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The xanthophyll cycle pool size controls the kinetics of non-photochemical quenching in *Arabidopsis thaliana*

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Abstract ***Arabidopsis*** **plants overexpressing β-carotene hydroxylase 1 accumulate over double the amount of zeaxanthin present in wild-type plants. The final amplitude of non-photochemical quenching (NPQ) was found to be the same in these plants, but the kinetics were different. The formation and relaxation of NPQ consistently correlated with the de-epoxidation state of the xanthophyll cycle pool and not the amount of zeaxanthin. These data indicate that zeaxanthin and violaxanthin antagonistically regulate the switch between the light harvesting and photoprotective modes of the light harvesting system and show that control of the xanthophyll cycle pool size is necessary to optimize the kinetics of NPQ.**
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Keywords: Xanthophyll cycle; Thylakoid membrane; Non-photochemical quenching; Photoprotection

1. Introduction

The reversible enzymatic interconversion between the carotenoids violaxanthin and zeaxanthin (the xanthophyll cycle) regulates the induction of photoprotective non-photochemical quenching (NPQ) in the thylakoid membranes of plants, the main component of which is the ΔpH-dependent, rapidly-reversible qE [1]. Xanthophyll cycle carotenoids are bound to the LHCII proteins [2–5], mostly at the peripheral V1 site [6,7]. There are two theories to explain the mechanism of action of these carotenoids in qE. Firstly, it has been proposed that zeaxanthin, but not violaxanthin, is a direct quencher of chlorophyll excited states [8]. Secondly, these carotenoids were suggested to allosterically regulate a quenching process that is intrinsic to LHCII [9,10]. Although there is experimental evidence in support of both theories, it has not been possible to conclusively prove that either one explains the role of the xanthophyll cycle *in vivo*.

The first evidence used to support the allosteric model was the phenomenon of "light activation" of qE – pre-illumination of leaves to convert violaxanthin into zeaxanthin shifted the ΔpH requirement for qE but had little effect on the maximum qE capacity [11]. Light activation was also recognised in kinetic effects: the rate of qE formation was faster in the presence of zeaxanthin but the rate of dark relaxation was slower [12,13]. These kinetic effects on qE were consistent with the observation that the rate of quenching of isolated LHCII was accelerated by addition of zeaxanthin but slowed down by violaxanthin [14,15]. It was therefore suggested that violaxanthin and zeaxanthin work antagonistically and competitively, the former as a qE inhibitor and the latter as a qE promoter [9,10]. In order to further test this hypothesis it is necessary to determine whether these effects arise from changes in zeaxanthin concentration or from the change in ratio of zeaxanthin to violaxanthin, expressed as the de-epoxidation state (DES). Here we have used *Arabidopsis* plants in which the expression of the enzyme β-carotene hydroxylase 1 has been increased; these plants accumulate 2–3 times the level of violaxanthin with little perturbation of the content of other pigments [16,17]. Comparing these plants to wild-type plants we show that NPQ kinetics depend upon DES. Furthermore, the data point to a new explanation of why the size of the xanthophyll cycle pool is subject to fine control according to external environmental signals.

2. Materials and methods

Arabidopsis thaliana, cv C24 (*wt*) and β-carotene hydroxylase 1 overexpressing lines (*sChyB*) derived from it [16] were grown for 8–9 weeks in Conviron plant growth rooms with an 8-h photoperiod at a light intensity of 100 μmol photons m^{-2} s^{-1} and a day/night temperature of 22/18 °C. The composition of carotenoids was determined by HPLC for leaf disks rapidly frozen in liquid N_2 [17]. To completely inhibit violaxanthin de-epoxidation, leaves were vacuum infiltrated with a 5 mM dithiothreitol (DTT) solution. Chlorophyll fluorescence kinetic analyses of whole leaves was carried out using a Walz PAM-100 fluorimeter at an actinic light intensity of 1000 μmol photons m^{-2} s^{-1}, with light saturation pulses applied as indicated in the figures. NPQ data analysis used a SigmaPlot software curve-fitting procedure (SPSS, Chicago, IL).

