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Lutein from de-epoxidation of lutein epoxide replaces zeaxanthin to sustain an enhanced capacity for non-photochemical chlorophyll fluorescence quenching in avocado shade leaves in the dark

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Abstract

Leaves of avocado (Persea americana Mill.) that develop and persist in deep shade canopies have very low rates of photosynthesis but contain high concentrations of lutein epoxide (Lx) that are partially de-epoxidized to lutein (L) after one hour exposure to 120 to 350 μmol photons m-2 s-1 increasing the total L pool by 5-10% (ΔL). De-epoxidation of Lx to L was near stoichiometric and similar in kinetics to de-epoxidation of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z). Although the V pool was restored by epoxidation of A and Z overnight, the Lx pool was not. Depending on leaf age and pretreatment, the pool of ΔL persisted for up to 72 h in the dark. Metabolism of ΔL did not involve epoxidation to Lx. These contrasting kinetics enabled us to differentiate three states of the capacity for nonphotochemical chlorophyll fluorescence quenching (NPQ) in attached and detached leaves: ΔpH-dependent (NPQΔpH) before de-epoxidation; after de-epoxidation in the presence of ΔL, A, and Z (NPQΔLAZ); and after epoxidation of A+Z but with residual ΔL (NPQΔL). The capacity of both NPQΔLAZ and NPQΔL was similar and 45% larger than NPQΔpH but dark relaxation of NPQΔLAZ was slower The enhanced capacity for NPQ was lost after metabolism of ΔL. The near equivalence of NPQΔLAZ and NPQΔL provides compelling evidence that the small dynamic pool ΔL replaces A+Z in avocado to "lock-in" enhanced NPQ. Results are discussed in relation to data obtained with other Lx-rich species and in mutants of Arabidopsis with increased L pools.

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Lutein replaces zeaxanthin in photoprotection of avocado

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Lutein from de-epoxidation of lutein epoxide replaces zeaxanthin to sustain an enhanced capacity for non-photochemical chlorophyll fluorescence quenching in avocado shade leaves in the dark

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ABSTRACT

Leaves of avocado (*Persea americana* Mill.) that develop and persist in deep shade canopies have very low rates of photosynthesis but contain high concentrations of lutein epoxide (Lx) that are partially de-epoxidized to lutein (L) after one hour exposure to 120 to 350 µmol photons m^{-2} s^{-1} increasing the total L pool by 5-10% (ΔL). De-epoxidation of Lx to L was near stoichiometric and similar in kinetics to de-epoxidation of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z). Although the V pool was restored by epoxidation of A and Z overnight, the Lx pool was not. Depending on leaf age and pretreatment, the pool of ΔL persisted for up to 72 h in the dark. Metabolism of ΔL did not involve epoxidation to Lx. These contrasting kinetics enabled us to differentiate three states of the capacity for nonphotochemical chlorophyll fluorescence quenching (NPQ) in attached and detached leaves: ΔpH-dependent ($NPQ_{\Delta pH}$) before de-epoxidation; after de-epoxidation in the presence of ΔL, A, and Z ($NPQ_{\Delta LAZ}$); and after epoxidation of A+Z but with residual ΔL ($NPQ_{\Delta L}$). The capacity of both $NPQ_{\Delta LAZ}$ and $NPQ_{\Delta L}$ was similar and 45% larger than $NPQ_{\Delta pH}$ but dark relaxation of $NPQ_{\Delta LAZ}$ was slower The enhanced capacity for NPQ was lost after metabolism of ΔL. The near equivalence of $NPQ_{\Delta LAZ}$ and $NPQ_{\Delta L}$ provides compelling evidence that the small dynamic pool ΔL replaces A+Z in avocado to “lock-in” enhanced NPQ. Results are discussed in relation to data obtained with other Lx-rich species and in mutants of Arabidopsis with increased L pools.

INTRODUCTION

The violaxanthin cycle (V-cycle) based on reversible inter-conversion of zeaxanthin (Z), antheraxanthin (A) and violaxanthin (V) stabilizes non-photochemical chlorophyll fluorescence quenching (NPQ) and confers photoprotection in most green plants and algae (Demmig et al 1987; Demmig-Adams and Adams 1992; Niyogi 1999; Förster et al 2001). Since Bungard et al (1999) an additional xanthophyll cycle (the Lx-cycle), involving the α-carotene (α-C) pathway pigments lutein (L) and lutein epoxide (Lx), has been recognized in many diverse taxa (García-Plazaola et al 2003; Matsubara et al 2003, 2008).

The Lx-cycle is distinctive in that in some species it was reversible with kinetics similar to that of the V-cycle (Bungard et al 1999; Matsubara et al 2001; Esteban et al 2010), whereas in others it was only very slowly reversible (Matsubara et al 2005; García-Plazaola et al 2007; Förster et al 2009). Leaves that developed and persisted in deep shade canopies of avocado (*P. americana* Mill.) showed particularly high concentrations of Lx (Esteban et al 2008). Although de-epoxidation of Lx occurred during a few hours of exposure to sunlight and made a small addition to the much larger pool of L (ΔL) little epoxidation of ΔL to Lx was observed overnight and Lx concentrations were only restored after 3 to 5 weeks in the shade (Förster et al 2009). In contrast, the V-cycle was largely reversible on a diel basis.

Slow reversibility of the Lx-cycle in some species led to the hypothesis that augmentation of the L pool by de-epoxidation of Lx might "lock-in" photoprotection (García-Plazaola et al 2003; Matsubara et al 2005). Evidence for persistent, enhanced capacity for NPQ due to ΔL, after A+Z had largely reverted to V, was reported during photosynthetic induction in-vivo with leaves of *Quercus rubra* and *Inga marginata* (García-Plazaola et al 2003; Matsubara et al 2008). These experiments provided the first direct evidence for potentially similar functional roles for ΔL and Z in photoprotection (García-Plazaola et al 2007; Horton et al 2008) and presaged the recent demonstration of a Z-like radical cation that appeared when NPQ was engaged in a Z-free, L-enriched Arabidopsis mutant (Avenson et al 2007; Li et al 2009). However, as yet there have been no quantitative comparisons of the components of NPQ associated with ΔL and A+Z.

Not surprisingly, shade leaves of avocado proved to be extremely responsive to light during treatment and assay. Photosynthetic induction curves at selected, often high, light intensities have been widely used to assess NPQ in relation to xanthophyll pigment composition in Arabidopsis and Chlamydomonas mutants (Niyogi et al 1998; Pogson et al 1998; Förster 2001), as well as in *Quercus* and *Inga*. These assays proved unsatisfactory for

quantitative comparisons of NPQ components in shade leaves of avocado, and were confounded by responses of stomata and CO_2 supply (Takayama et al 2008), by photosynthetic induction following treatment, and by xanthophyll de-epoxidation during the measurement. In contrast, the light response curve traditionally used to distinguish biochemical and physiological relationships in sun and shade plants (Björkman 1981), has found less application in these model systems (Russell et al 1996; Bailey et al 2001; Pérez-Bueno and Horton 2008). We tailored rapid light response curves (RLRC) using chlorophyll fluorescence (Schreiber et al 1994; White and Critchley 1999) to the requirements of avocado shade leaves. These minimized de-epoxidation during the assay and facilitated quantitative analysis of NPQ while monitoring opening of stomata and induction of photosynthetic metabolism following light treatments.

