Decreased photochemical efficiency of photosystem II following sunlight exposure of shade-grown leaves of avocado: because of, or in spite of, two kinetically distinct xanthophyll cycles?

Husen Jia  
*Australian National University*

Britta Forster  
*Australian National University*

W S Chow  
*Australian National University*

Barry James Pogson  
*Australian National University*

C Barry Osmond  
*University of Wollongong, cosmond@uow.edu.au*

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Decreased photochemical efficiency of photosystem II following sunlight exposure of shade-grown leaves of avocado: because of, or in spite of, two kinetically distinct xanthophyll cycles?

Abstract
This study resolved correlations between changes in xanthophyll pigments and photosynthetic properties in attached and detached shade-grown avocado (Persea americana) leaves upon sun exposure. Lutein epoxide (Lx) was deepoxidized to lutein (L), increasing the total pool by ΔL over 5 h, whereas violaxanthin (V) conversion to antheraxanthin (A) and zeaxanthin (Z) ceased after 1 h. During subsequent dark or shade recovery, de novo synthesis of L and Z continued, followed by epoxidation of A and Z but not of L. Light-saturated nonphotochemical quenching (NPQ) was strongly and linearly correlated with decreasing [Lx] and increasing [ΔL] but showed a biphasic correlation with declining [V] and increasing [A+Z] separated when V deepoxidation ceased. When considering [ΔL+ΔZ], the monophasic linear correlation was restored. Photochemical efficiency of photosystem II (PSII) and photosystem (PSI; deduced from the delivery of electrons to PSI in saturating single-turnover flashes) showed a strong correlation in their continuous decline in sunlight and an increase in NPQ capacity. This decrease was also reflected in the initial reduction of the slope of photosynthetic electron transport versus photon flux density. Generally longer, stronger sun exposures enhanced declines in both slope and maximum photosynthetic electron transport rates as well as photochemical efficiency of PSII and PSII/PSI more severely and prevented full recovery. Interestingly, increased NPQ capacity was accompanied by slower relaxation. This was more prominent in detached leaves with closed stomata, indicating that photorespiratory recycling of CO2 provided little photoprotection to avocado shade leaves. Sun exposure of these shade leaves initiates a continuum of photoprotection, beyond full engagement of the Lx and V cycle in the antenna, but ultimately photoinactivated PSII reaction centers.

Keywords
photosystem, xanthophyll, decreased, cycles, photochemical, following, efficiency, ii, sunlight, exposure, shade, grown, leaves, avocado, because, spite, two, kinetically, distinct

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Decreased Photochemical Efficiency of Photosystem II following Sunlight Exposure of Shade-Grown Leaves of Avocado: Because of, or in Spite of, Two Kinetically Distinct Xanthophyll Cycles\(^1\)[W]

Husen Jia, Britta Förster*, Wah Soon Chow, Barry James Pogson, and C. Barry Osmond

Plant Sciences Division, Research School of Biology (H.J., B.F., W.S.C., C.B.O.), and Australian Research Council Centre of Excellence in Plant Energy Biology (B.J.P.), Australian National University, Canberra, Australian Capital Territory 0200 Australia; and Institute for Conservation Biology and Environmental Management, School of Biological Sciences, University of Wollongong, New South Wales 2522, Australia (C.B.O.)

This study resolved correlations between changes in xanthophyll pigments and photosynthetic properties in attached and detached shade-grown avocado (Persea americana) leaves upon sun exposure. Lutein epoxide (Lx) was deepoxidized to lutein (L), increasing the total pool by $\Delta L$ over 5 h, whereas violaxanthin (V) conversion to antheraxanthin (A) and zeaxanthin (Z) ceased after 1 h. During subsequent dark or shade recovery, de novo synthesis of A and Z but not of L. Light-saturated nonphotochemical quenching (NPQ) was strongly and linearly correlated with decreasing $[\Delta L]$ but showed a biphasic correlation with declining [V] and increasing $[A+Z]$ separated when V deepoxidation ceased. When considering $[\Delta L+\Delta Z]$, the monophasic linear correlation was restored. Photochemical efficiency of photosystem II (PSII) and photosystem (PSI; deduced from the delivery of electrons to PSI in saturating single-turnover flashes) showed a strong correlation in their continuous decline in sunlight and an increase in NPQ capacity. This decrease was also reflected in the initial reduction of the slope of photosynthetic electron transport versus photon flux density. Generally longer, stronger sun exposures enhanced declines in both slope and maximum photosynthetic electron transport rates as well as photochemical efficiency of PSII and PSI/PSII more severely and prevented full recovery. Interestingly, increased NPQ capacity was accompanied by slower relaxation. This was more prominent in detached leaves with closed stomata, indicating that photorespiratory recycling of CO\(_2\) provided little photoprotection to avocado shade leaves. Sun exposure of these shade leaves initiates a continuum of photoprotection, beyond full engagement of the Lx and V cycle in the antenna, but ultimately photoinactivated PSII reaction centers.

