membranes, we considered the cfq mutant to be an ideal system to investigate these relationships. Further, due to the manner by which this mutant was selected (see details in the 'Introduction'), it was expected to show light intensity-dependent differences, allowing another means to check the relationships.

Intensity dependence of chlorophyll a *fluorescence transient and of non-photochemical quenching of fluorescence*

Intensity-dependent changes in NPQ and Chl *a* fluorescence transient of wt and cfq mutant *Arabidopsis* leaves are shown in Fig. 3. In these experiments, done at three selected light intensities (50, 250 and 750 μmol photons m^{-2} s^{-1}), intact attached leaves of equal size were dark-adapted for 10 min prior to Chl *a* fluorescence measurements with a PAM fluorometer (panels *A–F*) and PEA fluorometer (panels *G–I*).

Chlorophyll fluorescence induction curves

The fluorescence induction curves in Fig. 3 (labelled as 'F' in panels *A–F*; and traces in panels *G–I*) display significant differences between wt and cfq mutant leaves. At 50 and 250 μmol photons m^{-2} s^{-1} (panels *A*, *B*, *D* and *F*), Chl *a* fluorescence declines much more slowly in the cfq mutant than in the wt leaves, throughout the 60 s starting at the peak, *P*. This reflects changes in the so-called *P*-to-*S* or *T* transient (Papageorgiou 1975). To further evaluate the differences in details, fluorescence transients, measured with the PEA fluorometer, were plotted on a logarithmic time scale, up to 60 s (Figs 3*G–I*). No changes were observed in the F_o-*JIP* transient, i.e. there are no differences in the quantum yield of PSII [$=(F_m - F_o)/F_m$], or in the rate of filling of the plastoquinone pool (Strasser *et al.* 1995). However, the *P*-to-*S* decay in the cfq was clearly slower at 250 μmol photons m^{-2} s^{-1} than in the wt leaf (panel *H*). Further, at 50 μmol photons m^{-2} s^{-1} the fluorescence decay