Rapid light response curves were used to differentiate and quantitatively compare three components of the capacity for NPQ in avocado leaves that are expressed following inter-conversions of xanthophyll pigments. Semantic confusion that has emerged from use of the terms qE and qN (Horton et al 1996), particularly in reference to different components of energy-dependent quenching, distinguished on different criteria, in different species and treatments led us to a simple designation the three types of NPQ in shade leaves of avocado with subscripts as follows:

(i) $NPQ_{\Delta pH}$ in dark adapted, un-induced leaves with closed stomata that contain high concentrations of Lx and V and extremely low levels of A and Z. We presume this component is associated with development of ΔpH in chloroplast thylakoid membranes in the absence of external CO_2 (Krause et al 1982) during assay, prior to de-epoxidation of Lx and V.

(ii) $NPQ_{\Delta LAZ}$ assayed in leaves after modest light exposures that open stomata, induce photosynthetic metabolism and lead to partial but stoichiometric de-epoxidation in the Lx- and V-cycles. We presume this component is associated with the stabilization of NPQ by both ΔL and A+Z.

(iii) $NPQ_{\Delta L}$ in leaves treated as in (ii) but assayed after the 24-72 h in the dark following almost complete epoxidation of the V cycle (disappearance of A+Z) but not of Lx. We presume this component is associated with stabilization of NPQ by ΔL alone.

In this paper we provide the first direct evidence that the capacity of $NPQ_{\Delta L}$ is quantitatively similar to $NPQ_{\Delta LAZ}$ suggesting that a small addition (~5 - 10%) to the total L pool (ΔL) substitutes for A+Z and maintains the capacity of elevated NPQ for up to 72 h in the dark. The duration of elevated $NPQ_{\Delta L}$ is determined by the rate of dark metabolism of ΔL by processes other than epoxidation to Lx. Importantly, dark relaxation of $NPQ_{\mathbf{\Delta L}}$ is faster

than that of $NPQ_{\Delta LAZ}$ and similar to $NPQ_{\Delta pH}$. These observations are discussed in relation to a common mechanism of L and Z based NPQ recently proposed in other studies, and in relation to potential functions of L-enhanced photoprotection in shade leaves.

RESULTS

Optimization of light treatments to manipulate xanthophyll pigment composition in shade leaves of avocado and to avoid subsequent de-epoxidation during assays

Our previous studies of diel changes in Lx- and V-cycle pigment composition in avocado leaves suggested little de-epoxidation occurred at light intensities < 200 µmol photons m^{-2} s^{-1} (Förster et al 2009). The optimal light intensities needed to bring about quantitatively similar de-epoxidation of Lx and V in mature leaves of shade-grown avocado plants without photoinhibition were explored with a single large shade-grown avocado leaf detached mid afternoon and arranged with the petiole in water in the open greenhouse. The top third of the leaf was covered by aluminum foil (Al-foil) as a dark control and neutral density filters were placed on the remainder of the leaf to give 50 x 50 mm areas exposed to 80 and 163 µmol photons m^{-2} s^{-1}, leaving other parts of the leaf exposed to the weak afternoon sunlight (300 µmol photons m^{-2} s^{-1}) for 90 min. As shown in Fig. 1, leaf discs from dark control areas contained very little A (1.6 ± 0.2 mmol mol^{-1} Chl), only traces of Z (0.5 ± 0.5 mmol mol^{-1} Chl). Note that the 3-5 fold larger pools of L are shown reduced by 100 mmol mol^{-1} Chl (designated L*) in all figures unless stated otherwise to facilitate comparison of stoichiometry with other xanthophylls. Whereas de-epoxidation of Lx to L was first evident after exposure to 163 and 300 µmol photons m^{-2} s^{-1} (ΔL ~15 mmol mol^{-1} Chl), quantitatively similar de-epoxidation of V to A+Z occurred already at 80 µmol photons m^{-2} s^{-1}. Therefore, all subsequent actinic light treatments to manipulate the pool sizes of both xanthophylls cycles were done at 120 and 350 µmol photons m^{-2} s^{-1} for 60 min.

Measurements of pigments in discs punched at random from control and treated areas of attached mature leaves in 9 experiments showed quantitatively similar, almost stoichiometric de-epoxidation of Lx and V (Table I). Initial levels of A were close to the limits of detection (0.8 mmol mol^{-1} Chl) whereas Z was not detectable in most cases. Although levels of residual A+Z after 21 to 72 h recovery in the dark lab (0-5 µmol photons m^{-2} s^{-1}) were higher, residual A+Z and de-epoxidation (DS = A + Z / V + A + Z) was about the same as that produced from de-epoxidation of V during the optimized RLRC assays

described below. Importantly, measurements of F_v/F_m showed these light exposures caused little photoinhibition (Table I).

Some de-epoxidation of V, but not of Lx, always occurred during chlorophyll fluorescence assays. For example, contrary to expectations of Takayama et al (2008), imaging photosynthetic induction for 20 min at 100 µmol photons m^{-2} s^{-1} caused substantial de-epoxidation of V and increased DS from 0.04 to 0.24 (data not shown). Assuming that each of the 16 x 1 s saturating flashes used in this protocol delivered 5,000 µmol photons m^{-2}, this assay delivered a total of 200 mmol photons m^{-2}which is equivalent to the photons delivered in a 6 min photosynthetic induction assay at 300 µmol photons m^{-2} s^{-1} with the MINI-PAM (Fig. 2 A). This problem had been mitigated previously by infiltration of leaf discs with DTT, an inhibitor of violaxanthin de-epoxidase (Adams et al 1990) but clearly this was impracticable in our measurements with large attached leaves. Moreover, infiltration with DTT had been reported to substantially depress F_v/F_m (Matsubara et al 2008). Seeking a compromise between the actinic light intensity needed to produce good resolution of kinetic responses and that needed to minimize de-epoxidation during assays (Fig. 2 B), we chose a rapid light response curve (RLRC) protocol delivering about 60 µmol photons m^{-2} using 8 x 30 s steps with actinic light from 0 to ~ 400 µmol photons m^{-2} s^{-1}.

A persistent 5 to 10% addition (ΔL) to the L pool from de-epoxidation of Lx sustains enhanced capacity for NPQ after conversion of A+Z to V overnight

The less intrusive RLRC assays enabled us to follow enhancement of NPQ in the presence of ΔL under standardized conditions for up to 72 h in the dark. In addition, these curves routinely presented light response profiles of 1-qP (a measure of the redox state of the acceptor side of PSII) and photosynthetic electron transport (ETR). These data helped us ensure that other factors, such as stomatal opening and induction of photosynthesis during light treatments, did not confound the interpretation of NPQ data.

Light treatments caused an initial de-epoxidation of ~ 30% of both Lx and V pools with practically no change in PSII efficiency indicated by steady F_v/F_m (Fig. 3 A). Pigment composition changed little during 1 h dark following treatment. V-cycle DS declined by one-third, mainly due to epoxidation of about half the Z pool, without change in the pools of A or L. Although 1-qP was depressed by 20% at low PFD immediately after exposure, the profiles of control and exposed areas were the same after 1 h in the dark (Fig. 3 B), consistent with the

initial increase in ETR that also returned to control levels after 1 h in the dark (Fig. 3 C). These responses of 1-qP to light treatment and their relaxation to controls within 1 h dark were similar in all experiments and are not presented in subsequent graphs. Light treatment led to a relatively larger increase in capacity for NPQ at lower light intensities during assay, as well as a higher level of NPQ at light saturation (Fig. 3 D). Notably, in the presence of A+Z NPQ relaxed more slowly in the dark during assay (Fig. 3 D). The small decline of the NPQ profile in exposed areas during 1 h dark may reflect the partial epoxidation of the Z pool (Fig. 3 A).