Much contemporary biochemical and biophysical research in photosynthesis is focused on the mechanisms of photoprotection and photoinactivation of PSII in vivo when exposed to excess light (Horton et al., 1996; Niyogi, 1999; Li et al., 2009b; Jahns and Holzwarth, 2012; Ruban et al., 2012). The extent to which, the circumstances in which, and the mechanisms by which photoprotection actually mitigates the photoinactivation of PSII reaction center functions vary widely between species and with the growth light environment of growth and exposure to stress. An early turning point was marked by Björkman and Demmig-Adams (1994) with their conclusion: “Responses that in the past were thought to be indicative of photoinhibitory damage (reduced photon yield of CO\(_2\) uptake and oxygen evolution and quenching of maximum fluorescence yield) in many cases now appear to be reflections of protective processes.” Although the need for simultaneous evaluation of these two principal responses to excess light has been recognized for some time (Osmond, 1994; Logan et al., 2007), surprisingly few comprehensive studies have been published. Photoinactivation of PSII centers, in spite of full expression of xanthophyll-dependent nonphotochemical quenching (NPQ), has been observed in model plants such as wild-type Arabidopsis (Arabidopsis thaliana; Russell et al., 1995; Bailey et al., 2001), in some strains of very high-light-resistant mutants of Chlamydomonas reinhardtii (Förster et al., 2001, 2005), and evaluated in wild plants from contrasting light environments (Thiele et al., 1996, 1997).

Shade leaves in canopies of tropical species such as Inga spp. and avocado (Persea americana) and many other woody plants (García-Plazaola et al., 2007, 2012; Förster et al., 2009; Matsubara et al., 2009) contain high...
concentrations of lutein epoxide (Lx) that is a substrate for an additional, kinetically distinct xanthophyll cycle, in addition to the near universal violaxanthin (V) cycle (Demmig-Adams and Adams, 1992; Niyogi, 1999; Li et al., 2009b; Demmig-Adams et al., 2012). Studies have revealed NPQ associated with lutein (L) as well as with antheraxanthin (A) and zeaxanthin (Z) in these plants (Garcia-Plazaola et al., 2003; Matsubara et al., 2008; Förster et al., 2011), consistent with insights into the role of L in Arabidopsis transgenics (Pogson and Holzwarth, 2000) and with recent evidence from mutants (Li et al., 2009a). Partial deepoxidation of Lx in sunlight makes a small (approximately 10%), very slowly reversible addition to the pool of lutein (ΔL) that is augmented by further de novo synthesis of L from α-carotene as well as de novo synthesis of A and Z from β-carotene. In spite of these increases in [L] and large diel changes in the V cycle following sunlight exposure, quantum yield of photochemical energy conversion in PSII (QPSII) remained low (Förster et al., 2009).

Notwithstanding highly active research yielding insights into the biochemical and biophysical mechanisms of reversible down-regulation of the photochemical efficiency of PSII (FV/FM), the precise roles of the xanthophyll pigments, their locations in antenna complexes, and the mechanisms contributing to photoprotection remain controversial (Matsubara et al., 2007, 2011; Avenson et al., 2008; Horton et al., 2008; Li et al., 2009a; de Bianchi et al., 2010; Lepetit et al., 2010; Jahns and Holzwarth, 2012). Much of that work, with profound implications for the structural biology of the photosynthetic apparatus in sun and shade, is beyond the scope of this paper. However, Matsubara et al. (2005) found that Lx and L colocated with V in trimeric LHClI and LHCl from thylakoids isolated from Inga sapindoides. After deepoxidation of Lx and V, followed by epoxidation of A+Z, ΔL had replaced Lx in peripheral L1 and internal L2 sites of both monomeric and trimeric Lhcs (i.e. ΔL evidently replaced A+Z in Lhcs; Matsubara et al., 2007) to “lock in” a capacity for elevated NPQ in the dark. These shade leaves seemed ideally suited for an examination of the question of whether the decline in QPSII following sunlight exposure occurred because of, or in spite of, two kinetically distinct xanthophyll cycles in these plants.

The terminology used by various authors to describe component processes in photoprotection, photoinhibition, and photoinactivation (van Kooten and Snel, 1990; Horton et al., 1996; Maxwell and Johnson, 2000) remains a semantic minefield. Because we focus on the relationships between xanthophyll pigment composition and photoprotection in this paper, we will use the subscript designation of different components of NPQ introduced independently from several laboratories (Nilkens et al., 2010; Förster et al., 2011; Jahns and Holzwarth, 2012; Nichol et al., 2012).

In brief, the widely used nomenclature of Horton et al. (1996) has been refined by designating tristhylakoid ΔpH-dependent, xanthophyll-independent nonphotochemical fluorescence quenching (qE) as NPQΔpH and state transition-dependent nonphotochemical fluorescence quenching as NPQST. Preliminary experiments showed that the slow state transitions in avocado shade leaves were quantitatively similar to those reported by Havaux and Lannoye (1987) but also were an early casualty of sunlight exposure. The contribution of NPQST to NPQ overall was likely to be small (Cleland et al., 1990; Tikkanen et al., 2006) and was not considered further.

The different xanthophyll-dependent components of nonphotochemical fluorescence quenching can be distinguished on the basis of chlorophyll fluorescence kinetics associated with differences in pigment composition. Deepoxidation in the Lx and V cycles of avocado generates NPQΔLΔZ rather than NPQAZ in species with the V cycle alone (presumably equivalent to zeaxanthin-dependent nonphotochemical fluorescence quenching of Nilkens et al. [2010]). Conveniently, NPQΔLΔZ in avocado also accommodates the component of nonphotochemical fluorescence quenching attributed to persistently high A+Z (qI; Adams et al., 1994) and ΔL following incomplete epoxidation in the dark. The distinctive component NPQΔL associated with low-light treatments that led to persistent enhanced capacity for NPQ in the dark after epoxidation of A+Z but not L (Förster et al., 2011), could not be identified in the sunlight treatments applied here. We do not wish to be drawn into mechanistic issues; indeed, common mechanisms of pH sensing and xanthophyll pigment interconversions may be involved in NPQΔpH and all xanthophyll-stabilized forms of NPQ (Ruban et al., 2012).