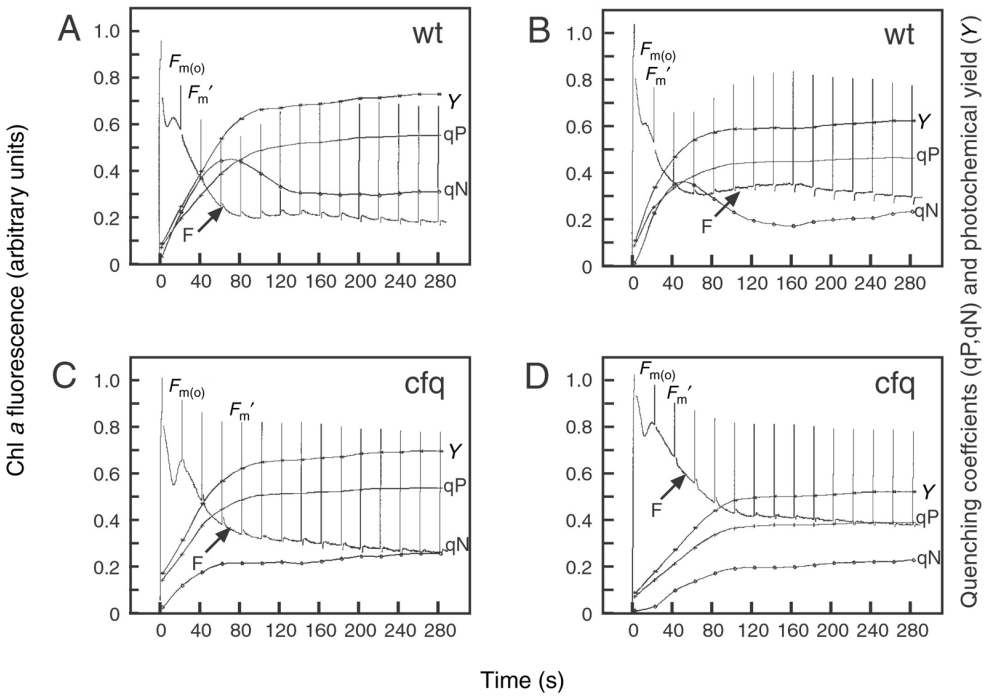

Fig. 2. Chl *a* fluorescence induction curves (labelled as 'F'), qN (a measure of NPQ [$(F_m - F_m')/(F_m - F_o)$]), qP (photochemical quenching of fluorescence [$(F_m' - F)/(F_m' - F_o)$]), and *Y* (photochemical yield [$(F_m' - F)/F_m'$]), as determined by PAM-2000 Chl fluorometer. Intensity of excitation was 50 μmol photons m^{-2} s^{-1}. Curves in *A* and *C* are with, and curves in *B* and *D* are without, determination of F_m prior to onset of measurement. Measurements of *Arabidopsis* wt (curves in *A* and *B*) and cfq mutant (curves in *C* and *D*) were performed on the same intact attached leaves following 10-min dark-adaptation.

from *P*-to-*S* followed more complex kinetics in the cfq than in the wt leaf (panel *G*). Based on the work of Briantais *et al.* (1979), the *P*-to-*S* (or *T*) fluorescence quenching is known to be related to the formation of ΔpH across the thylakoid membrane, with the lumen being acidified (see later discussion). At 750 μmol photons m^{-2} s^{-1} (Figs 3*C*, *F*, *I*), differences in *P*-to-*S* (or *T*) decay between cfq and wt leaves disappear, since it seems that at higher light intensities, the ΔpH becomes equal between the two. The above results are consistent with the conclusions of Gabrys *et al.* (1994) based on measurements of Δψ.

Non-photochemical quenching of chlorophyll a *fluorescence*

At 50 and 250 μmol photons m^{-2} s^{-1} on the same time scale as that of the Chl *a* fluorescence transient, the wt leaves displayed a distinct rise and decline in the NPQ $[(F_m - F_o)/(F_m' - F_o)$; see Fig. 3]. In the mutant leaves, the NPQ rises and levels off. At a higher light intensity (750 μmol photons m^{-2} s^{-1}), however, the differences in the kinetics of the energy-dependent quenching are almost absent (Figs 3*C*, *F*). This parallels the loss of differences in fluorescence transients discussed above.

There is a difference in the photochemical quenching parameter, qP $[(F_m' - F)/(F_m' - F_o)]$, as well as in photochemical efficiency, Y $[(F_m' - F)/F_m']$, between wt and the cfq mutant. The levels for both qP and Y were higher in the first 100 s of measurement in the cfq mutant than in the wt leaves at both 50 and 250 μmol photons m^{-2} s^{-1}. At these intensities, the NPQ values at approximately 150 s and beyond are also higher in the cfq than in the wt. However, the initial value in the wt exceeds that of the cfq. We do not know the reasons for these complexities. At 750 μmol photons m^{-2} s^{-1}, the observed differences in both qP and qN disappear.