In general, L was not metabolized during 24 h in the dark, whereas expoxidation of A+Z was practically complete (Fig. 3 A). Photosynthetic parameters in the Al-foil controls after 21 h in the dark were almost identical to the dark controls at the beginning of the experiment. Profiles of 1-qP (cf. Fig. 3 B, E) and ETR (cf. Figs. 3 C, F) in control and recovered areas were the same after 1 h and 21 h dark. Enhanced capacity for NPQ persisted in exposed areas after 21 h dark (Fig. 3 G), in spite of the epoxidation of A+Z. Importantly the kinetics of dark NPQ relaxation now were much the same in control and exposed areas (Fig. 3 G). The slower decline of NPQ in the dark following de-epoxidation of Lx and V, and its fast relaxation after epoxidation of A+Z, was observed in all experiments with attached leaves. This showed that although ΔL substituted for A+Z and sustained higher capacity for NPQ in the dark, it did not slow the dark relaxation of NPQ observed in the presence of A+Z.

Enhanced capacity for NPQ persists for up to 72 h in the dark without A+Z, while ever ΔL is detectable

Younger, fully-expanded leaves with lower Lx content (Fig. 4 A) showed much the same de-epoxidation of Lx and V after light treatments, and commencement of epoxidation of A+Z during 1 h and 3 h in the dark. Photosynthetic induction in the light treatment increased ETR during assay which declined to near control levels after 3 h dark (Fig. 4 B). Non-photochemical quenching increased following light treatment then declined slightly as of the level of Z began to decrease after 3 h in the dark, but the slower dark relaxation of NPQ persisted (Fig. 4 C). Epoxidation of A+Z was largely complete after 24 h dark but ΔL was still detectable (Fig. 4 D). Although ETR of treated areas was somewhat lower than controls after 24 h dark (Fig 4 E), persistent ΔL sustained higher capacity for NPQ (Fig. 4 F) with relaxation kinetics similar to controls. Metabolism of ΔL between 24 and 48 h re-established control levels of L (Fig. 4 C) and this was reflected in identical profiles of ETR and NPQ

(Fig. 4 G, H). Clearly, the capacity for enhanced NPQ persisted only as long as ΔL was detectable, and interestingly, the metabolism of ΔL between 24 and 48 h dark was not due to epoxidation to Lx.

The longest period of enhanced capacity for NPQ associated with sustained ΔL in our experiments was found in isolated leaf discs 72 h after the start of the actinic light treatment (Fig. 5). Light treatment produced a persistent 30% decrease in Lx and a less than stoichiometric, but still substantial, increase in ΔL after 48 and 72 h in the dark. The levels of V, A, and Z were the same as controls (Fig. 5 A) and A+Z formed during the light treatment was epoxidised to V within 24 h in the dark (data not shown). The ETR observed after 48 and 72 h in the dark was slightly greater than in controls (Fig. 5 B) showing that elevated NPQ capacity (Fig. 5 C) was not an artifact of reduced ETR. The relaxation of NPQ was the same as controls throughout.

We were able to eliminate sustained ΔL accumulation and the associated enhanced $NPQ_{\Delta L}$ by pretreatment of a plant in the dark lab for 11 days (0-5 µmol photons m^{-2} s^{-1}) prior to light treatment (1 h at 200 µmol photons m^{-2} s^{-1}). Prolonged dark pretreatment did not alter de-epoxidation of Lx and V during exposure, or epoxidation of A+Z overnight (Fig. 6 A). The response of ETR and NPQ to the light treatment was as found in all previous experiments (Fig. 6 B, C) as was the slower dark relaxation of NPQ in the presence of ΔL and A+Z. However, prolonged dark pretreatment specifically accelerated metabolism of the pool of ΔL. Both pools of ΔL and A+Z were metabolized overnight and no enhancement of NPQ was detected during assays (Fig. 6 B, C) and as expected, relaxation kinetics of NPQ were the same as controls.

DISCUSSION

Previous studies suggested that the presence of Lx- and V-cycles in shade canopy leaves of woody plants from Mediterranean (García-Plazaola et al 2003) and tropical American forests (Matsubara et al 2008) indicated that photoconversion of Lx to L may "lock-in" photoprotection overnight after restoration A+Z to V. The pronounced difference in the kinetics of epoxidation of L and A+Z in the Lx-rich shade leaves of avocado (Esteban et al 2008; Förster et al 2009) offered an ideal system in which to probe the role in photoprotection of the particular fraction of L (ΔL from Lx) that persists after epoxidation of A+Z. This paper presents the first comprehensive and quantitative analysis using non-intrusive rapid light response curves to differentiate three states of the capacity for NPQ

associated with distinct xanthophyll pigment compositions in these plants and substantially extends our understanding of this enigmatic role of L in-vivo.

The three pigment composition states were established by exposing shade leaves of avocado to modest, non-photoinhibitory light treatments (120-350 µmol photons m^{-2} s^{-1}). The transitions between $NPQ_{\Delta pH}$ (in the absence of de-epoxidation of xanthophylls), to $NPQ_{\Delta LAZ}$ (after stoichiometric de-epoxidation of Lx and V) and to $NPQ_{\Delta L}$ (persistence of ΔL following overnight epoxidation of A+Z) were assayed using sensitive RLRC in which a range of 30 s exposures to increasing PFD was tailored to minimize de-epoxidation of xanthophyll pigments during assay. The non-intrusive assays were supported with simple tests, such as the "Vaseline patch", that help monitor physiological (closure of stomata) and biochemical (induction of photosynthetic metabolism) processes with the potential to confound interpretations of NPQ capacity in relation to pigment composition in-vivo. We did not use routine photosynthetic induction curves to analyze changes in NPQ in shade-grown avocado leaves because they produced higher de-epoxidation of V (Fig. 2). As an aside, we strongly recommend that de-epoxidation should be routinely assessed during NPQ induction assays at high PFD.

Four functional aspects of photoprotection that have arisen from this investigation of avocado shade leaves will be discussed. First, we will explore comparative quantitative aspects of the substitution of A+Z by ΔL to sustain enhanced capacity for NPQ for up to 72 h in the dark and expand earlier hypotheses that de-epoxidation of Lx to L "locks-in" photoprotection (García-Plazaola et al 2003, 2007; Matsubara et al 2005, 2007, 2008). Second and contrary to expectations, we briefly discuss the demonstration that the duration of enhanced NPQ capacity was determined by metabolism of ΔL by processes other than its slow epoxidation to Lx. Third, we point out that in avocado leaves, de-epoxidation of V to A+Z slows the dark relaxation of NPQ during assay, whereas ΔL from de-epoxidation of Lx does not affect NPQ relaxation kinetics. Fourth, we suggest some implications of sustained NPQ capacity with the above properties for photoprotection in shade canopies and light use efficiency during sun-flecks.

Past research has been focused on the extent to which Z-independent components of chlorophyll fluorescence quenching ($NPQ_{\Delta pH}$) participate in NPQ even after de-epoxidation xanthophylls. There is a debate as to whether these components are additive or substitutive (Adams et al 1990; Bilger and Björkman 1994; Gilmore et al 1998; Li et al 2004), and whether common or separate mechanisms are involved (Pogson et al 1998; Finazzi et al

2004). Recent evidence suggests that both involve common antenna based mechanisms (Johnson et al 2009), and moreover, at least two distinct quenching mechanisms involving Z (type I and type II) seem to be involved. In the type I mechanism, Z in the L2 position of minor antenna complexes is a direct quencher of excited chlorophyll as demonstrated by a distinctive radical cation (Avenson et al 2008). In the type II mechanism, Z is an allosteric modulator of the ΔpH-sensitivity of NPQ involving xanthophyll exchanges in the L1 position of Lhcs (Johnson et al 2009). It is beyond the scope of our data to speculate further on these mechanistic details.