3. Results

As observed previously [16,17], the leaves of dark-adapted *sChyB* plants have nearly three times the content of violaxanthin

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Abbreviations: DES, de-epoxidation state of the xanthophyll cycle pool; DTT, dithiotheitol; LHCII, the main light harvesting complex of photosystem II; NPQ, Non-photochemical quenching; ΔpH, pH difference across the thylakoid membrane; qE, the ΔpH-component of NPQ; *wt*, wild-type plants

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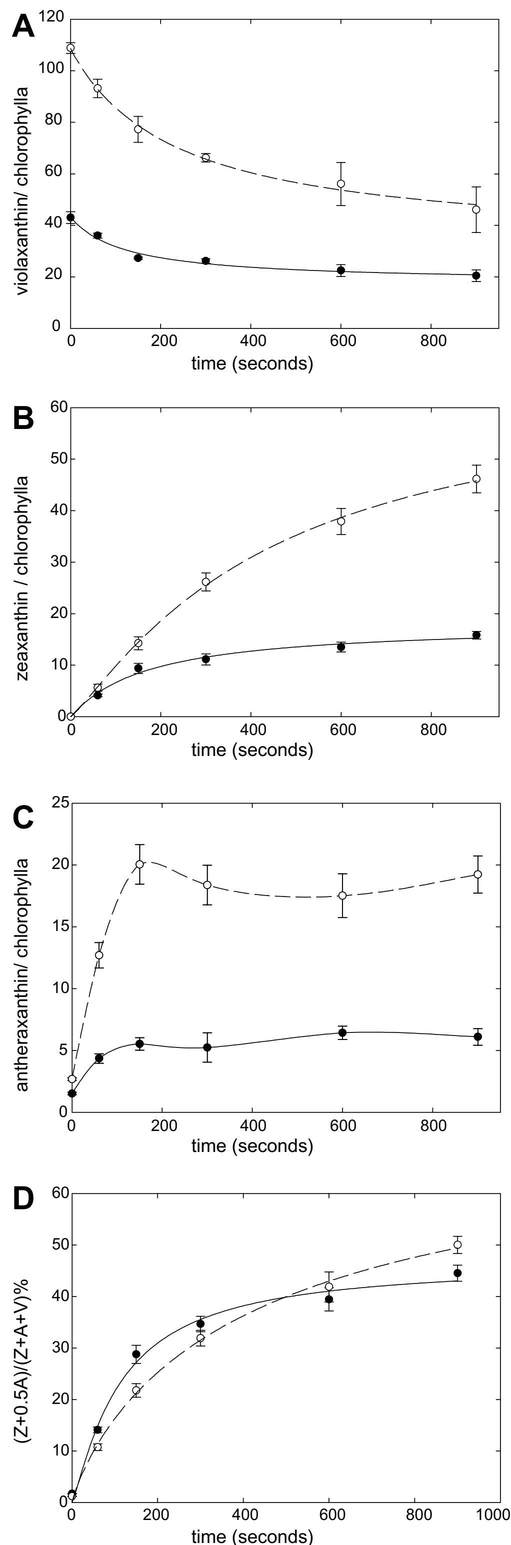

Fig. 1. Kinetics of de-epoxidation in *wt* (filled circles and line) and *sChyB*, (open circles and dashed line) leaves at 1000 μmol photons m^{-2} s^{-1}. (A) violaxanthin; (B) zeaxanthin; (C) antheraxanthin; (D) DES (Zx + 0.5Ax)/(Vx + Zx + Ax)%; A, B, and C values expressed as mmoles carotenoid per mole chlorophyll *a*. Data are means of three independent experiments ± S.E.M.

compared to the wild-type (*wt*) plants (Fig. 1A). Upon illumination, violaxanthin was de-epoxidised to antheraxanthin and zeaxanthin (Figs. 1A–C). During the first 300 s zeaxanthin accumulated rapidly in both *wt* and *sChyB*, after this point the *wt* zeaxanthin level saturated while in *sChyB* it continued to increase, slowing down only after about 15 min. The final levels of zeaxanthin and antheraxanthin were about three times larger in the *sChyB* plants compared to *wt*. Although the initial rate of zeaxanthin formation was apparently the same in *wt* and *sChyB*, the larger xanthophyll cycle pool size in the latter affected the rates of change in their DES (Fig. 1D). Thus, during the first 150 s of illumination the DES was significantly less in the *sChyB* plants compared to the wt (approx. 20% compared to 30%) (Fig. 1D), but after 15 min of illumination the DES was larger in the *sChyB* plants (approx. 50% compared to 40%). No further changes in DES were observed at longer illumination times (data not shown).