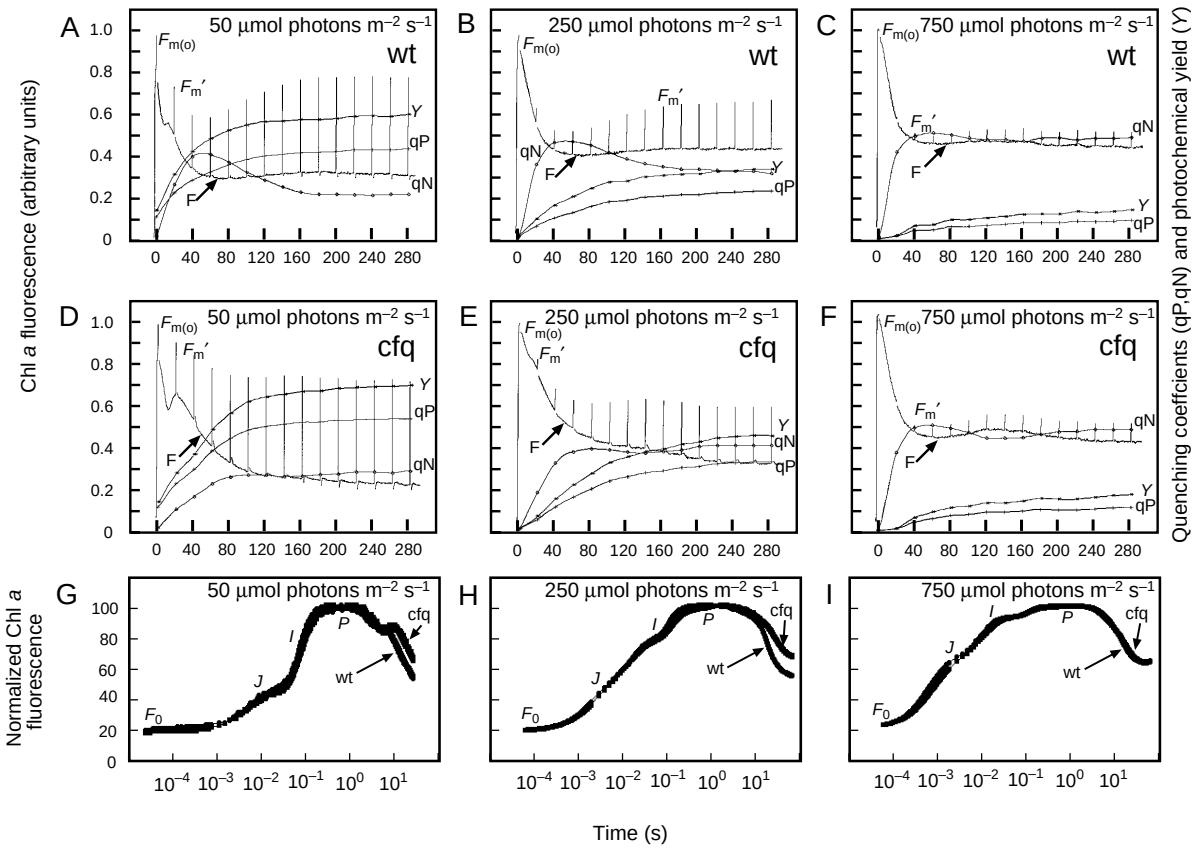

Fig. 3. Chl *a* fluorescence (arbitrary units) measurements using PAM and PEA fluorometers. PAM measurements show the on-line calculation of photochemical quenching (qP), NPQ (qN), and photochemical yield (Y) in intact, attached leaves of wt (*A*–*C*) and cfq mutant (*D*–*F*) *Arabidopsis*. Recordings of fluorescence transients by PAM began at time = 0 when the actinic, continuous light was switched on and followed by an 0.8-s saturating pulse 2 s later. *A* and *D* are for 50 μmol photons m^{-2} s^{-1}; *B* and *E* are for 250 μmol photons m^{-2} s^{-1}; *C* and *F* are for 750 μmol photons m^{-2} s^{-1}. The initial spike of the recording, $F_{m(0)}$, is nearly equal to the F_m measured in the dark prior to onset of actinic light (see Fig. 2). Following $F_{m(0)}$ analysis, saturating pulses determined F_m' at intervals of 20 s. PEA measurements (*G*–*I*) show the Chl *a* fluorescence transient curves of wt and the cfq mutant on a logarithmic time scale. Intact attached leaves were dark-adapted for 10 min, prior to exposure to light (wavelength, 650 nm) intensities of 50 (*G*), 250 (*H*), and 750 (*I*) μmol photons m^{-2} s^{-1}.

Effect of pre-exposure to low intensity red light, followed by variable dark time, on fluorescence transient

Gabrys *et al.* (1994) had observed that a 3-s pre-illumination with 65 μmol photons m^{-2} s^{-1} red light had caused the ΔA 518 nm absorbance change to be slowed to the level of dark-adapted change in the cfq mutant. Since this change is due to membrane potential, and thus pmf, we investigated the effect of this light treatment on the Chl *a* fluorescence transient, particularly the *P*-to-*S* decay that is related to concentration of protons in the lumen, and to ΔpH (Briantais *et al.* 1979).

When the Chl *a* fluorescence transient was measured at 50 μmol photons m^{-2} s^{-1} on the leaves of wt and cfq mutant leaves following a 3-s pre-illumination with red light at 65 μmol photons m^{-2} s^{-1} and compared with measurements from samples that were allowed to re-adapt in darkness for 2, 4, and 10 min, the following results were observed: (i) the *OJIP* transient (Strasser *et al.* 1995) showed an enhancement of the *O*-to-*J* rise and the subsequent fall to a dip, both in wt (Figs 4*A*, *C*, *E*) and cfq mutant (Figs 4*B*, *D*, *F*) leaves in the light-adapted (LA) samples; (ii) the decay of fluorescence from the *P* to the *S* level was markedly slowed in the cfq mutant upon 3 s of red-light adaptation (LA). This fluorescence transient, measured after 2 (panel *B*), 4 (panel *D*), or 10 min (panel *F*) of dark re-adaptation time, is faster than in the LA samples. This slowing in LA samples is