We took a simple approach to quantitative estimation of the relative NPQ capacity using the total areas under RLRC of NPQ vs PFD to compare $NPQ_{\Delta pH}$ and $NPQ_{\Delta LAZ}$ in avocado leaves. Comparison of controls with those after 1 h exposure and after 1 h dark with similar amounts of ΔL and A+Z, show an increase of 45.1 ± 1.5% (n = 4 experiments) in $NPQ_{\Delta LAZ}$ compared to $NPQ_{\Delta pH}$. In other words, in these assays the capacity of Z (and ΔL)-independent $NPQ_{\Delta pH}$ in control leaves was about 69% of $NPQ_{\Delta LAZ}$ after de-epoxidation of Lx and V. Compared to leaves with persistent ΔL after 21-24 h dark recovery, $NPQ_{\Delta L}$ was increased by 36.6 ± 2.5% over $NPQ_{\Delta pH}$. Even after 48 h dark recovery $NPQ_{\Delta L}$ was still 84% of $NPQ_{\Delta LAZ}$. Thus, our analyses provide evidence that ΔL effectively and almost completely substitutes for A+Z in stabilizing capacity for NPQ in avocado.

The mechanistic aspects of substitution of A+Z by ΔL are little known. Analyses of shade leaves of *Inga sapindoides* (Matsubara et al 2007) showed that Lx was the principal xanthophyll in LHCII complexes. In these experiments de-epoxidation of part of the Lx pool to L on exposure to 200 μmol photons m^{-2} s^{-1} for 30 min led to the replacement of Lx with L in the peripheral V1 site of LHCII trimers, as well as in the internal L2 site of both monomeric and LHCII trimers in both photosystems. It was concluded that the slowly reversible Lx↔L exchange took place at the same binding sites as reversible V↔A+Z exchange. We suggest the observed sustained high capacity for $NPQ_{\Delta L}$ is based on substitution of A+Z with L in these sites, but further evidence for the association of ΔL, A and Z with specific LHCII proteins in avocado is needed before this substitution can be integrated into general models of regulated excitation dissipation in antenna complexes (de Bianchi et al 2010).

Whereas the structural roles of L in the antennae of the photosynthetic apparatus have been the focus of much past research, the photoprotective function of L has remained enigmatic (Gilmore 2001). Analyses of Arabidopsis mutants for a decade or so had not

uncovered direct evidence of a role for L in NPQ. Although *lutein-deficient2 (lut2)* mutants show less NPQ (Pogson et al 1998) and although other mutants with lower than wild type L content show impaired NPQ, mutants with slightly enhanced L and enhanced NPQ (Pogson and Rissler 2000; Niyogi et al 2001) have been difficult to evaluate, principally because none were markedly deficient in Z. A recently constructed suppressor of the *npq1* mutant *suppressor of zeaxanthinless1 (slz1 npq1)* lacks Z, has low levels of V and A, accumulates more L and α-carotene than wildtype, and retains about 50% wild type NPQ (Li et al 2009). The authors state "*Analysis of the carotenoid radical cation formation and leaf absorbance changes strongly suggest that the higher amount of lutein substitutes for zeaxanthin in qE, implying a direct role in qE,...*". In the light of these observations in Arabidopsis it seems a compelling next step is to examine shade leaves of avocado in the $NPQ_{\Delta pH}$, $NPQ_{\Delta LAZ}$ and $NPQ_{\Delta L}$ states to discover whether distinctive leaf absorbance changes, and changes in carotene radical cation formation (Avenson et al 2008; Li et al 2009), persist in-vivo following the substitution of A+Z by ΔL.

A still unanswered key question is how the partitioning of the majority of the L pool to structural functions in Lhcs is regulated and how a small additional pool (ΔL) participates in excitation dissipation. This bears some analogy to the situation in the Arabidopsis mutants *lut2*, where V-cycle pigments substitute for the structural roles of L (Pogson et al 1996), and in *aba1-3* and *aba1-4* which are constitutively enriched in Z and depleted in V+A, but only a small fraction of the Z pool seems to be involved in NPQ photoprotection (Hurry et al 1997). Also, this may related to the still ill-defined fraction of the L and Z pools that mitigate photo-oxidation (Havaux and Niyogi 1999; Johnson et al 2007) and the contribution of these ROS scavenging activities to photoprotection (Förster et al 2005; Dall'Osto et al 2006). It remains to be seen if the recently described partitioning (Lepetit et al 2010) of xanthophylls in diatoms between protein-associated fractions (involved in NPQ) and lipid-dissolved fractions (involved in ROS scavenging) can be achieved with avocado thylakoids. Both L and Z serve similar roles in visual systems (Kim et al 2006), and photo-oxidation products of both xanthophylls have been identified in retinas of the human eye (Khachik et al 1997). We observed earlier that sudden exposure of shade-grown avocado leaves to sunlight was initially accompanied by a decline in the total L pool in spite of Lx de-epoxidation (Förster et al 2009), which could be explained by photo-oxidation of L. It would be of interest to investigate the occurrence of L (and Z) photo-oxidation products in avocado leaves under these conditions.

The transition from higher capacity $NPQ_{\Delta L}$ to lower capacity $NPQ_{\Delta pH}$ after prolonged darkness was associated with disappearance of ΔL. Against expectation, the decline of ΔL was not coupled to epoxidation of ΔL to Lx and restoration of the Lx pool. Interestingly, metabolism of ΔL occurred much faster when leaves were pretreated with 11 days in the dark lab prior to light exposure, when ΔL was fully metabolized in 24 h darkness without epoxidation (Fig. 6). In previous experiments (Förster et al 2009) slow recovery of Lx pools in prolonged shade after de-epoxidation in sunlight was not simply related to epoxidation of L or accumulation of the biosynthetic precursor α-carotene, as this pool remained constant and the total L pool declined more than twice as rapidly as the Lx pool recovered. Obviously, other pathways to metabolize ΔL became dominant in prolonged dark. Little is known about degradation of xanthophyll pigments, although carotenoid cleavage dioxygenases and non-enzymatic degradation have been implicated in some tissues (Cazzonelli and Pogson 2010). At present we cannot explain the dark-induction of L metabolism.

Another intriguing aspect of the NPQ-pigment relationships was that relaxation kinetics of NPQ were delayed specifically by the presence of A+Z, whereas both $NPQ_{\Delta pH}$ and $NPQ_{\Delta L}$ relaxation rates were equally fast in the absence of A+Z. This seems consistent with previous evidence that Z slows the relaxation of qE in isolated chloroplasts (Nocter et al 1991). More recently, Johnson et al (2008) reported that in wildtype Arabidopsis and a Z-accumulating β-carotene hydroxylase overexpression line (*sChy B*), slower relaxation in the dark after assay was not related to the absolute Z pool itself, but to higher DS. Other contributing factors to the slow relaxation of $NPQ_{\Delta LAZ}$ cannot be ruled out. For example, the slower decay of $NPQ_{\Delta LAZ}$ might reflect post-illumination interactions with leaf respiratory metabolism and could reflect slowly declining energization of chloroplast thylakoids in darkness, when chloroplast and/or mitochondrial ATP pools sustain NPQ in the dark through a ΔpH maintained by ATP hydrolysis rather than photosynthetic electron transport (Gilmore and Björkman 1995).

