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Rethinking the existence of a steady-state $\Delta\psi$ component of the proton motive force across plant thylakoid membranes

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Light-driven photosynthetic electron transport is coupled to the movement of protons from the chloroplast stroma to the thylakoid lumen. The resulting proton motive force that is generated is used to drive the conformational rotation of the transmembrane thylakoid ATPase enzyme which converts ADP (adenosine diphosphate) and Pi (inorganic phosphate) into ATP (adenosine triphosphate), the energy currency of the plant cell required for carbon fixation and other metabolic processes. According to Mitchell's chemiosmotic hypothesis the proton motive force can be parsed into the transmembrane proton gradient (ΔpH) and the electric field gradient ($\Delta\psi$), which are thermodynamically equivalent. In chloroplasts the proton motive force has been suggested to be split almost equally between $\Delta\psi$ and ΔpH [Kramer et al., 1999, Photosynth. Res. 60: 151–163]. One of the central pieces of evidence for this theory is the existence of a steady-state electrochromic shift (ECS) absorption signal ~515 nm detected in plant leaves upon illumination. The interpretation of this signal is complicated however by a heavily overlapping absorption change ~535 nm associated with the formation of photoprotective energy dissipation (qE) during illumination. In this study we present new evidence that dissects the overlapping contributions of the ECS and qE-related absorption changes in wild-type *Arabidopsis* leaves using specific inhibitors of the ΔpH (nigericin) and $\Delta\psi$ (valinomycin) and separately by using leaves of the *Arabidopsis lut2npq1* mutant that lacks qE. In

both cases our data show that no steady-state ECS signal persists in the light longer than ~60 seconds. The consequences of our observations for the suggesting parsing of steady-state thylakoid proton motive force between (ΔpH) and the electric field gradient (Δψ) are discussed.

Introduction

Photosynthesis in higher plants involves the light-induced splitting of water by photosystem II (PSII) and the transport of electrons via cytochrome b_6f (cyt b_6f) and photosystem I (PSI) to $NADP^+$, forming NADPH, a source of reducing power for the reduction of 1,3-bisphosphoglycerate to glyceraldehyde 3-phosphate in the Calvin cycle. Photosynthetic electron transport is coupled to the translocation of protons across the thylakoid membrane leading to their accumulation in the thylakoid lumen. Transmembrane electron transport and coupled proton translocation lead to differences in both the pH (ΔpH) and electric potential (Δψ) between the lumen and the chloroplast stroma. The potential energy stored in these gradients is used to drive the synthesis of the universal energy carrier molecule ATP from ADP and Pi by the CF_1- CF_0 ATP synthase. The proton motive force (*pmf*) used to drive ATP synthesis is the sum of the ΔpH and Δψ given by the equation:

$$pmf = \Delta\psi + \frac{2.3RT}{\mathrm{F}}\Delta\mathrm{pH}$$

where Δψ and ΔpH represent the differences in the electric potential and pH respectively between the thylakoid lumen and stroma of the chloroplast, *R* is the universal gas constant, *T* is the temperature, and *F* is Faraday's constant. According to Mitchell's hypothesis the ΔpH and Δψ components of the pmf are thermodynamically and kinetically equivalent (Mitchell, 1961, 1966). In mitochondria the *pmf* is stored mainly as ΔΨ, due to the low permeability of the mitochondrial inner membrane to ions, with a ΔpH contribution of ~0.5 pH units (Mitchell, 1966). In contrast, in chloroplasts recorded values of ΔpH have varied depending upon the method of measurement employed. Using the distribution of ^{14}C-labelled methylamine (Rottenberg et al., (1972), pH microelectrodes (Remis et al., (1986) and pH-sensitive EPR spectra of imidazoline- and imidazolidine-based nitroxide spin-robes (Tikhonov et al., 2007) gave ΔpH values between 1.8 and 2.1 at saturating light intensity. Whereas calibration of ΔpH using $P700^+$ reduction kinetics (Tikhonov et al., 1981) or quenching of the fluorescent dye 9-aminoacridine gave values of 3.0-4.0 pH units (; Schuldiner et al., 1972; Pick et al., 1974;; Solvacek and Hind, 1981). Such a large ΔpH would be thermodynamically incompatible with a significant contribution of Δψ to *pmf* in chloroplasts. Indeed, evidence from experiments involving electrode impaled giant chloroplasts of *Peperomia metallica* and specific ionophores indicated that virtually all $\Delta\psi$ component of the *pmf* was rapidly dissipated under continuous illumination leaving a steady-state $\Delta\psi$ of <30 mV (Bulychev et al., 1972; Vredenberg and Bulychev, 1976; van Kooten et al., 1986). Ion-specific electrode studies on spinach chloroplasts indicated that the thylakoid membrane was much more permeable to ions than the mitochondrial inner membrane with an influx of Cl^- and efflux of Mg^{2+} (and small amounts of K^+) accompanying the light-driven influx protons into the thylakoid lumen (Hind et al., 1974). Similar conclusions were drawn by Dilley and Vernon (1965) and Chow et al., (1976) using direct measurements of the ion contents of the thylakoid lumen before and after illumination and by Barber et al., (1974) using specific ionophores to dissipate ΔpH. The ionic permeability of the thylakoid was explained in Patch-clamp studies on isolated thylakoid membranes which identified both voltage-gated anion (Schönknecht et al., 1988) and cation (Pottosin and Schönknecht, 1996) channels. Recently the genes encoding the voltage-gated ion channel proteins have been identified in plants (Marmagne et al., 2007) and cyanobacteria (Checchetto et al., 2012).