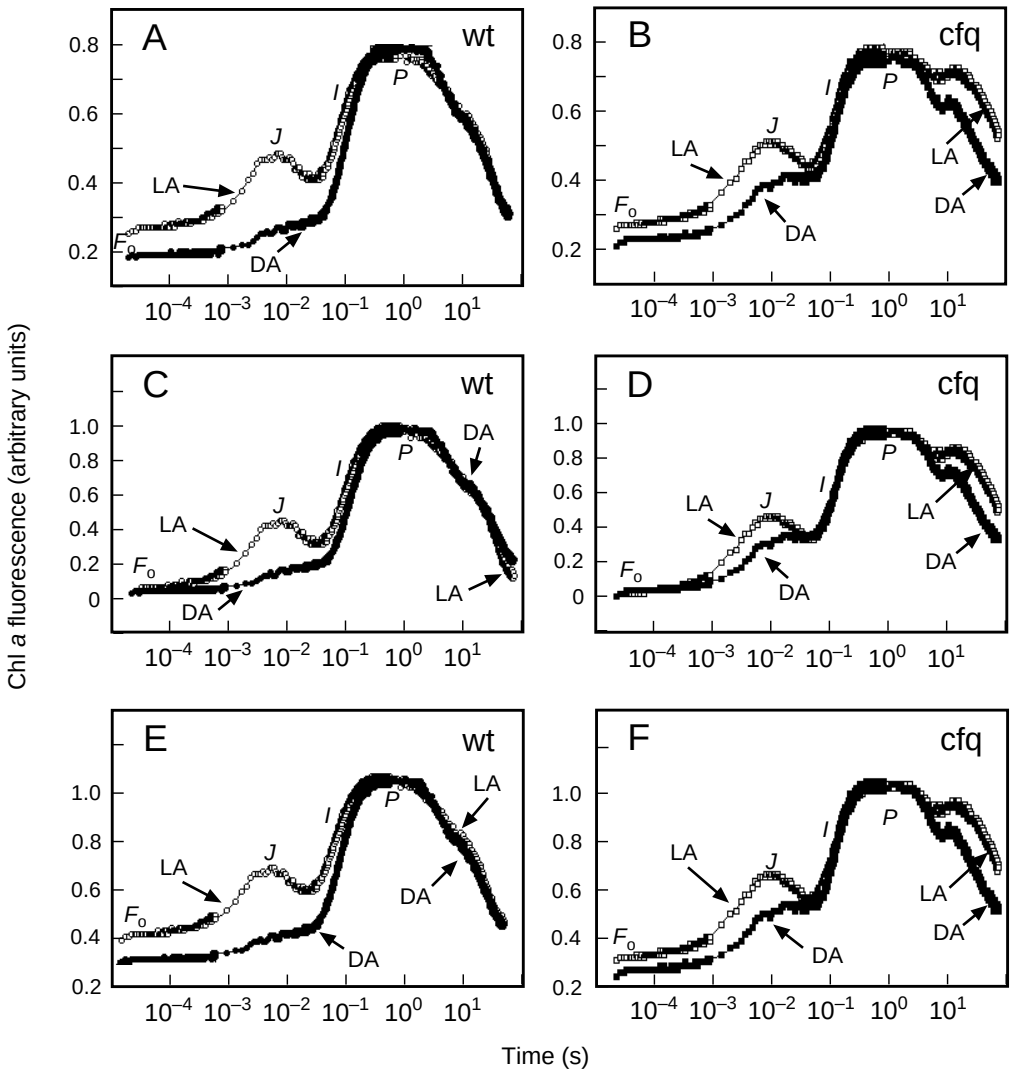

Fig. 4. Effect of dark-adaptation time on Chl *a* fluorescence transient curves of wt (*A*, *C*, *E*) and cfq mutant (*B*, *D*, *F*) of *Arabidopsis* leaves, plotted on a logarithmic time scale. Plants were first dark-adapted for 10 min, and illuminated for 3 s with 65 μmol photons m^{-2} s^{-1} red light (LA) prior to measurement within 1 s. Plants were again dark-adapted for 10 min, illuminated for 3 s at 65 μmol photons m^{-2} s^{-1}, and allowed to dark-re-adapt for 2 (*A*, *B*), 4 (*C*, *D*), and 10 min (*E*, *F*) (DA) and then measured. All measurements were made at 50 μmol photons m^{-2} s^{-1}.