The component of qI associated with photo-inactivated PSII centers is designated as NPQΔpH and must be distinguished using other biochemical and/or biophysical criteria. PSII is well known to be susceptible to radiation damage, and β-carotene has a key role in the quenching of singlet oxygen in the reaction center (Telfer, 2002). However, it is now clear that photoinactivation of PSII occurs under many circumstances (Takahashi and Badger, 2011) and generates a new population of rapidly quenching centers (Matsubara and Chow, 2004). Although the component mechanisms of the D1 repair cycle of PSII now are agreed to in detail (Chow and Aro, 2005), the particular steps or stages of the repair cycle that serve as these rapidly quenching centers are unknown. In this paper, we sought to distinguish the contributions of different components of NPQ to the decline in FV/FM during sunlight exposure in shade leaves of avocado and subsequent recovery in the dark and shade. Chlorophyll fluorescence analyses based on optimized, nonintrusive rapid light-response curves (RLLC) that minimized V and Lx deepoxidation during the assay (Förster et al., 2011) were used to separate qE (NPQΔpH) from the three forms of nonphotochemical fluorescence quenching (NPQAZ, NPQΔLΔZ, and NPQΔL). Very slow rates of photosynthesis in avocado shade leaves prevented accurate estimates of oxygen yield in single-turnover flashes, so another more sensitive method, based on

Photoprotection/Photoinactivation of PSII in Shade Leaves
the kinetics of delivery of electrons from PSII to PSI as measured from F700 \( ^{-} \) rereduction kinetics (Losiciale et al., 2008; Chow et al., 2012), was used to estimate independently the functional fraction of PSII centers in vivo (PSII/PSI) and thereby assess NPQ \( \text{II} \).

We found that progressively stronger sunlight exposures of attached and detached leaves led to persistently higher levels of \( \Delta L \) and \( \text{A+Z} \), to changes in the light response profile of the redox state of the primary electron-accepting plastoquinone of PSII (\( Q_A \)) pool monitored as photochemical fluorescence quenching (1 – \( qP \)) and photosynthetic electron transport (ETR), and to persistently high NPQ that relaxed very slowly in the dark during assay. The slowly reversible decrease in \( F_v/F_m \) was also associated with a slowly reversible decline in the functional fraction of PSII centers. The results are discussed holistically in terms of component xanthophyll-dependent and -independent processes of photoprotection in shade leaves that are overwhelmed in sunlight, leading to photoinactivation of PSII and followed by its partial recovery on return to the shade. These physiological observations underpin other analyses of acclimation (Matsubara et al., 2012) and of inner and outer canopy photosynthesis in general (Nichol et al., 2012) and advance the understanding of these processes in avocado canopy management practices in particular (Whiley et al., 2002).

**RESULTS**

Four experiments are reported using attached (experiments 1 and 2) and detached (experiments 3 and 4) shade leaves. Sun exposures in experiments 1 and 3 were similar (200 min at 750 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) and 300 min at 800 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\), respectively; measured with a LICOR 190 quantum sensor) on cloudless fall days (March and April; Southern Hemisphere) in a temperature-controlled glasshouse (28°C day/18°C night). Confirmatory experiments 2 and 4 were done in the same glasshouse under longer, stronger sun exposures (March and May). The experiments used three or four leaves of equivalent age from different branches in small avocado canopies that were arranged horizontally to ensure equivalent sun exposure. Leaves were partly covered with black cloth and with aluminum foil to minimize heating and to serve as dark controls.

Leaf discs were removed at intervals for pigment analysis during 200 min (experiment 1), 300 min (experiments 2 and 3), and 90 min (experiment 4) of sun exposure for measurements of changes in the reduction state of \( Q_A \) (1 – \( qP \)), ETR, and NPQ using rapid light response curves of chlorophyll fluorescence as well as independent measurements of \( F_v/F_m \) and the functional fraction of PSII. Preliminary experiments with attached leaves showed no response to the “Vaseline patch” test predawn (Supplemental Fig. S1), confirming that stomata were closed. When the test was applied following induction in the shade and during sun exposure, marked inhibition of ETR and increase in NPQ confirmed that stomata were open (had been occluded by Vaseline). In contrast, detached leaves showed no response of ETR or NPQ to the test during sun exposure, confirming that stomata were closed.

**Early Cessation of Deepoxidation Distinguishes the V Cycle from the Lx Cycle in Attached Avocado Shade Leaves Exposed to Sunlight**

Changes in xanthophyll and carotenoid pigments in attached, fully expanded leaves of a shade-grown avocado plant were assayed predawn and after 3 and 4 h of induction in the shade prior to transfer to sunlight (experiment 1). Control leaves sampled predawn, as well as those in which photosynthesis was induced in the shade enclosure and discs taken from the shaded areas of exposed leaves, showed no change in \( F_v/F_m \) or change in deepoxidation status of xanthophyll pigment pools (\( \text{DES}_{Lx} = (\Delta L)/(\Delta L + \Delta L_x) \); \( \text{DES}_{V} = (\text{A+Z})/(\text{V+A+Z}) \)). Note that the chemical conventions of [V] to specify the concentration of a xanthophyll pigment, in this case V, [\( \text{L} \)] to indicate a change in [L], and [L*] to indicate [L-100] in mmol mol\(^{-1}\) chlorophyll to facilitate stoichiometric comparison in graphical data are used throughout.