An alternative and far-less invasive method of monitoring the $\Delta\psi$ compared to electrode studies is the use of the electrochromic shift (ECS) absorption change peaking at ~515 nm, described by Duysens (1954) and Junge and Witt (1968). The ΔA_{515} signal is the result of a transient red shift of the chlorophyll and carotenoid Soret absorption bands in response to the $\Delta\psi$ (Witt, 1971). This early work followed the rapid response of the ΔA_{515} kinetics to brief flashes of actinic light that largely avoided the slow non-specific absorption changes. These slow absorption changes include cytochrome *f* oxidation/ reduction, zeaxanthin synthesis and so-called 'light-scattering'. A single turnover actinic light pulse caused a rapid microsecond rise in the ΔA_{515} signal in leaves or chloroplasts which then decayed on a millisecond timescale to the dark baseline (Witt, 1971). Using a diffused optics flash spectrophotometer, that pre-scattered the measuring light to minimise the contribution from the 'scattering' changes and the known kinetic differences between the various absorption changes, Kramer and co-workers attempted to separate their contributions to the ΔA_{515} signal in *Solanum nigram* leaves (Kramer and Sacksteder, 1998). Under continuous illumination Kramer and Sacksteder's (1998) deconvoluted ΔA_{515} signal showed the typical rapid microsecond rise, previously reported in the flash spectrophotometry experiments (Junge and Witt, 1968), was followed by a rapid decay with a half-time of ~5 seconds. The decay of the ΔA_{515} signal was then followed by a secondary slow rise with a half-time of ~10 seconds to a steady-state level ~50% of the initial rapid response amplitude (Kramer and Sacksteder, 1998). When the actinic light was then switched off the ΔA_{515} signal showed a rapid (microsecond) decay below the dark baseline, which was followed by a slow rise back to the initial dark baseline within ~10 seconds (Kramer and Sacksteder, 1998). The rapid decay of the ΔA_{515} signal below the dark baseline upon switching off the light was attributed to the inverted $\Delta\psi$ formed by the continued efflux of protons from the lumen to the stroma via the ATPase despite the rapid cessation of electron transport. The subsequent slow rise back to the dark baseline reflects the movements of counterions down the membrane potential gradient from the stroma to lumen collapsing the inverted $\Delta\psi$. These observations led Kramer and co-workers to suggest a significant fraction of the *pmf* in chloroplasts can in fact be stored as $\Delta\psi$ under steady-state conditions (Kramer and Sacksteder, 1998; Cruz et al., 2001; Kramer et al., 2003; Avenson et al., 2004; Takizawa et al., 2007).

In parallel with the developments in understanding the extent and duration of the ECS signal in plants much progress has been made in the last two decades on the origins of the so-called 'light-scattering' changes. The light-induced scattering changes peaking at ~535 nm in chloroplasts were first described by several groups in the 1960s (e.g. Neumann and Jagendorf, 1965; Deamer et al., 1966, Heber, 1969) and linked to ultrastuctural changes (membrane thinning and lumen shrinkage) in the thylakoid membranes that occur upon light-induced formation of the *pmf* (Murakami and Packer, 1970a, 1970b). Krause (1973) linked the scattering changes to the rapidly-reversible non-photochemical quenching of chlorophyll fluorescence (qE), a photoprotective process occurring in the light harvesting complexes of PSII (LHCII) induced by the build-up of the ΔpH across the thylakoid membrane (see Ruban et al., 2012 for a recent review). Two observations confirmed the link between light-scattering and NPQ *per se,* rather than ΔpH. Bilger et al., (1989) showed that light scattering was severely reduced when synthesis of the carotenoid zeaxanthin, known to promote qE (Demmig-Adams, 1990), was inhibited by dithiothreitol; while Horton et al., (1991) demonstrated that the scattering changes were abolished if qE was inhibited with antimycin, despite ΔpH still being present. Horton et al., (1991) suggested that the membrane structural changes monitored by the light scattering change were linked to the process LHCII aggregation during qE. The similarity between the light scattering changes and the aggregation of the carotenoid zeaxanthin in solution led Ruban et al., (1993a) to speculate that the light scattering change may in fact belong to a true absorption change caused by a red-shift of zeaxanthin. Further evidence of xanthophyll involvement was provided by Noctor et al., (1993) who found that the absorption maxima was downshifted to ~525 nm when qE was induced in the absence of zeaxanthin. This was later confirmed by resonance Raman spectroscopy which showed that a light-induced resonance Raman change belonging to zeaxanthin could be detected with the 528.7 nm laser line (Ruban et al., 2002), that was absent in the *Arabidopsis* PsbS mutant that lacks qE.

The wide range of carotenoid mutants created by the groups of Niyogi and Pogson enabled the absorption changes associated with qE (previously referred to as light scattering) to be investigated in greater detail (Johnson et al., 2009). In addition to the absorption maxima peaking at 535 nm, a series of minima were observed at ~430, 465 and 495 nm (Johnson et al., 2009), the positions of all of these bands were sensitive to the composition of carotenoids present in the LHCII complexes in the various mutants. In the *Arabidopsis npq1* mutant lacking zeaxanthin, qE is drastically reduced (Niyogi et al., 1998), the qE-related absorption changes were similarly attenuated in amplitude and the absorption maxima was down shifted to ~520-525 nm (Johnson et al., 2009). In the *lut2npq1* mutant which lacks both lutein and zeaxanthin qE is completely absent (Niyogi et al., 2001) and no qE-related absorption change was observed (Johnson et al., 2009). In the present work we compared the absorption signals associated with qE and the ECS in wild-type *Arabidopsis* and in a mutant lacking qE to discover the extent of spectral overlap between them in an attempt to provide new information on the extent of the Δψ contribution to *pmf*.

Materials and methods

Plants and growth conditions

Wild-type *Arabidopsis thaliana* cv *Columbia* and *lut2npq1* mutant (lacking lutein and unable to de-epoxidase violaxanthin to zeaxanthin) (Niyogi et al., 2001) were grown for 8-9 weeks in Sanyo plant growth cabinets with a 8-h photoperiod at a light intensity of 100 μmol photons m^{-2} s^{-1} and a day/night temperature of 22/15 °C.