reminiscent of slowed ΔA 518, observed by Gabrys *et al.* (1994), although measured at a different time scale; (iii) the *P*-to-*S* decay in wt leaves was unaffected by light treatment (LA) or dark re-adaptation (DA). It decayed faster than in cfq mutant, confirming the results discussed earlier. However, the absence of the difference in *P*-to-*S* decay between LA and DA wt samples is not in agreement with ΔA 518 differences between LA and DA wt samples (Gabrys *et al.* 1994). Although further experiments are necessary for full understanding of the phenomenon, the ΔA 518 is not a measure of ΔpH, and it is the latter that is correlated with the *P*-to-*S* decay.

Kinetics of recovery of non-photochemical quenching from light to dark

Experiments were conducted to examine the rates of recovery of NPQ in wt and cfq mutant leaves. Earlier studies had associated the recovery of NPQ with the status of the electric transmembrane potential and lumen acidity (see, for example, van Voorthuysen *et al.* 1995). In our fluorescence experiments, plants were dark-adapted for 10 min, and measurements were made at 50 μmol photons m^{-2} s^{-1}. Light-to-dark changes were made 60 s after actinic illumination had begun, and then from dark-to-light at 180 s [Figs 5*A* (wt), *B* (cfq)]. Figure 5*C* shows the $(F_m - F_o)/(F_m' - F_o)$ values of wt and the cfq mutant. These data show significant differences in the kinetics of NPQ between wt and cfq upon exposure to light. Figure 5*D* shows NPQ, plotted as F_m'/F_m values, for cfq and wt during light-to-dark transition. Once the continuous actinic light is turned off, the quenching of the cfq mutant returns to a steady-state more quickly (20 s) than that of the wt (40 s; Figs 5*C* and *D*, respectively). The differences in the relaxation of the NPQ, or the rise of F_m'/F_m to a steady-state level, are influenced by the return of lumen acidity to a 'non-energized' level. There is a quick recovery in Chl fluorescence parameters, observed here, that is apparently in agreement with the quick recovery observed in the activation of ATPase parameters (Gabrys *et al.* 1994).

Effects of an electron transfer inhibitor, an electron acceptor, and a protonophore

DCMU and methyl viologen

The electron-transfer inhibitor, DCMU, which functions by displacing Q_B (Velthuys 1981; Wraight 1981), showed a 50- to 100-times faster Chl *a* fluorescence rise, and abolished the *P*-to *S*-decay in both wt and cfq mutant leaves (data not shown). This is interpreted simply by suggesting that in both cases, reduced electron flow (faster Chl *a* fluorescence rise; see Duysens and Sweers 1963) led to