As expected, deepoxidation of both Lx and V commenced following exposure to sunlight, and time courses of changes in xanthophyll pigments are given in Figures 1 and 2. The slowly reversible Lx cycle in avocado was characterized by a decline in [Lx] and increases in [L] (Fig. 1A) and in \( \text{DES}_{Lx} \) (Fig. 2A) that showed little change in the dark overnight and in the shade enclosure the next day. An unusual feature of avocado shade leaves in this and other experiments (Förster et al., 2009; Matsubara et al., 2012) was an initial small decline (not significant here) of [L] in the first 10 min in sunlight, despite some deepoxidation of Lx. These dynamics were reflected in the estimate of \( \text{DES}_{Lx} \), which was initially negative (data not shown) due to loss of L, but then increased throughout the experiment (Fig. 2A). The increase in [L] eventually exceeded the decline in [Lx] by a factor of 2, suggesting that about one-half of the increase in [L] was due to de novo synthesis rather than deepoxidation. However, \( \alpha \)-carotene remained virtually unchanged (Fig. 1C), and there was little change of [Lx], [L], or \( \alpha \)-carotene after the plant was returned to the shade enclosure.

Quantitatively greater and more rapid deepoxidation of V commenced immediately in sunlight but ceased after 60 min, after which it too continued to be augmented by slow de novo synthesis of Z without further decline in [V] (Fig. 1B). Despite full recovery of [V] overnight, some residual [A+Z] remained 28 h after commencement of the experiment (when leaves had recovered during 24 h in the shade enclosure), and the residual [A+Z] level was very similar to [\( \Delta L \)]. In contrast to \( \text{DES}_{Lx} \), \( \text{DES}_{V} \) showed little increase after 60 min in sunlight but declined by approximately 50% overnight (Fig. 2A). The different dynamics of \( \text{DES}_{Lx} \) and...
DESV presumably were a reflection of differences both in deepoxidation/epoxidation kinetics as well as de novo syntheses. Surprisingly, none of this complexity in pigment dynamics was reflected in the continued steady decline in $F_v/F_m$ and the functional fraction of PSII (PSII/PSI) throughout sun exposure or in the nearly complete recovery of both of these parameters (Fig. 2B).

Changes in Photosynthetic Capacity and Properties of NPQ following the Transition from Deepoxidation and de Novo Synthesis of Xanthophylls in Sunlight

The changes in xanthophyll pigment composition in experiment 1 were associated with profound changes in photosynthesis in vivo, especially in the capacity and properties of NPQ after the transfer of shade-grown avocado leaves to sunlight. For convenience, the photosynthetic data from RLRC are presented (Fig. 3) in panels corresponding to three periods of major change in pigment composition. Following the nomenclature outlined above, these corresponded to (1) the transition from NPQ$_{aP}$ at predawn and after photosynthetic induction in the shade to NPQ$_{AL}$ until the cessation of deepoxidation of V, (2) the amplification of NPQ$_{AL}$ during continued Lx deepoxidation and augmented de novo syntheses of xanthophylls, and (3)

Figure 2. Photochemical efficiency of PSII continues to decline after deepoxidation of V ceases. A, Times courses of change in deepoxidation status of the V cycle ($DESV = [A+Z]/[V+A+Z]$) and the Lx cycle ($DESLx = [DL]/[Lx+DL]$) in experiment 1. B, Decline in the photochemical efficiency of PSII ($F_v/F_m$) in sunlight in experiment 1 and in the functional fraction of PSII, indicated by the arbitrary ratio PSII/PSI measured as the capacity of a single-turnover saturating flash to deliver electrons to PSI. Values are means ± SE, $n = 3$; error bars appear when SE exceeds the symbol size.
the transition from maximum NPQ_{MLAZ} after prolonged sun exposure to recovery overnight and in the shade enclosure the next day. Whether NPQ_{pH} occurred during this experiment is discussed in detail below.

Predawn (i.e. overnight dark-adapted) photosynthetic properties of avocado shade leaves (black triangles) were closely similar to those reported previously (Förster et al., 2011). Photosynthetic induction was evident in period 1 after 4 h in very low light in the shade enclosure (gray squares), and this continued after transfer to sunlight (white circles), as indicated by a small depression in the profile of QA reduction (1 - qP) and a substantial increase in ETR (Fig. 3, A and B). In the absence of deepoxidation (DES, actually declined from 0.09 to 0.05 during induction), there was a small depression of NPQ_{pH} (Fig. 3C), presumably associated with lower ΔpH due to increased ETR following the opening of stomata in the shade. A decline in $F_v/F_m$
and in PSII/PSI, already evident within 10 and 20 min in sunlight (Fig. 2B), was accompanied by substantial deepoxidation of V (Fig. 1B) and an increase in the initial slope and maximum rate of NPQ (data not shown). After 42 min, the increase in these properties of NPQ$_{\text{LAZ}}$ (Fig. 3C) was clearly associated with the de novo synthesis of L and a 2-fold greater decline in [V] due to deepoxidation (Fig. 1, A and B). There was little further depression in 1 - qP (Fig. 3A) or change in the light-limited or maximum rate of the ETR profile (Fig. 3B).