Absorption changes in whole leaves

Absorption changes in the 410-560 nm region were measured using a SLM DW2000 dual wavelength spectrophotometer. Whole *Arabidopsis* leaves were detached from plants dark-adapted for 30 min and the petioles wrapped in moist filter paper. The leaves were inserted into a 1cm^2 transparent cuvette at 45º to the DW2000 measuring light path. An optic fiber, at 90° to the DW2000 measuring light, delivered actinic light (700 μmol photons m^{-2} s^{-1}) illuminating the leaf at 45º and was defined using a Corning 2-58 filter. The photomultiplier was protected using a Corning 4-96 filter and an OCLl Cyan T400-570 mirror. The instrument slit-width was 5 nm and the scan rate was 4 nm s^{-1}. ΔA_{535} and ΔA_{515} kinetics were recorded using the wavelength pairs 535-560 nm and 515-560 nm respectively, 560 nm being an isobestic point for the cytochrome *f* associated absorption changes (Bendall et al., 1971). The sample compartment was water-cooled to maintain the leaf temperature at 22ºC. Where mentioned in the text, leaves were vacuum -infiltrated using a 50 ml syringe with 30μM valinomycin or 50 μM nigercin prepared by diluting 10 mM stocks of these ionophores dissolved in ethanol with a suitable amount of 10 mM HEPES buffer pH 8.0.

Measurement of ΔpH in intact chloroplasts.

Intact chloroplasts were isolated from the wild-type and *lut2npq*1 mutants according to Crouchman et al., (2006). 1.4 ml of intact chloroplasts was suspended in a quartz cuvette at a concentration of 35 μM chlorophyll under continuous stirring. Actinic illumination was provided by arrays of 635 nm LEDs to induce ΔpH. The reaction medium contained 0.45 M sorbitol, 20 mM HEPES pH 8.0, 10 mM EDTA, 10 mM $NaHCO_3$, 0.1% BSA, 5 mM $MgCl_2$, 1μM 9-aminoacridine (9-aa). ΔpH was determined from the measurement of 9-aa fluorescence using the Dual-ENADPH and Dual-DNADPH modules for the Dual-PAM-100 chlorophyll fluorescence photosynthesis analyzer (Walz, Germany). Excitation was provided by 365 nm LEDs and fluorescence emission was detected between 420 and 580 nm.

Results

Individual dark-adapted leaves from wild-type *Arabidopsis* plants were illuminated at a light intensity of 700 μmol photons m^{-2} s^{-1}. Following 15 seconds, 1 minute, 2 minutes or 5 minutes of illumination a spectrum was recorded of each leaf in the 420-560 nm region, the scan taking approximately 30 seconds to complete. Upon completion of the scan, the light was switched off and was followed by a 5 minute period of darkness after which a second scan was completed. Since the enzymatic epoxidation of zeaxanthin back into violaxanthin has a half-time of ~90 minutes (Yamamoto et al., 1962) the light-*minus*-dark recovery difference spectra are free from the contribution from zeaxanthin synthesis. Light-*minus*-dark recovery difference spectra are shown in Figure 1. The spectra showed a gradual red-shift in the positions of both the maximum above 500 nm and the minima below 500 nm. In the 15s difference spectrum the peak maximum was at 518 nm, with minima at 478 and 438 nm, consistent with the shape of the ECS spectrum previously reported (Duysens, 1954; Junge, 1977,;; Kramer and Sacksteder, 1998). The 1 min light-*minus*-dark difference spectrum was red-shifted with a maximum at 528 nm and minima at 425, 464 and 487 nm. The 2 min spectrum showed a further red-shift with a maximum positioned at 532 nm and minima at 497, 472 and a broad band at 425-438 nm. In the 5 min difference spectrum the maximum red-shifted to 535 nm, with minima at similar positions as the 2 min spectrum, at 498, 473 and a broad band at 420-440 nm (Fig. 1)

The spectra in Figure 1 illustrate the extent of the spectral overlap between the ECS and qE-related absorption changes. This issue is illustrated when monitoring the light-induced absorption changes using the wavelength pair ΔA535-560 nm (Fig. 2A), which shows a rapid rise as the light is switched on due to the contribution from the ECS signal, before falling as the ECS decays and then rising again in line with the formation of the qE absorption change. Similarly, upon switching off the light, the

ΔA_{535} signal contains a rapid decay component attributable to the inverted $\Delta\psi$ field and a slower decay component attributable to the relaxation of qE (Fig. 2A). Following a period of darkness, if the leaf was re-illuminated the rise in the ΔA_{535} signal was accelerated to the extent that the ECS contribution to the signal was much harder to distinguish (Fig. 2A). This observation is consistent with the well known acceleration of the qE absorption change formation following light activation and zeaxanthin synthesis (Bilger et al., 1989; Ruban et al., 1993b; Johnson et al., 2009). When a measurement is taken using the wavelength pair ΔA515-560 nm the kinetics are dominated instead by the ECS signal, which shows a sharp rise as illumination commences before decaying to less than 50% of its initial amplitude over the next 30 seconds (Fig. 2B). However, as with the ΔA_{535} signal in Fig. 2A the ΔA_{515} signal shows a slow rise component that saturates within ~100 seconds (Fig. 2B). When the light is switched off the ECS signal shows a sharp fall below the dark baseline as the $\Delta\psi$ is inverted before returning to the dark baseline over the next 30 seconds or so (Fig. 2B). Previously, attempts have been made to remove the contribution of the qE-related changes by 'pre-scattering' the measuring light. However, as previously noted this approach is inconsistent with the evidence discussed in the Introduction that these bands arise from specific xanthophyll absorption changes. An alternative approach is to vacuum infiltrate the leaves with ionophores that selectively remove the contribution of the ΔpH (and so qE) or $\Delta\psi$ (and so ECS) from the observed signals. Nigericin is a monovalent cation-transporting ionophore that acts as an electroneutral antiporter that equilibrates K^+ and H^+ across the membrane, dissipating ΔpH but preserving $\Delta\psi$ (Reed, 1979). By dissipating ΔpH, nigericin inhibits both qE and the associated absorption changes (Krause, 1973). The kinetics of the ΔA_{515} signal in wild-type leaves infiltrated with nigericin are devoid of the 'slow' component seen in untreated leaves (Fig. 2C). In contrast the kinetics still showed the sharp rise and decay upon switching the light on and the sharp fall below the dark baseline when the illumination ceased (Fig. 2C). This result suggests that the 'slow' component of the ΔA_{515} signal in untreated leaves depends on the presence of ΔpH. We attempted to further dissect the 'fast' and 'slow' components using the ionophore valinomycin that equilibrates K^+ across membranes collapsing $\Delta\psi$ but leaving ΔpH present (Reed, 1979). In leaves infiltrated with valinomycin the 'fast' component of the ΔA_{515} signal was absent (Fig. 2D), or more likely the collapse of the $\Delta\psi$ was so rapid as to render it undetectable with our time-resolution. In contrast the kinetics of the 'slow' component in valinomycin infiltrated leaves seemed undisturbed and showed the same accelerated formation during the second illumination cycle as was observed for the ΔA_{535} signal in untreated leaves (Fig 2D, arrow). When leaves were infiltrated with both nigericin and valinomycin both the 'slow' and 'fast' components were nearly completely eliminated (Fig. 2E). Taken together these data indicate that the 'fast' components in the ΔA_{515} signal arise from the $\Delta\psi$ dependent ECS absorption changes, while the 'slow' components arise from ΔpH dependent qE related absorption changes.