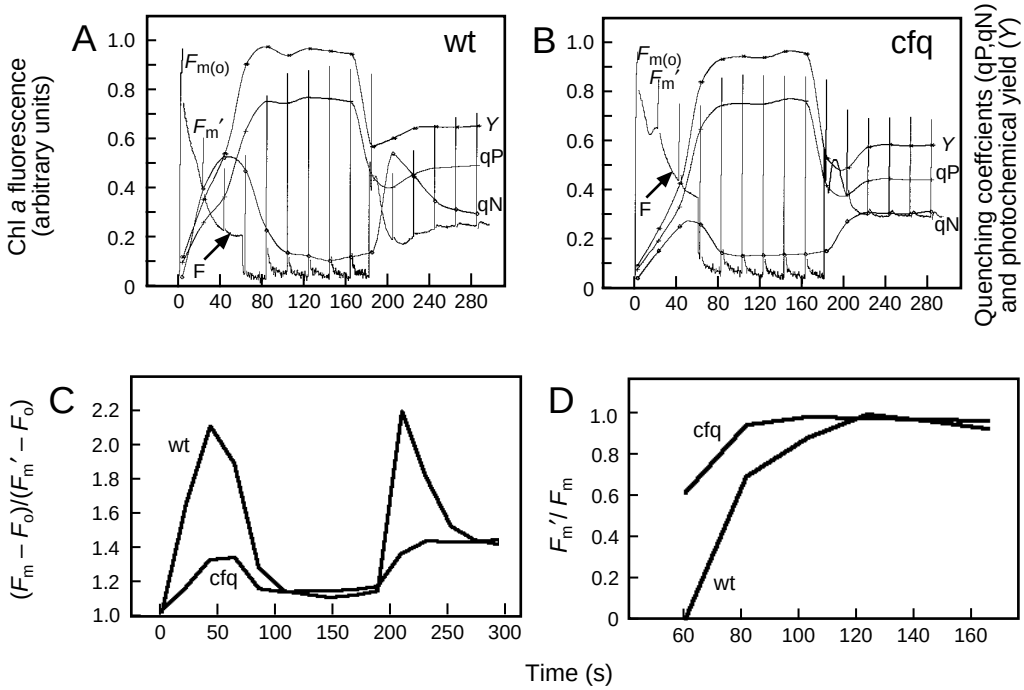

Fig. 5. Reversal of NPQ after actinic light is turned off. Chl *a* fluorescence induction curves (curves labelled 'F'), qP, qN and *Y*, as determined by PAM-2000 Chl fluorometer, are shown in *A* (wt) and *B* (cfq mutant). After 1 min of continuous light following the onset of measurement, the light was turned off. After another 1 min of darkness, the light was turned back on. Plants were dark-adapted for 10 min prior to measurement. *C* shows the $(F_m - F_o)/(F_m' - F_o)$ ratio (inverse of NPQ), and *D* shows F_m'/F_m ratio, which is related directly to NPQ.

reduced ΔpH (lack of *P*-to-*S* decay). However, methyl viologen, which accelerates electron flow and decreases the *P* level of Chl *a* fluorescence (for example see Munday and Govindjee 1969*a*, *b*), decreased the *P* level to approximately the same degree in both wt and cfq mutant leaves (data not shown). This is interpreted simply by suggesting that no significant differences exist in the mutant that are related to accessibility of PSI to externally added chemicals.

Effects of nigericin, which dissipates proton gradients

Briantais *et al.* (1979) had established that the *P*-to-*S* fluorescence decay was due to ΔpH. The best way to check this in wt and the cfq mutant of *Arabidopsis* was to add nigericin, which dissipates proton gradients (see discussion in Jagendorf 1975), and observe its effect on the *P*-to-*S* fluorescence decay, as well as on qP, qN and *Y*. For details of treatment method and the concentration used, see 'Materials and methods'. Data for the highest concentration of nigericin used are shown in Fig. 6. When nigericin was added to *Arabidopsis* leaves, the *P*-to-*S* (or *T*) fluorescence was slowed in PAM measurements (*cf.* 'F' in Fig. 6*B* with *A* for wt; *E* with *D* for cfq). This was confirmed in PEA measurements (Fig. 6*C* for wt, and *F* for cfq mutant). These observations confirm that slower *P*-to-*S* decay in cfq means less ΔpH in the cfq mutant. Further, the fluorescence did not reach the maximal levels present in untreated leaves [Figs 6*C* (wt) and *F* (cfq)]. This was accompanied by nigericin-induced reductions in qP, qN and *Y* in both wt and