Deepoxidation of V had ceased by 60 min, and period 2, between 60 and 160 min, was characterized by relatively stable DES$_V$, continued increase in DES$_{Lx}$ (Fig. 2A), but continued declines in F$_v$/F$_m$ and PSII/PSI (Fig. 2B). The profile of 1 - qP became more complex at lower PFD and continued to decline at high PFD, indicating that QA became more readily oxidized with increasing time in sunlight (Fig. 3D). There was little further induction of ETR (Fig. 3, B and E), but NPQ$_{\text{LAZ}}$ evidently increased markedly (Fig. 3F) as de novo syntheses of L and A+Z continued (Fig. 1, A and B). Measurements after 90 min of exposure were intermediate between those at 60 and 120 min. Because there was little change in DES$_V$ during this time, it seems plausible that most of the increase in NPQ$_{\text{LAZ}}$ was associated with the doubling in DES$_{Lx}$, driven by a quantitatively similar increase in L from deepoxidation and de novo synthesis.

Period 3 begins with data from the last sun-exposed sample collected after 200 min (white triangles) and compares the recovery of photosynthetic parameters in exposed areas of the leaves after 24 h in the dark overnight and in the shade the next day (gray circles; 28 h after commencement of experiment 1) with an equivalent unexposed but induced leaf on another plant in the shade enclosure (gray squares). Aluminum-covered, dark control areas of leaves measured after 250 min had photosynthetic properties similar to the original dark control measured predawn (data not shown). The 200-min sunlight exposure showed a continuation of the trends observed in Figure 3D toward more oxidized QA, lower initial slope of ETR, and strongly increased NPQ (Fig. 3, compare D–F and G–I). After 24 h of recovery, the sunlight-exposed areas showed features of both controls and sunlight-exposed leaf areas. The PFD response of the QA reduction reverted from its more oxidized condition after 200 min in sunlight to levels of QA similar to shade-induced leaves (Fig. 3, A and G). On the other hand, maximum ETR rates did not differ in leaves sun exposed for 200 min, in exposed leaves after 24 h of recovery, or in unexposed but shade-induced leaves (Fig. 3H). However, exposed tissues had a clearly lower initial slope of ETR versus PFD that did not recover.

The PFD profiles of NPQ in period 3 were particularly informative (Fig. 3, G–I). Tissue exposed to 200 min of sunlight showed the most rapid increase and the highest level of NPQ$_{\text{LAZ}}$ (white triangles) achieved during continued accumulation of L and Z. Shade-induced controls were unchanged compared with the starting point of the experiment (Fig. 3, C and I, gray squares), consistent with the absence of deepoxidation of Lx or V (Fig. 1). After 24 h of recovery, NPQ of 200-min sun-exposed leaves (gray circles) declined by about one-half (Fig. 3I) to closely resemble the profile of NPQ$_{\text{LAZ}}$ after 60 min of sun exposure (Fig. 3F). However, the xanthophyll pigment compositions of the leaves at these times were markedly different, with DES$_{Lx}$ = 0.66 after recovery (versus 0.42 at 60 min) and DES$_V$ = 0.32 after recovery (versus 0.64 at 60 min) and with residual [DL] = 34.5 after recovery (versus 24.3 after 60 min) and residual [A+Z] = 16.1 after recovery (versus 26.9 after 60 min). These data suggest the possibility that ΔL may replace A+Z during recovery in the shade and sustain an elevated capacity for NPQ$_{\text{LAZ}}$. Interestingly, both independently measured F$_v$/F$_m$ and PSII/PSI also recovered to precisely the same levels as those recorded after 60 min of sun exposure (Fig. 2B). The clear implication is that if NPQ$_{\text{LAZ}}$ occurred during sun exposure, it too recovered in the shade.

A second experiment (experiment 2) with attached, induced shade leaves (summarized in Table I) confirmed that deepoxidation of V, but not of Lx, was complete after 60 min of exposure at 1,100 μmol photons m$^{-2}$ s$^{-1}$. In contrast to experiment 1, leaf clips were applied after 300 min of sun exposure to check for recovery in the dark over the next 19 h (24 h after commencement of the experiment). There was no change in [Lx] or [L] in the dark, whereas epoxidation of A and Z largely restored [V] (DES$_V$ = 0.61 recovered to 0.24). As in experiment 1, the decline in light-saturated 1 - qP in sunlight (more oxidized QA) was reversed in the dark. Interestingly, light-limited ETR declined during sun exposure but did not recover, whereas the maximum (light-saturated) rate remained only slightly depressed in the dark. Again F$_v$/F$_m$ and PSII/PSI declined during 300 min of sun exposure, but importantly, none of these parameters recovered in the dark under the leaf clips deployed in this experiment. As in experiment 1, initially low NPQ$_{\text{plp}}$ in the induced shade leaves was transformed during sun exposure to faster and markedly higher NPQ$_{\text{LAZ}}$, but this did not decline during dark recovery. Moreover, the single exponential fit ($r^2 > 0.98$) to normalized relaxation of NPQ in the dark after these assays was always slower for NPQ$_{\text{LAZ}}$ (half-life $t_{1/2}$ of approximately 150 s) compared with NPQ$_{\text{plp}}$ and NPQ$_{\text{LH}}$ ($t_{1/2}$ of approximately 90 s). Residual NPQ in this experiment may have had a component associated with NPQ$_{\text{pp}}$ but further experiments were needed to explore this possibility.