Another way to distinguish the ECS and qE-associated absorption changes is by using an *Arabidopsis* mutant deficient in qE. The *lut2npq1* mutant, which lacks lutein and is unable to convert violaxanthin into zeaxanthin; it therefore lacks qE and the associated absorption changes (Niyogi et al., 2001; Johnson et al., 2009). The *npq4 Arabidopsis* mutant that lacks the PsbS protein and thus rapidly-forming qE is still able to slowly form a photoprotective state resembling qE and still shows some absorption changes in the 535 nm region (Johnson and Ruban, 2010), hence this mutant was avoided. To check that the level of ΔpH was unaffected in the *lut2npq1* mutant compared to the wild-type we compared the quenching of 9-aminoacridine in *lut2npq1* and wild-type chloroplasts (Fig. 3). The data confirmed that the lack of qE in *lut2npq1* leaves could not ascribed to any reduction in the ability to generate ΔpH, since it showed similar levels of 9-aminoacridine quenching as the wild-type between light intensities of 0 and 700 μmol photons m^{-2} s^{-1} (Fig. 3). Light-*minus*-dark recovery difference spectra using the conditions for wild-type leaves in Figure 1 were recorded for *lut2npq1* leaves (Fig. 4). The overall shape and amplitude of the *lut2npq1* 15s light-*minus*-5 min dark spectrum bears a strong resemblance to the wild-type 15s light-minus-5 min dark spectrum, and the well known ECS spectrum, with a maximum at 515 nm and minima at 420, 449, 479 nm. The slight differences in the position of the ECS bands compared to the wild-type likely reflect the altered xanthophyll composition in this mutant (Niyogi et al., 2001). However, the 5 min light-*minus*-5 min dark spectrum is essentially featureless in the 420-530 nm region, consistent with previous results indicating the complete absence of the qE-related absorption changes in this mutant (Johnson et al., 2009). To investigate the duration of the ECS signal following the onset of illumination, the light-induced absorption changes measured using the wavelength pairs ΔA535-560 nm and ΔA515-560 nm were measured in *lut2npq1* leaves (Fig. 5A and B). The ΔA_{535} signal kinetics showed a sharp rise upon switching the light on followed by decay back to the dark baseline within ~ 30 seconds, when the light was switched off, a sharp dip below the dark baseline was just detectable above the noise level (Fig. 5A). The ΔA_{515} signal showed a much larger rise when the light was turned on reaching a peak within ~5 seconds before decaying back to the dark baseline within ~60 seconds (Fig. 5B). When the light is switched off there was a sharp decline below the dark baseline which then returned back to the same baseline within ~ 30 seconds (Fig. 5B). Comparison of the sharp rise upon switching the light on observed in the first illumination and second illumination cycles revealed that the ΔA_{515} signal decays much more rapidly in pre-illuminated leaves (Fig. 6) The half-time of the decay of the ΔA_{515} signal in the light was reduced from ~15 s in dark adapted leaves to ~5 s in pre-illuminated leaves, whereas the kinetics of the sharp decay below the dark baseline upon switching-off the light did not significantly change (Fig. 6). Multiple subsequent cycles of illumination and dark adaptation did not appreciably accelerate the kinetics decay of the further (Fig. 5B). In contrast to the wild-type no 'slow' changes were observed in the ΔA_{515} signal of *lut2npq1* leaves, providing further support to the theory that these changes arise from qE in the wild-type (Fig. 5B). Infiltration of *lut2npq1* leaves with nigericin did not appreciably affect the 'fast' component of the ΔA_{515} signal (Fig. 5C), while valinomycin infiltration removed the 'fast' component (Fig. 5D) and in combination neither 'fast' nor 'slow' components were observed (Fig. 5D).

Discussion

In this study we sought to distinguish between the ECS and qE-related contributions to the absorption spectra and kinetics in the 420-560 nm region using a combination of $\Delta\psi$ and ΔpH specific ionophores and an *Arabidopsis* mutant lacking qE. Kramer and Sacksteder (1998) have previously attempted to dissect the contribution of the ECS from the 'scattering' changes in *Solanum nigram* leaves by pre-scattering the measuring light. They used this approach to show that a signal they attributed to a steady-state ECS could persist in the light for minutes. Using the wavelength pair ΔA515-560 nm the light induced absorption change kinetics we recorded on wild-type *Arabidopsis* leaves contained similar 'fast' and 'slow' components as those observed in *Solanum nigram* leaves by