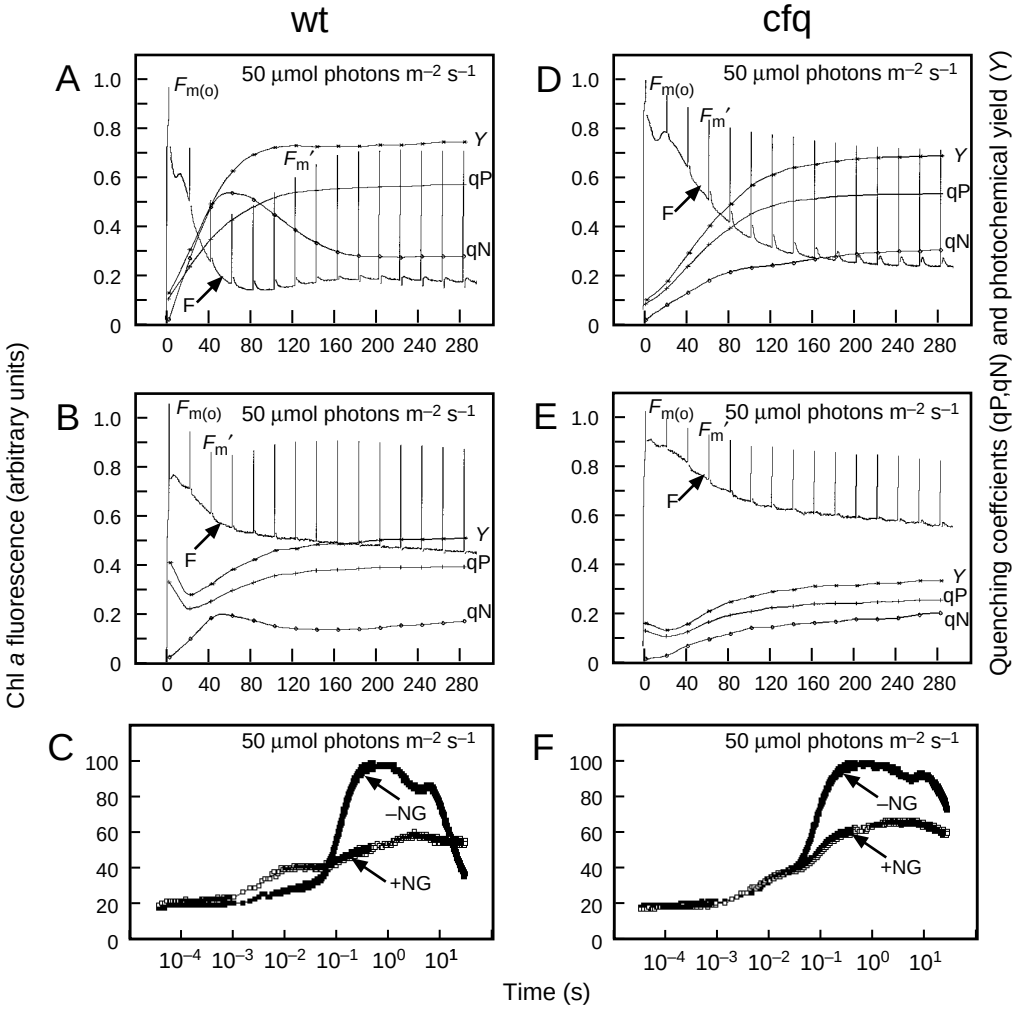

Fig. 6. Effect of nigericin on Chl *a* fluorescence transients and NPQ in intact, attached leaves of wt and the cfq mutant of *Arabidopsis*. PAM-2000 measurements are shown in *A* (wt) and *B* (wt with nigericin), and in *D* (cfq mutant) and *E* (cfq mutant with nigericin). *C* (wt) and *F* (cfq mutant) are Chl *a* fluorescence transients, obtained by PEA fluorometer. For details of nigericin treatment, see 'Materials and methods'. Data for the highest concentration used are shown here. Leaves were dark-adapted for 10 min. All measurements were made at a light intensity of 50 µmol photons m^{-2} s^{-1}.

cfq mutant leaves (Figs 6*A*, *B*, *D*, *E*). Since the steady-state fluorescence was lowered upon addition of nigericin, its effect cannot be explained solely as inhibition of electron flux (see Heber and Walker 1992). In any case, our experiments establish a correlation between ΔpH and NPQ, using a ATP synthase mutant of *Arabidopsis*.

Concluding remarks

In this paper, we have accepted the simplest possible interpretations. To us, the importance of this paper is: (i) it is the first study of the photoprotective energy dissipation involving a mutant of the pH gradient. It establishes that protons play an important role in the pattern of NPQ of Chl *a* fluorescence; and (ii) we show that the differences between the cfq mutant (that has differences in ΔpH) and the wt leaves are observed only at subsaturating light intensities, and are strongest in the initial few minutes of the induction period. In view of the limited data available thus far, it is difficult to eliminate other possible interpretations, but it is equally pointless to add further speculations. First, we recognize that the differences between the mutant and the wt may involve not only differences due to turnover rates of ATP synthase, but may also be due to effects of thiol regulation. Second, no direct determination of the xanthophyll cycle pigments is available. Thus, our paper opens up the need for further research on this and other ΔpH mutants. Parallel measurements on the kinetics of pH changes, ΔA 518 (518 nm absorbance changes), NPQ, and rates of electron flow, are needed to fully understand the molecular mechanism of the observed differences between the cfq mutant and wt leaves of *Arabidopsis*.

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