Persistent, ΔL-enhanced capacity for ($NPQ_{\Delta L}$) was first recognized in shade leaves of *Quercus* and *Inga* examined in the field (García-Plazaola et al 2003, Matsubara et al 2008) and has been confirmed in some *Inga* spp. in the Eden Project in Cornwall, UK (Nichol and Osmond, unpublished) as well as avocado shade leaves in orchards in tropical Eastern Australia (Osmond, Förster and Leonardi, unpublished). The deeply shaded canopies of the latter retain up to 30 leaves on shoots with photosynthetic capacities and pigment compositions that are comparable to those of shade-grown plants examined here. The modest

light treatments used to bring about xanthophyll pigment conversions in our greenhouse experiments correspond reasonably to changes in shade canopy light environment following upper canopy disturbance in storms, and to changes following routine orchard canopy pruning practices (Whiley et al 2002; Osmond, Förster and Leonardi, unpublished). The 30s steps in our RLRC curves reasonably correspond to sun light fleck experiences in tropical canopies (Pearcy 1990) and to sun fleck measurements in avocado orchards in California (Mickelbart, Stilwell, Arpaia and Heath, unpublished). The effect of ΔL on "locking-in" higher capacity of $NPQ_{\Delta L}$ in the shade for several days, without the penalty of slower relaxation associated with the presence of Z, may offer a lower penalty in light energy use efficiency during sequences of sun flecks than the slower relaxing $NPQ_{\Delta LAZ}$ (Zhu et al 2004). These consequences of the slowly reversible Lx-cycle need to be evaluated in relation to the reversible engagement of V-cycle in these plants. For example, we found previously that sunlight exposure of avocado shade leaves results in sustained low F_v/F_m (Förster et al 2008) and persistently higher L and A+Z. Separation of the contributions of slowly reversible Z and/or L-related NPQ from xanthophyll-independent, low-efficiency photoinactivated PSII centers to these observations will be discussed in a subsequent paper.

In conclusion, this study established improved assay methods to analyze xanthophyll pigment-NPQ relationships in avocado shade leaves that resolved three different NPQ components based on the associated xanthophyll de-epoxidation state. Based on these distinctions we were able to provide novel, direct evidence for a special role of the fraction ΔL of the L pool that had been generated by Lx de-epoxidation in conferring higher capacity for NPQ induction while allowing rapid dark relaxation, which implies a direct role of L in photoprotection and may improve the ability of avocado shade leaves to persist during frequent sun flecks. Evaluation of these processes during shade to sun acclimation in avocado shade leaves under laboratory and field conditions will be presented elsewhere.

MATERIALS AND METHODS

Plant material and growth conditions

Seedlings of avocado (*Persea americana* Mill. cv Edranol) were purchased from Vallance's Nursery (Mullumbimby NSW 2482 Australia) and maintained in 20 L containers of potting soil with regular irrigation and additions of slow release nutrients. The seedlings were kept in a shaded section of a temperature controlled glasshouse for 24 months (18°C

night/ 29°C day), and pruned to the main stem 6-12 months prior to experiments. The light environment of upper and lower canopy leaves in the shade enclosure, measured with a LI-190 sensor (www.licor.com), was 30-50 and 15-30 μmol photons m^{-2} s^{-1} respectively, when horizontal full sun in the outer greenhouse was 1,100 μmol photons m^{-2} s^{-1} at noon.

Fully expanded leaves ranged from 20-35 cm in length and were thinner and deeper green in the shade than in the sun. Transverse sections confirmed that shade-grown avocado leaves were heterobaric to the level of 4th class veins with a single layer of palisade cells above 2-4 layers of spongy mesophyll cells and had numerous small stomata restricted to the underside. Imaging experiments showed this anatomy conferred markedly heterogeneous, small scale differences in NPQ during photosynthetic induction and light response curves. However this heterogeneity was effectively integrated over areas of 1-2 cm^2, and similar results were obtained from imaging and spot measurement techniques (Takayama et al 2008). Photosynthetic parameters were routinely measured from the upper epidermis, but measurements from the lower epidermis were not significantly different.

Experimental protocols

Small shade-grown avocado trees were moved to the dark lab overnight prior to light treatments to alter xanthophyll pigment composition, but one experiment was done with a plant maintained in the dark lab for 11 d. Sets of two or three adjacent attached leaves of similar size and age in the canopy were part covered with Al-foil and/or black cloth prior to illumination of the rest of the leaf at 120 to 350 μmol photons m^{-2} s^{-1} for 60 min using wide-beam high-efficiency fluorescent spotlights (Philips PAR38 23W; 2700K warm white). A small leaf disc was punched from foil control and exposed areas of each leaf (or from the larger leaf discs) for pigment analysis before light treatment, and again at the end of the treatment, then after 1 to 3 h dark to relax photosynthetic induction following light treatment and after 21 to 72 h recovery in darkness. As found previously (Förster et al 2009), large avocado leaves were remarkably robust with respect to sampling for pigment analysis. Moreover, photosynthetic parameters measured adjacent to, or remote from, disc sampling areas were similar in both short-term (hours) and long-term (days) experiments.

Preliminary gas exchange measurements of photosynthesis in leaves of shade-grown plants during induction at 100 μmol photons m^{-2} s^{-1} at 25 ºC were very noisy, even at the slowest practicable flow rates. Stomata opened slowly to low conductance that was responsive to CO_2 (20 mmol m^{-2} s^{-1}, 7 mmol m^{-2} s^{-1} and 3 mmol m^{-2} s^{-1} in 100, 400 and 700ppm CO_2

respectively after 20 min). Photosynthetic CO_2 assimilation saturated at 1.5 to 2.0 µmol m^{-2} s^{-1} after 5 min in 700 ppm CO_2, reached 1.0 µmol m^{-2} s^{-1} after 20 min in 400 ppm CO_2, and remained negative in 100 ppm CO_2. Chlorophyll fluorescence imaging during the above experiments confirmed that NPQ was sensitive to [CO_2] and increased most rapidly to highest values in 100 ppm (Takayama et al 2008). Mature leaves taken from the shade enclosure during the light period showed higher NPQ when a "Vaseline patch" was applied to the lower epidermis. Indeed, we used this simple technique to confirm stomata were closed in leaves dark adapted overnight (no response of ETR or NPQ to a "Vaseline patch") and that stomata were open and photosynthesis was induced following the above actinic light treatments (decreased ETR and increased NPQ in response to a "Vaseline patch").

Chlorophyll fluorescence assays of photosynthetic parameters in mature leaves of shade-grown avocado were made with the Photosynthesis Yield Analyzer MINI-PAM fitted with leaf clip holder 2030-B (www.Walz.com). Photosynthetic induction curves with Arabidopsis grown under similar light environments have been routinely run at 1,000 to 1,500 µmol photons m^{-2} s^{-1} (Pogson et al 1998). Previous light response curve assays using chlorophyll fluorescence have ranged from dwell times of 10 s at PFD from 0 to 2,500 µmol photons m^{-2} s^{-1} (White and Critchley 1999; peas grown at 200 µmol photons m^{-2} s^{-1}) to 180 s at PFD from 0 to 2,000 µmol photons m^{-2} s^{-1} (Pérez-Bueno and Horton 2008; Arabidopsis grown at 120 µmol photons m^{-2} s^{-1}). Leaves of shade-grown avocado were extremely responsive to actinic light intensity and duration during measurement and these protocols led to photoinhibition, manifest as reductions in ETR during photosynthetic induction curves with >300 µmol photons m^{-2} s^{-1} and in rapid light response curves with dwell times of 60 to 120 s at each PFD. More important was our observation that measureable de-epoxidation of V, but not of Lx, occurred during both assays and was always greater during induction assays. A compromise was reached between the actinic light intensity needed to produce acceptable kinetic responses in ETR and NPQ during RLRC assays and that causing the least de-epoxidation of V. We settled for eight steps of 30 s duration over the range from 0 to 400 µmol photons m^{-2} s^{-1}, followed by measurements at four intervals during 240 s in the dark.