Kramer and Sacksteder (1998). Our data showed that the fast and slow components of ΔA_{515} signal showed different sensitivity to the ionophores valinomycin and nigericin. The 'fast' component was sensitive to valinomycin and thus could be attributed to light-induced changes in the $\Delta\psi$ across the thylakoid membrane, but was insensitive to nigericin, and thus was unaffected by the absence of the ΔpH. In contrast, the 'slow' component was insensitive to valinomycin but was sensitive to nigericin. The similarity in the kinetics of the qE related absorption changes seen clearly with the ΔA_{535} signal and the slow component of the ΔA_{515} signal also suggests they share a common, qE-related and thus ΔpH sensitive origin. Consistent with this suggestion, in leaves of the *lut2npq1 Arabidopsis* mutant that lacks qE and the associated absorption changes, only the 'fast' component of the ΔA_{515} signal was observed. There was no significant difference detected in the amount of ΔpH, as quantified by 9-aminoacridine quenching in isolated chloroplasts, between the wild-type and *lut2npq1* that could account for the observed differences. We thus conclude that the slow component attributed by Kramer and Sacksteder (1998) as a 'steady-state' ECS signal observed is in fact the overlapping qE-related absorption change, the position of which varies depending on the xanthophyll content of the leaves between 525 and 540 nm (Johnson et al., 2009). We suggest that the attempt to remove its contribution by pre-scattering the light in fact would be unsuccessful because it is not light scattering but an electronic absorption. (Ruban et al., 2002; Ilioaia et al., 2011).

The accelerating effect of pre-illumination on the rate of decay of the 'fast' component of the ΔA_{515} signal revealed in our data has, to our knowledge, not previously been reported for leaves under continuous illumination. Pre-illumination was previously shown to accelerate the decay of $\Delta\psi$ in flash-induced ΔA_{515} experiments on *Zea Mays* (Morita et al., 1982), cucumber (Kramer and Crofts, 1989) and sunflower (Kramer et al., 1990) leaves and in electrode studies on chloroplasts of *Anthoceros* (Bulychev et al., 1984). In this case the accelerated decay of $\Delta\psi$ in the dark was attributed to the light-activation of the ATPase (Morita et al., 1982; Junesch and Gräber, 1985). However in our results under continuous illumination the accelerated decay of 'fast' component of ΔA_{515} signal in the light probably reflects the light-activation of coupled photosynthetic electron transport due to activation of the Calvin cycle leading to a more rapid establishment of ΔpH and thus dissipation of $\Delta\psi$. This explanation also suggests that the voltage-gated counterion channels in the thylakoid membrane could be light-activated. Our results also show that under the high light intensity used in this study (700 μmol photons m^{-2} s^{-1}) that the inverted ΔA_{515} signal upon switching the light-off can be almost as large as the positive ΔA_{515} signal when the light is switched on; suggesting significant post-illumination transient proton diffusion potential is built-up. The extent of the inverted ΔA_{515} signal has been shown to depend upon light intensity and CO_2 concentration, consistent with its description of the size of the steady-state *pmf* in the proceeding illumination period (Avenson et al., 2004; Takizawa et al., 2007). Since the permeability of the thylakoid membrane to protons is much higher than other counterions above the threshold for ATP synthesis (Schönfeld and Neumann, 1977), such a transient proton diffusion potential would be expected when a significant steady-state *pmf* is present.

Our results lead to the conclusion that the $\Delta\psi$ contribution to the *pmf* in chloroplasts under the conditions used in our study is negligible. Instead our data favours the view represented in the earlier literature that the $\Delta\psi$ is rapidly dissipated in chloroplasts by counterion movements, particularly Mg^{2+} and Cl^-. It was interesting to note that the ΔA_{515} signal in *lut2npq1* leaves thus strongly resembles the ΔA_{515} signal previously recorded in isolated spinach thylakoids measured under ionic strengths above 10 mM where the ECS and thus $\Delta\psi$ was completely dissipated (Cruz et al., 2001). Our results therefore suggest that the ionic strength of the chloroplast stroma is probably above 10 mM as suggested in earlier studies (Barber, 1976; Schröppel-Meier and Kaiser, 1988) and consistent with the role of cations in thylakoid membrane stacking (Barber, 1982). Our results also explain why valinomycin was found to act as an effective uncoupler of ATP synthesis only during the initial onset of illumination (Ort and Dilley, 1976), when $\Delta\psi$ is still present and thus contributing to *pmf*. Nigericin in contrast was sufficient to completely abolish ATP synthesis in the light (Shavit et al., 1968). Kramer and co-workers have suggested that the *pmf* is differentially parsed into $\Delta\psi$ and ΔpH depending upon the light intensity and availability of CO_2 via dynamic regulation of the ionic concentration of the chloroplast, providing evidence that the contribution of the 'slow' component to the steady state ΔA_{515} signal is smaller under low CO_2 conditions (Avenson et al., 2004). We note that low CO_2 conditions would also promote zeaxanthin de-epoxidation (Takizawa et al., 2007) and thus red-shift the peak position of the qE-related absorption change (see Fig 1 and Johnson et al., 2009), which would have the same effect of diminishing the contribution of the 'slow' component to the

ΔA_{515} signal. The suggested advantage of parsing the *pmf* between $\Delta\psi$ and ΔpH is to keep the lumen pH in a moderate range (pH ~5.8) optimal for the oxidation of plastoquinol at cytochrome b_6f and the stability of the oxygen evolving complex of photosystem II (Kramer et al., 2003). We agree with the logic of Kramer and co-workers in advantages of maintaining moderate lumen pH but suggest in light of our results that it may be achieved in a different way. Using changes in the EPR spectra of pH-sensitive spin probes to measure ΔpH in bean chloroplasts Tikhonov et al., (2007) gave luminal pH values of 5.4-5.7 under conditions of qE induction and photosynthetic control and in the presence of an excess of ADP and Pi of >5.7-6.2. Tikhonov (2012) calculates that if the ΔG_{ATP} (phosphate potential) in chloroplasts is ~40-50 kJ mol^{-1} as measured by Giersch et al., (1980), and the ratio of H^+/ATP is either 4 as suggested by *in vitro* biochemical assays (Berry and Rumberg, 1996; Steigmiller et al., 2008) or 4.67 as suggested by the 14 c-subunits/ CF_0 revealed in atomic force microscopy structural studies of the spinach ATP synthase (Seelert et al., 2003), and the stromal pH is ~7.8-8.0 (Werdan et al., 1975), then the steady-state lumen pH under conditions of ATP synthesis would be in the range of 5.7-6.2 and the ΔpH 1.8-2.1 (~125 mV). Kaim and Dimroth (1998) have argued that some (~30 mV) $\Delta\psi$ is necessary for ATP synthesis since ΔpH and $\Delta\psi$ are not kinetically equivalent in their ability to drive ATP synthesis by the chloroplast ATPase when reconstituted into liposomes. We cannot rule out that such a small contribution of $\Delta\psi$ to the *pmf* is undetectable using the ΔA_{515} signal. However based on the calculations of Tikhonov (2012) it seems that moderate values of ΔpH are certainly capable of supporting ATP synthesis in the absence of a large (>25%) $\Delta\psi$ contribution to *pmf*.