The kinetics of induction of NPQ in dark-adapted leaves of *sChyB* plants were different than in those of the *wt* (Figs. 2A and B). In both cases there was a similar initial fast phase of qE formation, reflecting the capacity for qE formation driven by ΔpH formation but without de-epoxidation. This is followed by a second slower phase of NPQ formation which is associated with zeaxanthin accumulation. This phase was much slower in the *sChyB* plants than in the *wt*: at around 100–200 s there was approx. 30% less NPQ in the *sChyB* plants compared to the *wt*. The final amplitude of NPQ obtained was not significantly different, even if the illumination period was extended (data not shown), as previously reported [16,17]. This

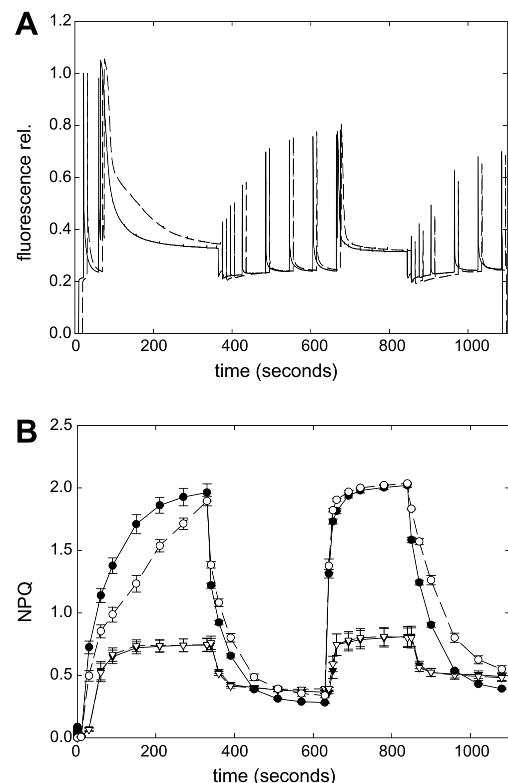

Fig. 2. Fluorescence induction curves (A) and NPQ (B) in *wt* (solid line and filled symbols) and *sChyB* (dashed line and open symbols) leaves at 1000 μmol photons m^{-2} s^{-1} actinic light. Control leaves (circles) leaves infiltrated with DTT (triangles). Arrows indicate actinic light on and off.

was observed over a range of actinic light intensities from 50 to 2000 μmol photons $m^{-2} s^{-1}$ (data not shown).

Following a dark interval to relax the qE-component of NPQ, pre-illumination results in a greatly accelerated induction of NPQ, apparently the same, in both plant types. The relaxation of qE following both illumination periods was slower in the *sChyB* plants compared to the *wt*. Although many other features of photosynthetic activity are different when comparing a dark-adapted leaf with a pre-illuminated one it is unlikely that such effects could be responsible for the differences between the *wt* and *sChyB* plants. When violaxanthin de-epoxidation was inhibited by DTT [18], the kinetics of induction and relaxation of qE were identical in the *wt* and *sChyB* plants (Fig. 2B), showing that the differences in qE formation kinetics in Fig. 2A were due only to differences in de-epoxidation kinetics.

Experiments were carried out to determine the effects of the de-epoxidation state and zeaxanthin concentration on the kinetics of the formation and relaxation of NPQ. Leaves from wt and *sChyB* plants were pre-illuminated with saturating light for varying periods to induce different extents of violaxanthin de-epoxidation. It was found that as the pre-illumination period was lengthened, the subsequent rate of NPQ formation increased for both *wt* and *sChyB* plants (Fig. 3A). However, differences in the relative rate of NPQ formation were found; in *sChyB* (dashed lines) it was considerably slower relative to *wt* (black lines) in dark-adapted leaves (0 s pre-illumination), $t1/2$ = 136 s compared to 56 s respectively, and those pre-illuminated for 60 s ($t1/2$ 31 s and 23 s) or 150 s (18 s and 11 s). However, after longer pre-illumination times the $t1/2$ values