Calculation of photosynthetic parameters

The automated PIC and RLRC protocols in the MINI-PAM fluorimeter measured intrinsic chlorophyll fluorescence (F) and maximum fluorescence yield during a saturating flash (F_m) in dark adapted leaves to calculate the maximum quantum yield of photochemical

energy conversion $F_v/F_m = (F_m-F)/F_m$. Photosynthetic electron transport was calculated from fluorescence yield in saturating flashes under actinic light (F_m') from the quantum yield of photochemical energy conversion $\Delta F/ F_m' = F_m'-F/F_m'$, and the PFD measured at that spot using the quantum sensor of the leaf clip (adjusted for absorptance of 0.85 and assuming equal light absorption in PSII and PSI). After light exposures to alter the xanthophyll pigment composition of leaves all NPQ data were recalculated using F_m measured on the leaf kept in the dark overnight and/or F_m from Al-foil shaded areas of the leaf during and after treatment. Likewise, $1\text{-}qP=1-((F_m'-F)/(F_m'-F_o'))$ was recalculated using the minimum value of F obtained within 100 s after actinic light was switched off as F_o'.

Pigment analyses

Leaf discs (1 cm diameter) were punched from treated and control areas of leaves at the times specified in each experiment. Discs were wrapped in foil and immediately frozen in liquid nitrogen. Pigments were extracted from individual discs in a microfuge tube with 0.6 mL of ethyl acetate:acetone (60:40 v/v) and shaken at 30 Hz for 2 min with a stainless steel ball (2 mm diameter) before addition of 0.5 mL of water prior to 5 min centrifugation at 13,000 rpm. The pigment containing upper layer was transferred to a fresh microfuge tube, centrifuged as before, and 0.1 mL of the pigment solution placed in vials for HPLC analysis on an Agilent 1100 fitted with Waters Spherosorb ODS2 column, using a linear gradient from 100-33% acetonitrile:water (90:10 v/v with 0.1% triethanolamine) into ethyl acetate over 31 min. Pigments were identified by retention times and spectra, and carotenoid concentrations were calculated using conversion factors for absorbance at 440 nm obtained with pure pigments, determined by Dr Shizue Matsubara, ICG-III: Phytosphäre, Forschungszentrum Jülich, 52425 Jülich, Germany. The L pool in shade leaves of avocado was three to five-fold greater than that of the other xanthophylls, so values for L have been reduced by 100 mmol mol^{-1} Chl (marked L* in graphical presentations) to facilitate more sensitive comparisons of stoichiometric relationships. As found previously (Förster et al 2009) there were scarcely detectable changes in neoxanthin, α- or β-carotene pool or Chl *a/b* ratio in the course of any of the 24 to 72 h experiments. In most cases only Lx- and V-cycle pigment concentrations are reported.

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FIGURE LEGENDS

Figure 1: Light intensity dependence of Lx-and V-de-epoxidation in a detached avocado leaf on water. Note that values for L* have been reduced by 100 mmol mol^{-1} Chl to facilitate stoichiometric presentation of data (i.e., the scale for L* corresponds to 100-160 mmol mol^{-1} Chl). Parts of the leaf were shaded with Al-foil as control (dark), neutral density filters were used to vary light intensity (80 and 163 µmol photons m^{-2} s^{-1}), and the remainder of the leaf was un-shaded during 90 min exposure to weak afternoon sunlight (300 µmol photons m^{-2} s^{-1}) in a glasshouse at 28ºC. (Mean ± SE; n = 3).

Figure 2: De-epoxidation of V in detached avocado leaves during chlorophyll fluorescence assay of photosynthetic parameters. Pigment composition of mature shade leaves before and after (A) photosynthetic induction assay at 300 µmol photons m^{-2} s^{-1} for 6 min and (B) rapid light response curves (RLRC) assay from 0 to 410 µmol photons m^{-2} s^{-1}. Note that values for L* have been reduced by 100 mmol mol^{-1} Chl to facilitate stoichiometric presentation of data as in Fig. 1 (i.e., the scale for L* corresponds to 100-160 mmol mol^{-1} Chl). (Mean ± SE; n = 4).

Figure 3: Pigment composition in foil covered (cont) and light exposed areas of attached avocado leaves (A) measured immediately following exposure to 150 µmol photons m^{-2} s^{-1} for 1 h (exp 1h), after recovery for 1 h dark (exp +1h dk), and in foil covered (cont 21h) and exposed areas (rec 21h) again after 21 h in the dark. Note the scale for L* corresponds to 100 to 170 mmol mol^{-1} Chl. Changes in photosynthetic parameters measured in RLRC at each of these pigment sampling times are shown in (B, E) 1-qP, (C, F) ETR and (D, G) NPQ, with the time course of NPQ relaxation in the dark shown in panels on the right of (D) and (G). (Mean ± SE, n = 3; error bars appear whenever SE exceeds symbol size).

Figure 4: Enhancement of NPQ is lost when ΔL is metabolized after 48 h dark. (A) Pigment composition measured in Al-foil control (cont) after exposure to 120 µmol photons m^{-2} s^{-1} (exp) and after 1 h and 3 h in the dark. Note the scale for L* corresponds to 100 to 160 mmol mol^{-1} Chl. (B) ETR and (C) NPQ assayed in RLRC in control and treatment at times as in (A). Pigment compositions (D) and ETR (E) and NPQ (F) measured again after 24 h and (G) and (H) 48 h recovery in the dark. The time course of NPQ relaxation in the dark following the RLRC is shown in columns on the right of (C), (F) and (H) (Mean ± SE; n = 4; error bars appear whenever SE exceeds symbol size).

Figure 5: Enhanced NPQ and retention of ΔL from Lx persists for up to 72 h in the dark in leaf discs in the absence of A+Z. Discs from four leaves on a plant dark adapted overnight were floated lower epidermis uppermost and exposed to white LED from below (1 h at 220 µmol photons m^{-2} s^{-1}) then allowed to recover on water in the dark. (A) Pigment composition of controls and of samples after 48 and 72 h; samples were collected after RLRC. Note that L* has been adjusted by 50 mmol mol^{-1} Chl, so the scale for L corresponds to 0 to 120 mmol mol^{-1} Chl. (B) Photosynthetic parameters assayed in RLRC on controls and after 48 and 72 h recovery in the dark. (Mean ± SE; n = 4; error bars appear whenever SE exceeds symbol size).

Figure 6: Prolonged dark pretreatment promotes metabolism of ΔL overnight and prevents sustained NPQ. Pigment compositions (A) show ΔL is metabolized within 24 h. Note that the scale for L* corresponds to 100 to 150 mmol mol^{-1} Chl. Measurements of photosynthetic parameters in RLRC confirm normal induction of ETR (B) and enhanced NPQ (C) following de-epoxidation. However, following overnight metabolism of ΔL without de-epoxidation and epoxidation of A+Z, ETR and NPQ return to control levels (D, E). The time course of NPQ relaxation in the dark is shown in panels on the right of C and E. (Mean ± SE; n = 3; error bars appear whenever SE exceeds symbol size).

Table I: Changes in Lx and V-cycle pigments in attached mature leaves of shade-grown avocado following light treatments (1 h at 223 ± 27 µmol photons m^{-2} s^{-1}) in nine experiments. (Means ± SE; n = 9-13; 2-3 replicate leaves in each experiment).