Sun Exposure of Detached Shade Leaves with Closed Stomata Increased the Capacity of NPQ, Exacerbated the Decline in F$_v$/F$_m$ and PSII/PSI, and Impaired Dark Recovery

Detached leaves were examined (experiment 3) using similar sunlight treatments to those described above.
The initial objective was to conserve the stock of shade-grown tissues. Despite our best efforts to maintain the transpiration stream during detachment, stomata closed during sun exposure (Supplemental Fig. S1). Detached leaves presumably were exposed to sunlight at the CO₂ compensation point ([CO₂] < 100 μL L⁻¹) and would have experienced substantially greater light stress. Inadvertently, there was an opportunity to examine the extent to which internal recycling of photosynthetic CO₂ conveyed photoprotection in shade leaves.

As observed previously in attached leaves, deep-oxidation of both Lx and V was essentially complete within 60 min, and little change in [Lx], [L], or DESLx was observed overnight (Fig. 4, A and C). Although substantial epoxidation of Z and partial recovery of [V] occurred in the dark (Fig. 4B), detached leaves maintained higher DESLx than attached leaves (compare Figs. 2A and 4C). However, there was less de novo synthesis of xanthophylls. Again, Fv/Fm and PSII/PSI continued to decline after deepoxidation ceased (Fig. 4D), and neither showed much recovery in the dark. Compared with attached leaves, there was a larger decline, and less recovery, in PSII/PSI and Fv/Fm in detached leaves (Figs. 2B and 4D).

Photosynthetic parameters in sun-exposed detached leaves (Fig. 5) showed interesting differences compared with attached leaves (Fig. 3). The PFD profile of QA reduction did not recover in the weak laboratory light (Fig. 5A). The profiles in sun-exposed and 24-h shade-recovered leaves were the same and showed a distinctive transient of more reduced QA at low PFD prior to the expected more oxidized QA at higher PFD (compare Figs. 3C and 5A). The ETR profiles were markedly different. Although dark control detached leaves had the same capacity for photosynthetic electron transport as attached leaves, once exposed to sunlight, detached leaves showed more extensive decline in both light-limited and maximum rate of ETR, and neither was restored during 24 h of shade recovery in weak laboratory light (compare Figs. 3, E and H, and 5B). The profiles of NPQₘₚ in dark control detached and attached leaves were similar (data not shown), but 60 and 300 min of sun exposure produced more rapid increases at lower PFD to similarly high NPQₘₚ at approximately 430 μmol photons m⁻² s⁻¹ followed by approximately 50% recovery overnight (Figs. 1, F and I, and 5C). Relaxation of NPQₘₚ in the dark after assay was much slower after sun exposure (t₁/₂ = 890 s), became slightly faster during recovery (t₁/₂ = 580 s), but remained well above NPQₘₚ in induced shade leaves (t₁/₂ = 160 s).

These responses in pigments and photosynthetic parameters were confirmed after 60 and 90 min of sun exposure in another detached leaf experiment (experiment 4) at 1,200 μmol photons m⁻² s⁻¹ (Table II). Deepoxidation of both V and Lx was similar in and ceased within 60 min under these extreme light stress conditions under low [CO₂], and some de novo synthesis of both L and Z had commenced. As before, whereas there was some epoxidation of A and Z during recovery in weak laboratory light, Lx and L pools remained unchanged. Measurements of 1 − qP indicated sustained highly reduced QA at low PFD throughout this experiment. There was a striking depression and even less recovery of both the light-limited and maximum rate of ETR. The sustained depression and limited recovery of Fv/Fm in this experiment was

### Table 1. Summary of changes in xanthophyll pigment composition and associated changes in photosynthetic parameters measured from rapid light-response curves in attached, sun-exposed avocado shade leaves in experiment 2

<table>
<thead>
<tr>
<th>Pigments (mmol mol⁻¹ chlorophyll)</th>
<th>Attached Leaves: Experiment 2</th>
<th>Induced Shade Leaf</th>
<th>60 min of Sunlight</th>
<th>300 min of Sunlight</th>
<th>Recovered 24 h in the Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Lx]</td>
<td>29.6 ± 0.1</td>
<td>18.8 ± 2.8</td>
<td>11.7 ± 1.1</td>
<td>12.0 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>[L]</td>
<td>127.8 ± 0.1</td>
<td>141.3 ± 1.5</td>
<td>145.5 ± 4.0</td>
<td>147.0 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>DESLx</td>
<td>0</td>
<td>0.42</td>
<td>0.61</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>[V]</td>
<td>34.0 ± 0.7</td>
<td>14.7 ± 0.8</td>
<td>14.1 ± 1.1</td>
<td>27.6 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>[A]</td>
<td>0.2 ± 0.3</td>
<td>6.2 ± 0.8</td>
<td>9.2 ± 0.8</td>
<td>2.8 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>[Z]</td>
<td>1.4 ± 0.4</td>
<td>15.8 ± 1.8</td>
<td>13.2 ± 0.8</td>
<td>5.8 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>DESLx</td>
<td>0.07 ± 0.02</td>
<td>0.60 ± 0.02</td>
<td>0.61 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Photosynthetic parameters from initial slope and saturation at PFD shown (μmol photons m⁻² s⁻¹)

- 1 − qP (69) 0.45 ± 0.04
- 1 − qP (432) 0.79 ± 0.02
- ETR (69) 13.1 ± 1.7
- ETR (432) 25.3 ± 3.0
- NPQ (69) 0.19 ± 0.07
- NPQ (432) 1.13 ± 0.11

Photosynthetic parameters from independent measurements

- Fv/Fm 0.81 ± 0.01
- PSII/PSI (arbitrary ratio) 0.70 ± 0.03

Values are means ± s (n = 4).