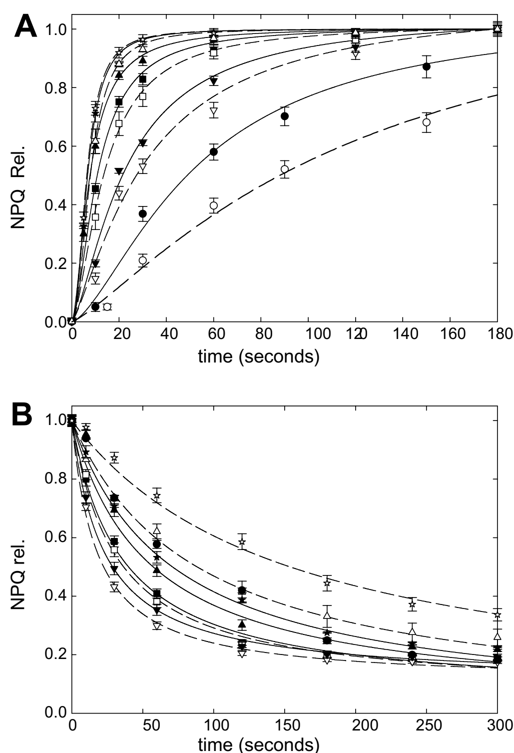

Fig. 3. Kinetics of NPQ formation (A) and relaxation (B) following different periods of pre-illumination. Light intensity, 1000 μmol photons $m^{-2} s^{-1}$. Periods of pre-illumination circles (0 s), inverted triangles (60 s), squares (150 s), triangles (300 s), stars (900 s), as marked on the figure, *wt*, filled symbols, *sChyB* open symbols with solid and dashed lines respectively as the best fits. Data means of three independent experiments ± S.E.M.

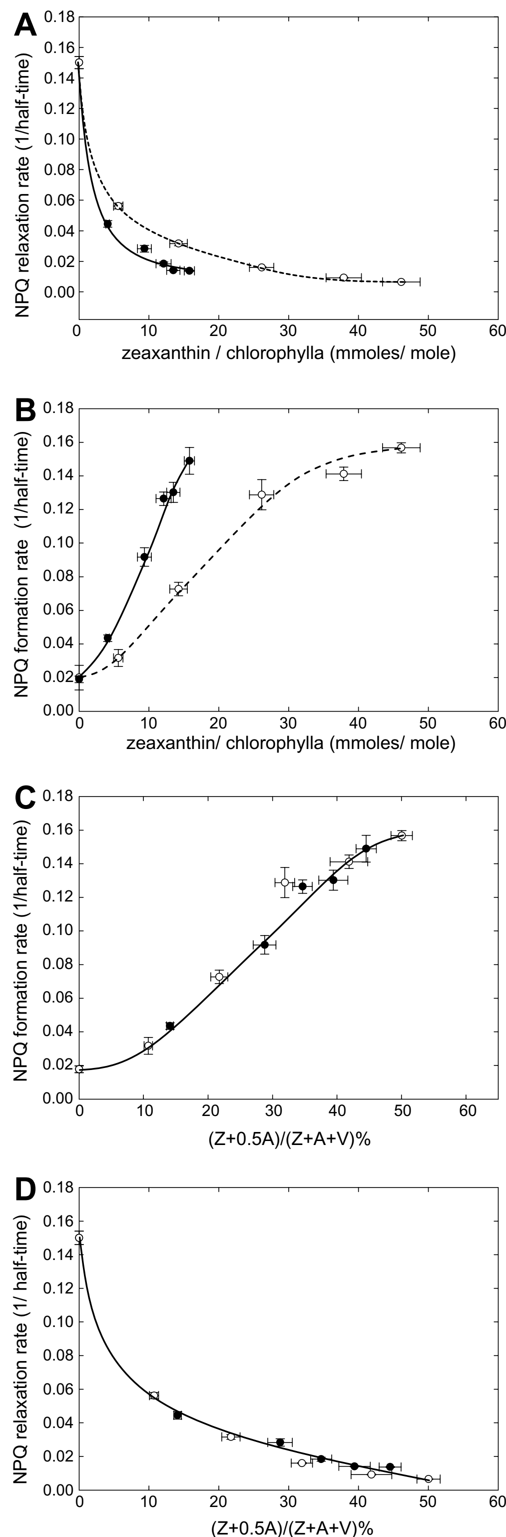

Fig. 4. Rate of NPQ formation (B and C) and relaxation (A and D) versus zeaxanthin content per chlorophyll a (A and B) and DES (C and D)*wt*, black circles and line; *sChyB*, white circles and dashed line. Data are mean of three independent experiments ± S.E.M.

for *sChyB* and *wt* were 7 s and 8 s (300 s) and 6 s and 7 s (900 s), respectively.

The kinetics of relaxation also depended on the length of the pre-illumination period; this time, as the pre-illumination period increased, the relaxation of NPQ became slower. Again *wt* and *sChyB* plants behaved differently (Fig. 3B). With pre-illumination of 60 or 150 s there were faster relaxation times in *sChyB* plants with $t1/2$ values 18 s and 32 s compared to 23 s and 35 s in *wt*. However, for pre-illumination times of 600 and 900 s the relaxation became slower in *sChyB* compared to *wt*, with values of 108 s and 153 s in the former and 54 s and 72 s in the latter.