Parameter	Pigments, mmol mol^{-1} Chl			
	ΔLx	ΔL	ΔV	ΔA+Z
De-epoxidation (1 h)	-11.8 ± 2.1	+10.2 ± 0.6	-12.5 ± 1.1	+11.7 ± 1.1

Epoxidation (>24 h)	A+Z mmol mol^{-1} Chl	$\frac{A+Z}{V+A+Z}$	F_v/F_m
Initial dark control	0.8 ± 0.3	0.03 ± 0.01	0.808 ± 0.002
After treatment	12.5 ± 0.6	0.37 ± 0.03	0.787 ± 0.002
After recovery	3.4 ± 0.6	0.11 ± 0.03	0.789 ± 0.003

Figure 1

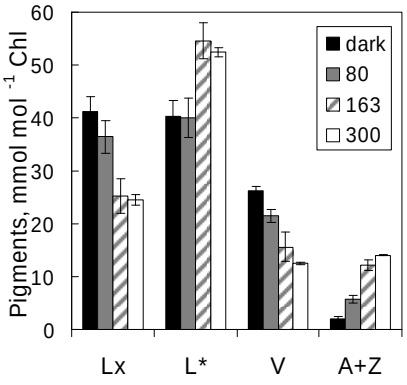

Pigments, mmol mol -1 Chl
60
50
40
30
20
10
0
dark
80
163
300
Lx
L*
V
A+Z

Figure 2

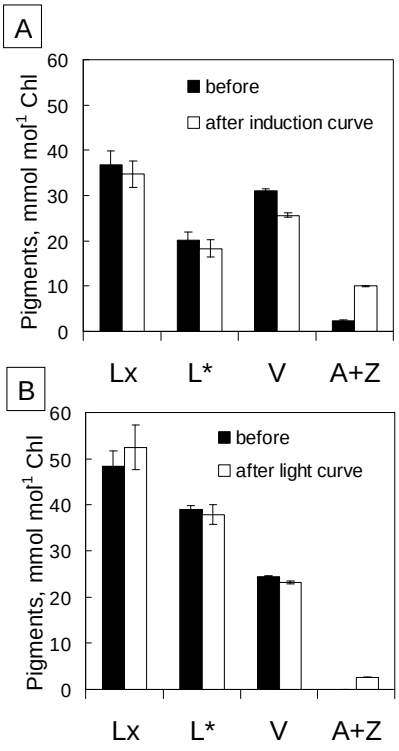

A
Pigments, mmol mol^{-1} Chl
60
50
40
30
20
10
0
before
after induction curve
Lx
L*
V
A+Z
B
Pigments, mmol mol^{-1} Chl
60
50
40
30
20
10
0
before
after light curve
Lx
L*
V
A+Z

Figure 3

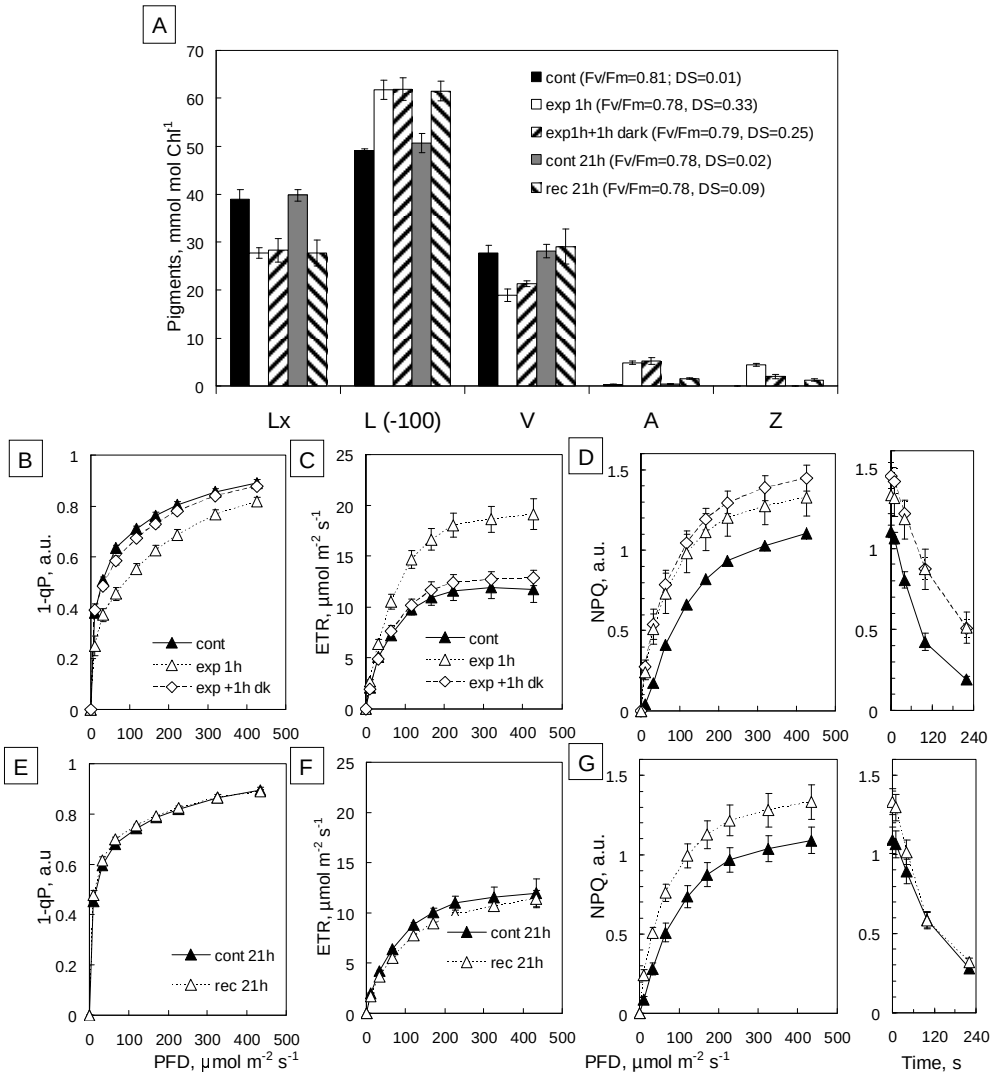

A
Pigments, mmol mol Chl-1
cont (Fv/Fm=0.81; DS=0.01)
exp 1h (Fv/Fm=0.78, DS=0.33)
exp1h+1h dark (Fv/Fm=0.79, DS=0.25)
cont 21h (Fv/Fm=0.78, DS=0.02)
rec 21h (Fv/Fm=0.78, DS=0.09)
Lx
L (-100)
V
A
Z
B
1-qP, a.u.
cont
exp 1h
exp +1h dk
C
ETR, µmol m-2 s-1
D
NPQ, a.u.
E
cont 21h
rec 21h
F
G
PFD, µmol m-2 s-1
Time, s