It may be asked what the evolutionary advantage of storing *pmf* in chloroplasts entirely or almost entirely as ΔpH? It can be argued that since the external pH of the environment around a bacterial cell can vary so dramatically (indeed ΔpH can even be inverted in alkaline environments) that pmf must be stored mainly as $\Delta\psi$, a feature which persists in mitochondria. In contrast, the photosynthetic membranes inside chloroplasts and possibly inside cyanobacterial cells are separated from the external environment and thus not subject to this limitation. However the photosynthetic membrane must cope with a different set of pressures, including the negative effect of $\Delta\psi$ in promoting charge recombination between $P680^+$ and electron acceptors in the PSII reaction centre thus forming the P680 triplet state and sensitizing the cell to singlet oxygen formation and photo-oxidative damage (Bennoun, 1994). Indeed recently, a cyanobacterial mutant lacking the potassium cation channel in cyanobacteria was shown to suffer from photoinhibition in high light (Checchetto et al., 2012). Storage of *pmf* as ΔpH also allows photoinhibition to be avoided via concomitant regulation of the chlorophyll excited state lifetime of the antenna system of PSII by qE. Allosteric regulation of qE by the xanthophyll cycle de-epoxidation state (itself regulated by lumen pH) matches the pK of qE induction to the extent to which the lumen pH regularly exceeds the threshold level for ATP synthesis of ~5.9 thus acting as a molecular memory of the prevailing light conditions (reviewed in Ruban et al., 2012). A third possibility is that the regulatory effects of the counterion movements themselves, particularly Mg^{2+}, are indispensible for the proper regulation of the Calvin cycle enzymes.

Conclusions

The results of this study suggest that the ‘slow’ component of the ΔA_{515} signal in wild-type *Arabidopsis* leaves that was previously used to support the notion of a steady-state ECS signal and thus steady-state Δψ component of *pmf* in chloroplasts actually belongs to the overlapping qE-related absorption changes peaking at 535 nm. Our data therefore supports the view that *pmf* is stored almost entirely as ΔpH in *Arabidopsis* chloroplasts.

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Figure Legends

Figure 1. Light induced absorption spectra in the Soret region in wild-type *Arabidopsis* leaves. Spectrum 1, 15 seconds light-minus-5 minutes dark relaxation; Spectrum 2, 1 minute light-minus-5 minutes dark relaxation; Spectrum 3, 2 minutes light-minus-5 minutes dark relaxation; Spectrum 4, 5 minutes light-minus-5 minutes dark relaxation. Light intensity used was 700 μmol photons m^{-2} s^{-1}.

Figure 2. Light induced absorption changes in wild-type *Arabidopsis* leaves.

(A) ΔA_{535} signal induced by light/ dark cycles as indicated by the black (light off)/ white (light on) bars. (B) ΔA_{515} signal in untreated wild-type leaves (C) ΔA_{515} signal in 50 μM nigericin vacuum infiltrated wild-type leaves (D) ΔA_{515} signal in 30 μM valinomycin vacuum infiltrated wild-type leaves (E) ΔA_{515} signal in 50 μM nigericin and 30 μM valinomycin vacuum infiltrated wild-type leaves. Light intensity used was 700 μmol photons m^{-2} s^{-1}.

Figure 3. Effect of light intensity on 9-aminoacridine quenching in wild-type (black circles) and *lut2npq1* (white triangles) *Arabidopsis* chloroplasts. Data are average of 3 independent experiments ± S.E.M.

Figure 4. Light induced absorption spectra in the Soret region in *lut2npq1 Arabidopsis* leaves. Spectrum 1, 15 seconds light-minus-5 minutes dark relaxation; Spectrum 2, 5 minutes light-minus-5 minutes dark relaxation. Light intensity used was 700 μmol photons m^{-2} s^{-1}.

Figure 5. Light induced absorption changes in *lut2npq1 Arabidopsis* leaves.

(A) ΔA_{535} signal induced by light/ dark cycles as indicated by the black (light off)/ white (light on) bars. (B) ΔA_{515} signal in untreated *lut2npq1* leaves. (C) ΔA_{515} signal in 50 μM nigericin vacuum infiltrated *lut2npq1* leaves (D) ΔA_{515} signal in 30 μM valinomycin vacuum infiltrated *lut2npq1* leaves (E) ΔA_{515} signal in 50 μM nigericin and 30 μM valinomycin vacuum infiltrated *lut2npq1* leaves. Light intensity used was 700 μmol photons m^{-2} s^{-1}.

Figure 6. Comparison of ΔA_{515} signal in dark adapted (thick black line) and pre-illuminated (thin black line) untreated *lut2npq*1 leaves induced by light/ dark cycles as indicated by the black (light off)/ white (light on). Light intensity used was 700 μmol photons m^{-2} s^{-1}.

Figure 1.