The sustained depression and limited recovery of Fv/Fm in this experiment was...
greater than previously recorded, as was the depression of PSII/PSI, and neither recovered. Overall, it seemed plausible that NPQ$_{hi}$ was a large contributor to higher, slowly reversible NPQ and sustained lower $F_v/F_m$ and PSII/PSI in this experiment. Despite sustained high $[\Delta L]$ and $[A+Z]$ from deepoxidation and de novo synthesis, photorespiratory recycling of CO$_2$ behind closed stomata was evidently inadequate for photoprotection in these detached shade leaves in sunlight.

Although Lx Cycle Pigments Linearly Correlated $[A+Z]$ with NPQ, Correlation with V Cycle Pigments Was Biphasic before and after Deepoxidation of V Ceased

Correlations between changes in xanthophyll pigments and NPQ in attached leaves were examined using data from frequent sampling at intervals after shade-to-sun transfer in experiment 1 (Fig. 6). Increasing light-saturated NPQ was linearly correlated with the decline in $[Lx]$ and increase in $[L^*]$ throughout the experiment (Fig. 6A). The difference slope highlights additional L from de novo synthesis throughout the experiment. In marked contrast, all V cycle components showed linear but biphasic correlations with NPQ. Increase in NPQ was positively correlated with DESV (Fig. 6B), $[A+Z]$ (Fig. 6C), and $[Z]$ alone (Fig. 6D) and was negatively correlated with $[V]$ (data not shown). In each case, there was a break point in these correlations after 42 and 60 min as deepoxidation of V ceased followed by a substantial increase in slope when de novo synthesis of Z began. Interestingly, the correlation of NPQ with the sum $[\Delta L] + [\Delta Z]$ was linear, with a slope similar to that of $[\Delta Z]$ during deepoxidation, suggesting the possibility of complementation and/or augmentation of the effects of these deepoxidized pigments on NPQ (Fig. 6D).

Detailed time courses of changes in NPQ and pigment composition were not studied during recovery in the shade. However, pooled data from all experiments showed some indication that during epoxidation from the end of sun exposure until sampling after dark recovery, the decrease in NPQ capacity was rather more weakly correlated with $\text{DESV} (r = 0.519)$ and $[A+Z] (r^2 = 0.646)$ than during deepoxidation. Note that $\Delta L$ was not epoxidized during recovery, so that its potential contribution to NPQ presumably remained unchanged, and that factors in addition to xanthophyll pigment composition, such as repair of photoinactivated PSII centers, may have been involved. Additional experiments are needed to explore these relationships during recovery.

Decline in $F_v/F_m$ and PSII/PSI in Sunlight Was Strongly Correlated with Increased Light-Saturated NPQ and Transients in the PFD Profile of $1 - qP$

The increased capacity of light-saturated NPQ during sun exposure was also strongly correlated with an independently measured decline in the functional
fraction of PSII (Fig. 7A). The maximum efficiency of PSII determined from $F_v/F_m$ (Fig. 7B), using chlorophyll fluorescence measurements independent of those used to estimate NPQ, was also very tightly correlated with the functional PSII fraction. These correlations held almost as strongly during sun exposure as in dark recovery (data not shown).

The profiles of $Q_A$ reduction ($1 - qP$ with PFD in Fig. 3, A, D, and G) were also highly correlated with the functional fraction of PSII (Fig. 2B) under both light-limited and light-saturated conditions in experiment 1 (123 and 433 μmol m$^{-2}$ s$^{-1}$), respectively. The same fraction of functional PSII was associated with about three times more $Q_A$ reduction in saturated light than in low light, which was almost proportional to the difference in light intensity. By extension, it follows that these properties of $Q_A$ oxidation were also well correlated with the decline in $F_v/F_m$ and the increase in NPQ.

### DISCUSSION

This paper builds on earlier observations of large, slowly reversible changes in $\Phi_{PSII}$ of avocado shade leaves that showed complex relationships with deep-oxidation of Lx to ΔL and of V to A+Z during short- and long-term acclimation to sunlight (Förster et al., 2009). Here, we examined the short-term responses of shade leaves to sunlight exposure in more detail to disentangle some of the complex pigment-photosynthesis relationships, which led to some essential new insights. Interestingly, deep-oxidation of V ceased after about 60 min in sunlight, and the subsequent increase in both [L] and [A+Z] from de novo synthesis produced a 2- to 5-fold increase in the capacity for NPQ.$\Delta$L.$\Delta$A over that due to NPQ.$\Delta$P.$\Delta$H. The extent of recovery overnight and in the shade depended on the duration and intensity of sun exposure. Recovery was

Table II. Summary of changes in xanthophyll pigment composition and associated changes in photosynthetic parameters in rapid light-response curves in detached, sun-exposed avocado shade leaves in experiment 4