Figure 4

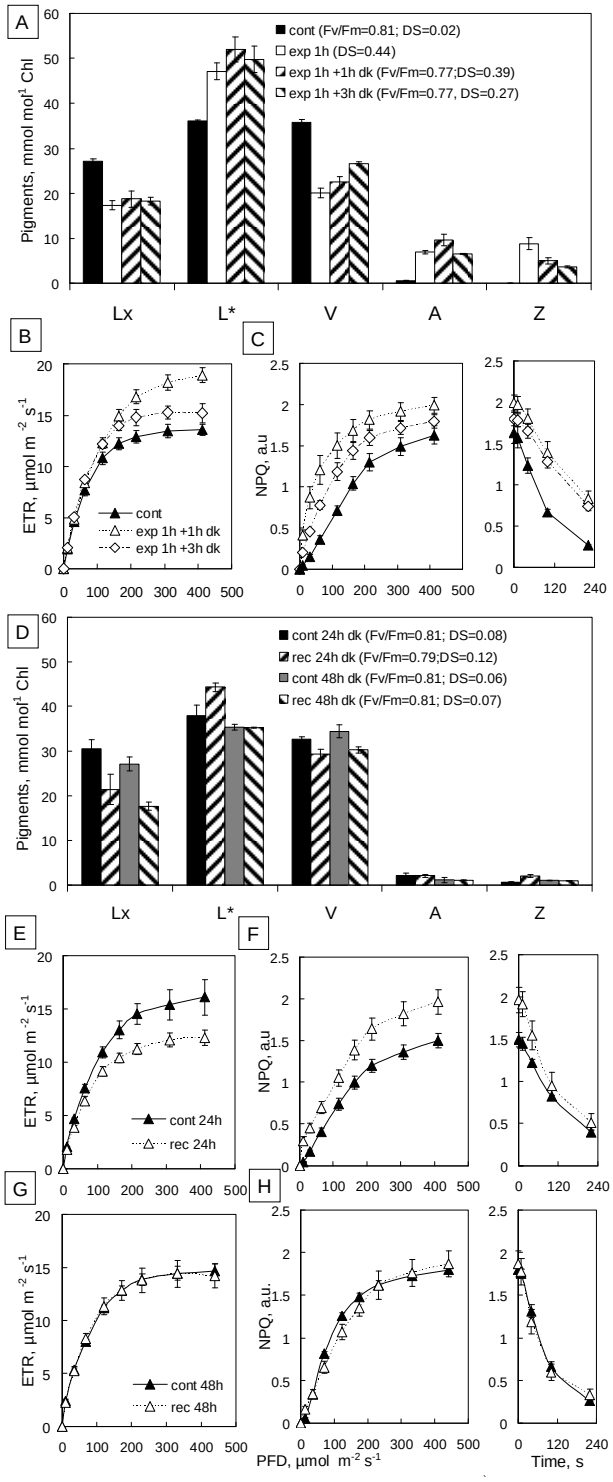

A
cont (Fv/Fm=0.81; DS=0.02)
exp 1h (DS=0.44)
exp 1h +1h dk (Fv/Fm=0.77;DS=0.39)
exp 1h +3h dk (Fv/Fm=0.77, DS=0.27)
Pigments, mmol mol-1 Chl
Lx
L*
V
A
Z
B
ETR, µmol m-2 s-1
cont
exp 1h +1h dk
exp 1h +3h dk
C
NPQ, a.u
D
cont 24h dk (Fv/Fm=0.81; DS=0.08)
rec 24h dk (Fv/Fm=0.79;DS=0.12)
cont 48h dk (Fv/Fm=0.81; DS=0.06)
rec 48h dk (Fv/Fm=0.81; DS=0.07)
E
cont 24h
rec 24h
F
G
cont 48h
rec 48h
H
PFD, µmol m-2 s-1
Time, s

Figure 5

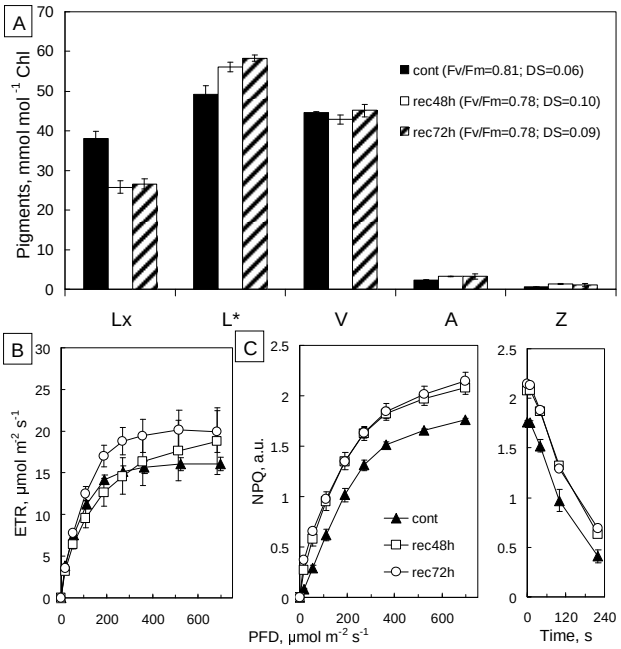
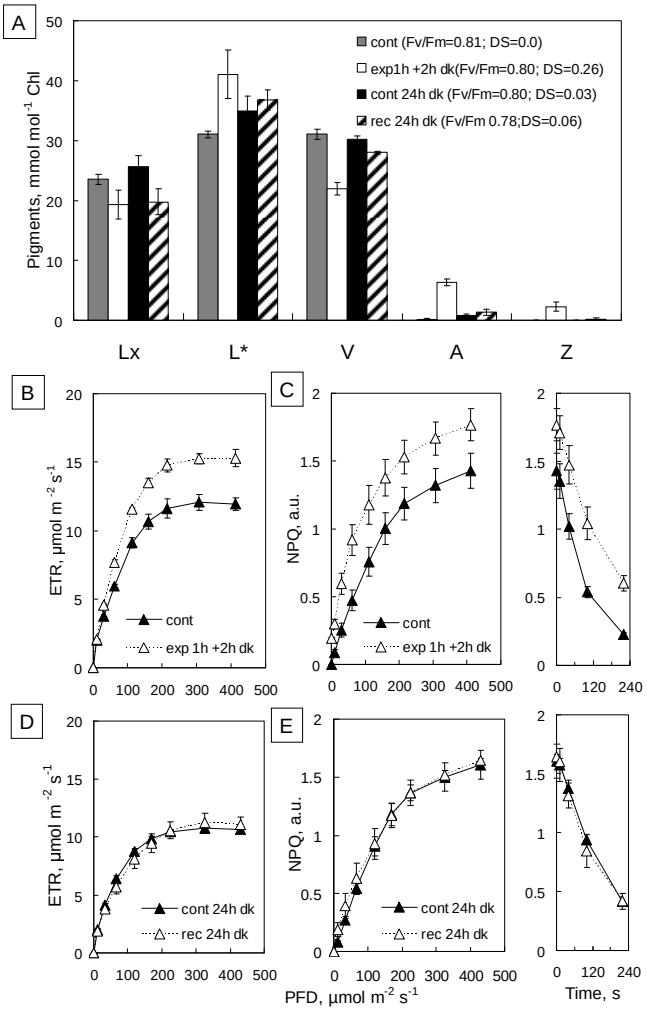

A
Pigments, mmol mol-1 Chl
cont (Fv/Fm=0.81; DS=0.06)
rec48h (Fv/Fm=0.78; DS=0.10)
rec72h (Fv/Fm=0.78; DS=0.09)
Lx
L*
V
A
Z
B
ETR, µmol m-2 s-1
C
NPQ, a.u.
cont
rec48h
rec72h
PFD, µmol m-2 s-1
Time, s

Figure 6


A
Pigments, mmol mol-1 Chl
cont (Fv/Fm=0.81; DS=0.0)
exp1h +2h dk(Fv/Fm=0.80; DS=0.26)
cont 24h dk (Fv/Fm=0.80; DS=0.03)
rec 24h dk (Fv/Fm 0.78;DS=0.06)
Lx
L*
V
A
Z
B
ETR, µmol m-2 s-1
cont
exp 1h +2h dk
C
NPQ, a.u.
cont
exp 1h +2h dk
D
ETR, µmol m-2 s-1
cont 24h dk
rec 24h dk
E
NPQ, a.u.
cont 24h dk
rec 24h dk
PFD, µmol m-2 s-1
Time, s