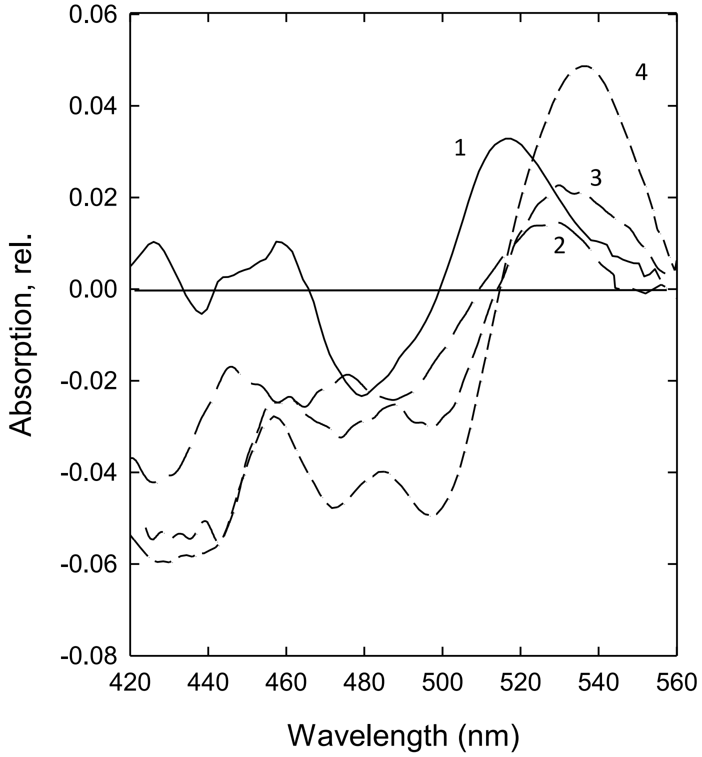

0.06
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
Absorption, rel.
420
440
460
480
500
520
540
560
Wavelength (nm)
1
2
3
4

Figure 2

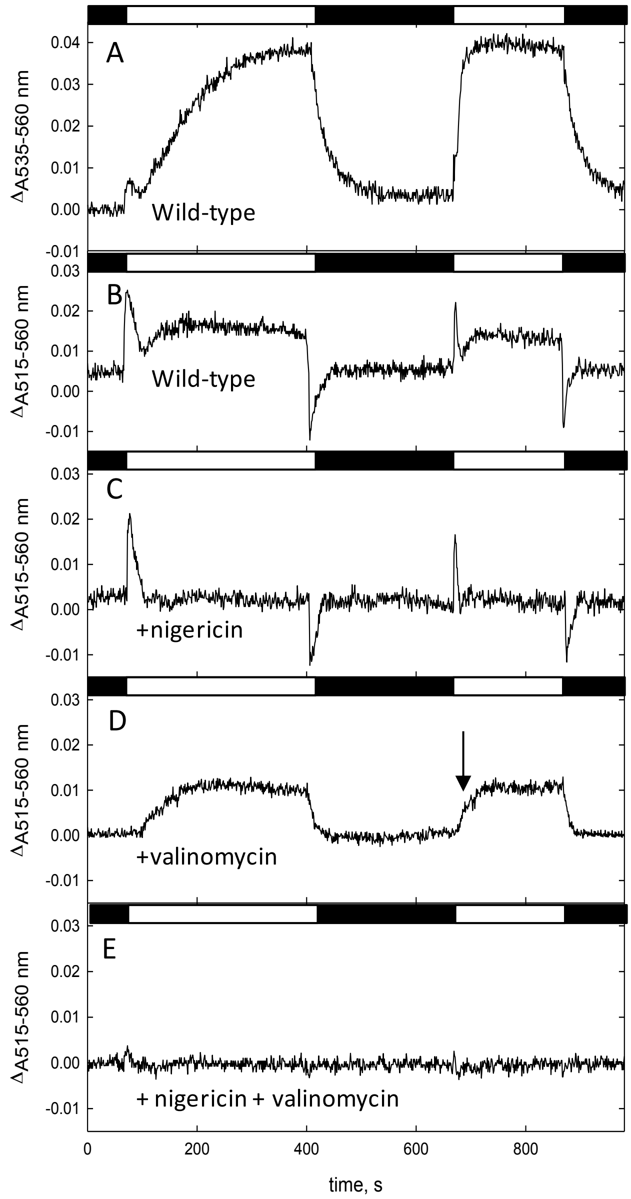

A
Wild-type
B
Wild-type
C
+nigericin
D
+valinomycin
E
+ nigericin + valinomycin
ΔA535-560 nm
ΔA515-560 nm
ΔA515-560 nm
ΔA515-560 nm
ΔA515-560 nm
0.04
0.03
0.02
0.01
0.00
-0.01
0
200
400
600
800
time, s