These kinetics were compared to the measured de-epoxidation states and the zeaxanthin concentrations obtained after different pre-illumination periods. When plotted against zeaxanthin concentration, distinct relationships were observed for *wt* and *sChyB* in both formation (Fig. 4B) and relaxation kinetics (Fig. 4A). It took approximately twice as much zeaxanthin in *sChyB* to achieve a given half-time as in *wt*. However when the kinetics were plotted against de-epoxidation state, a single relationship was found (Figs. 4C and D).

4. Discussion

The amplitude of NPQ has been correlated with the light-dependent de-epoxidation of violaxanthin under a variety of conditions and in different plant species [1]. Here, by comparing plants with different xanthophyll cycle pool size but identical qE capacity, we have been able to show that formation and relaxation of qE is controlled not by the concentration of zeaxanthin, but by the extent of de-epoxidation of the pool.

The formation of NPQ upon illumination of dark-adapted leaves was slower in the *sChyB* plants than in the *wt* despite the appearance of a higher content of zeaxanthin and antheraxanthin. The gradual increase in DES matched the increase in NPQ amplitude in both plant types. Thus, the difference in NPQ formation was explained by the fact that the change in DES is slower because the pool size is larger. It was thus demonstrated that the extent of the rapidly-reversible qE-component of NPQ is determined by the DES, not by the concentration of zeaxanthin.

We have also documented how the rates of formation and relaxation of NPQ were altered by pre-illumination: the longer the pre-illumination, the faster the formation and the slower the relaxation. By comparing the responses in *sChyB* and *wt* plants, we have shown that rates of the transitions between the quenched and unquenched states depend not upon the zeaxanthin concentration but the DES, implying competition between zeaxanthin and violaxanthin in their effects on qE. Previously, using the *in vitro* quenching of LHCII as a model for *in vivo* qE, antagonistic effects of violaxanthin and zeaxanthin on the rate of quenching were described [14,15]. Consistent with this, we now suggest that violaxanthin similarly inhibits the ΔpH-dependent formation of qE, while zeaxanthin promotes it, with the reverse effects on the rate of relaxation. In both cases, a high DES is associated with stabilization of the quenched state. The transition into the quenched state of isolated LHCII involves a conformational change [19], also found *in vivo* upon qE formation [20], which brings about energy transfer from chlorophyll to the S1 state of Lutein 1 [20]. The control of the rate of the transition into and out of this state by the DES is consistent with the proposed role of the xanthophyll cycle carotenoids as allosteric regulators of qE [9,10]. The data could also be accommodated within a model in which the only role of zeaxanthin is as the direct quencher [8], either bound to PsbS [21], LHCII [22] or to a minor antenna complex [23,24]. However, important new features would need to be invoked: there must be competition between violaxanthin and zeaxanthin for the quenching site; and the rate of binding and release from this site must be rate limiting for qE formation and relaxation respectively. Further experiments are needed to verify these aspects.

The demonstration that the extent and kinetics of NPQ are controlled by the DES gives a new insight into the factors which determine the size of xanthophyll cycle pool. Plants exposed to excess light increase the size of the xanthophyll cycle pool [25–27], a response assumed to increase stress tolerance. Indeed, *sChyB* plants have a greater tolerance to photo-oxidative stress compared to *wt* plants as a result of the increased antioxidant activity from zeaxanthin [16,17]. However, such a beneficial effect of a larger xanthophyll cycle pool in *sChyB* plants left unexplained why the pool was not constitutively larger in the wild-type plants, since an altered expression of only one gene was involved. The data shown here provide an explanation: a large xanthophyll cycle pool retards both the kinetics of formation and relaxation of qE. The former effect may predispose the plants to greater photoinhibition following sudden increases in light intensity, whilst the latter could result in photosynthetic yield losses upon transition from high to low light intensity [28]. Thus regulation of the xanthophyll cycle pool has to balance the positive effect of increased antioxidant activity from a high zeaxanthin concentration with the negative effect of compromised qE dynamics. In high light grown plants, this negative effect is presumably ameliorated by other features of the acclimation of the thylakoid membrane that control qE: an increase in PsbS concentration [29], a change in antenna composition [30] and alteration in grana stacking [31]. Together these allow rapid qE kinetics while also affording increased antioxidant protection of thylakoid lipids by the larger xanthophyll cycle pool size. In contrast, in low light grown plants, these features of the thylakoid membrane are optimized for efficient light utilization, a state which is consequently incompatible with a large xanthophyll cycle pool size.

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