Figure 3

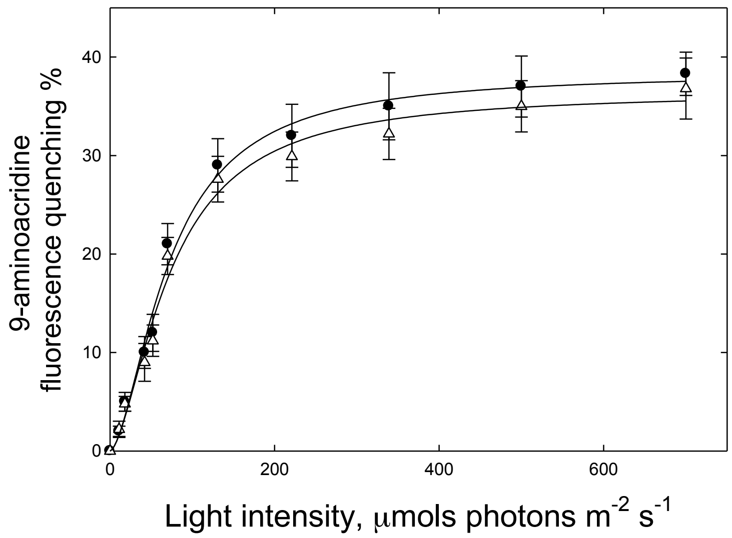
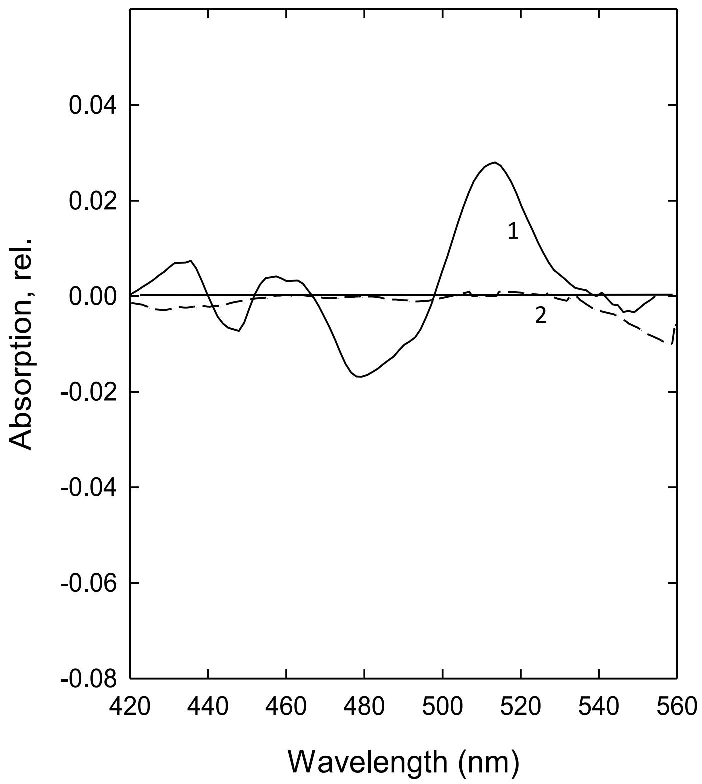
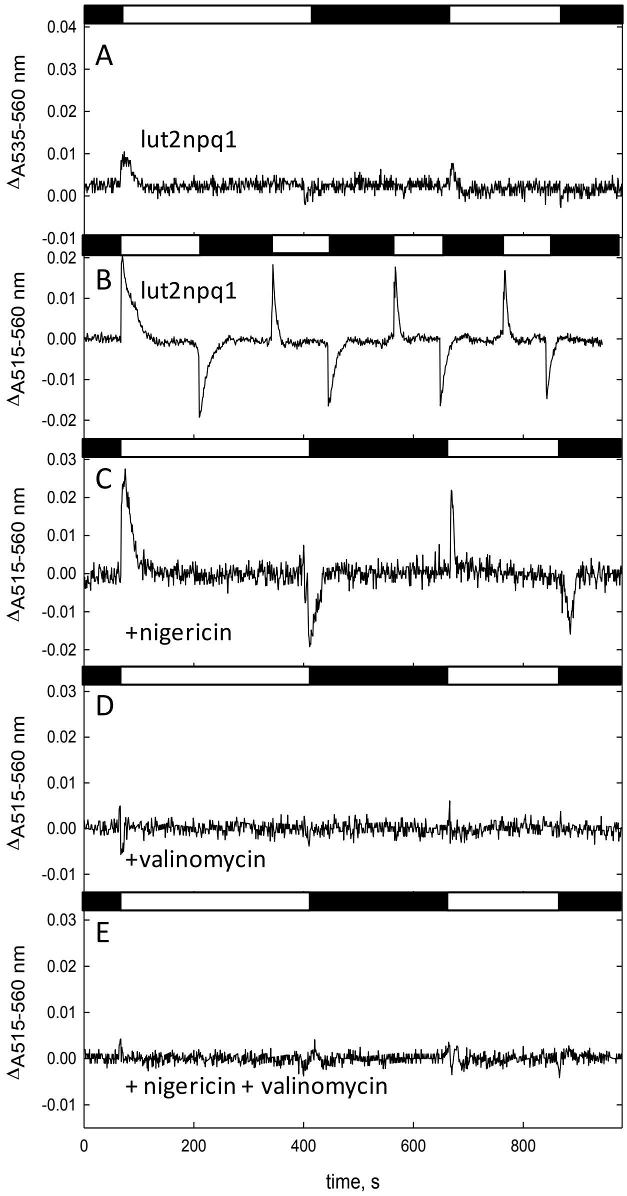

9-aminoacridine
fluorescence quenching %
40
30
20
10
0
0
200
400
600
Light intensity, μmols photons m-2 s-1

Figure 4



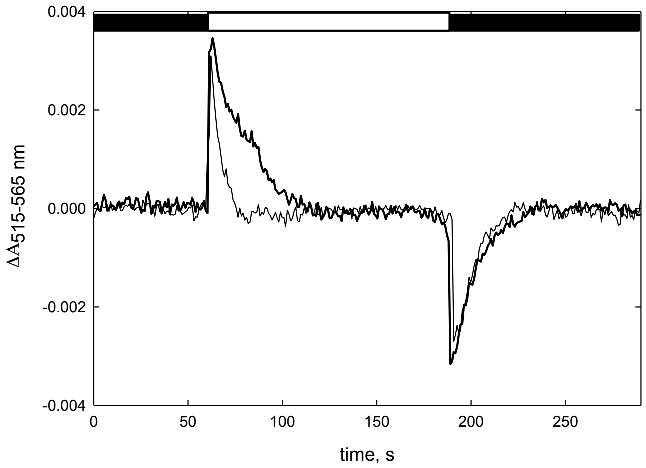
0.04
0.02
0.00
-0.02
-0.04
-0.06
-0.08
Absorption, rel.
1
2
420
440
460
480
500
520
540
560
Wavelength (nm)

Figure 5



A
lut2npq1
ΔA535-560 nm
0.04
0.03
0.02
0.01
0.00
-0.01
B
lut2npq1
ΔA515-560 nm
0.02
0.01
0.00
-0.01
-0.02
C
+nigericin
ΔA515-560 nm
0.03
0.02
0.01
0.00
-0.01
-0.02
D
+valinomycin
ΔA515-560 nm
0.03
0.02
0.01
0.00
-0.01
E
+ nigericin + valinomycin
ΔA515-560 nm
0.03
0.02
0.01
0.00
-0.01
0
200
400
600
800
time, s

Figure 6


0.004
0.002
0.000
-0.002
-0.004
$\Delta A_{515-565}$ nm
0
50
100
150
200
250
time, s