<table>
<thead>
<tr>
<th>Pigments (mmol mol$^{-1}$ chlorophyll) and deepoxidation states</th>
<th>Detached Leaves; Experiment 4 (1,200 μmol photons m$^{-2}$ s$^{-1}$)</th>
<th>Shade Leaf Predawn</th>
<th>60 min of Sunlight</th>
<th>90 min of Sunlight</th>
<th>Recovered 22 h in the Shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Lx]</td>
<td>30.4 ± 2.3</td>
<td>12.4 ± 0.6</td>
<td>10.5 ± 0.9</td>
<td>11.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>[L]</td>
<td>104.7 ± 1.1</td>
<td>133.9 ± 0.8</td>
<td>130.9 ± 2.3</td>
<td>130.1 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>DES$_{Lx}$</td>
<td>0</td>
<td>0.68</td>
<td>0.71</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>[V]</td>
<td>31.8 ± 0.2</td>
<td>10.4 ± 0.2</td>
<td>9.8 ± 0.2</td>
<td>16.3 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>[A]</td>
<td>0.2 ± 0.2</td>
<td>2.5 ± 0.3</td>
<td>3.0 ± 0.1</td>
<td>7.9 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>[Z]</td>
<td>2.4 ± 0.4</td>
<td>29.1 ± 0.5</td>
<td>28.4 ± 1.6</td>
<td>17.5 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>DES$_{V}$</td>
<td>0.08 ± 0.0</td>
<td>0.76 ± 0.0</td>
<td>0.76 ± 0.01</td>
<td>0.61 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Photosynthetic parameters from initial slope and saturation at PFD (μmol photons m$^{-2}$ s$^{-1}$)

1 − qP ($\Phi_{PSII}$)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Shade Leaf Predawn</th>
<th>60 min of Sunlight</th>
<th>90 min of Sunlight</th>
<th>Recovered 22 h in the Shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 - qP$ (94)</td>
<td>0.78 ± 0.01</td>
<td>0.91 ± 0.01</td>
<td>0.95 ± 0.02</td>
<td>0.88 ± 0.01</td>
</tr>
<tr>
<td>$1 - qP$ (467)</td>
<td>0.92 ± 0.01</td>
<td>0.94 ± 0.01</td>
<td>0.98 ± 0.03</td>
<td>0.85 ± 0.03</td>
</tr>
<tr>
<td>ETR (94)</td>
<td>5.3 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>ETR (467)</td>
<td>9.4 ± 1.2</td>
<td>1.7 ± 0.5</td>
<td>0.3 ± 0.3</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>NPQ (94)</td>
<td>0.74 ± 0.02</td>
<td>2.59 ± 0.08</td>
<td>3.83 ± 0.23</td>
<td>3.45 ± 0.47</td>
</tr>
<tr>
<td>NPQ (467)</td>
<td>1.38 ± 0.03</td>
<td>3.13 ± 0.08</td>
<td>4.48 ± 0.26</td>
<td>4.22 ± 0.47</td>
</tr>
</tbody>
</table>

Photosynthetic parameters from independent measurements

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Shade Leaf Predawn</th>
<th>60 min of Sunlight</th>
<th>90 min of Sunlight</th>
<th>Recovered 22 h in the Shade</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_v/F_m$</td>
<td>0.79 ± 0.0</td>
<td>0.18 ± 0.0</td>
<td>0.14 ± 0.03</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>PSII/PSI (arbitrary ratio)</td>
<td>0.56 ± 0.01</td>
<td>0.22 ± 0.0</td>
<td>0.28 ± 0.03</td>
<td>0.32 ± 0.06</td>
</tr>
</tbody>
</table>
least in detached leaves with closed stomata, in which excitation pressure was presumably increased when photosynthetic metabolism was limited to recycling of photorespiratory CO$_2$. Independent measures of the concurrent decline in light-saturated ETR and the capacity of PSII to deliver electrons to PSI in saturating single-turnover flashes during sun exposure, neither of which recovered in the shade, point to photoinactivation (NPQ$_{\text{P}}$), in spite of the complex pigment interactions presumed to be associated with the stabilization of photoprotection.

De Novo Syntheses of L and Z Sustain Increased Capacity for NPQ after Cessation of V Cycle Deepoxidation in Sunlight

Sunlight exposure of attached avocado shade leaves led to cessation of the V cycle, but not the Lx cycle or deepoxidation, within 60 min (Fig. 1, A and B; Supplemental Fig. S1). Incomplete deepoxidation of the V pool in strong light is not unprecedented (Jahns and Holzwarth, 2012) and seems common in shade plants (Watling et al., 1997; Thiele et al., 1998) as well as in Arabidopsis exposed to 2,000 μmol photons m$^{-2}$ s$^{-1}$ (Russell et al., 1995), but the timing had not been resolved in those studies. At this stage, we have no explanation for the early cessation of V deepoxidation in sunlight, but two lines of argument seem relevant. First, feedback on the capacity to maintain the high lumen pH needed to promote further deepoxidation (Pfündel et al., 1994) in the face of increasing NPQ and/or declining PSII/PSI seems plausible. Consistent with such a possibility, mutations of the D1 protein in _C. reinhardtii_ that slowed PSII electron transfer from QA to the secondary electron-accepting plastoquinone of PSII produced phenotypes that accumulated levels of Z in strong light similar to those in the wild type but were unable to generate similarly high NPQ, possibly because impaired PSII was unable to maintain an effective D$_{p}$H (Förster et al., 2001).

Second, compartmentalization of xanthophyll pools could explain the incomplete deepoxidation. compartmentalization of Z was invoked by Hurry et al. (1997) to explain the lack of effective photoprotection with increasing [Z] in Arabidopsis _aba_ mutants. These could include certain LHC-bound pigments in non-functional PSII, dissociated antennae, or elsewhere. Jahns et al. (2001) suggested that certain pools of V are physically inaccessible to the deepoxidase due to their location in the chloroplast. Subsequently, it was demonstrated in vitro and in vivo that the conversion efficiency of the V pool depended on the sublocalization were found between the increase in NPQ and the increase in [A+Z]. D, Interestingly, the correlation between NPQ and [Z] was biphasic, whereas with [ΔZ]+[ΔL], it was monophasic. Values are means ± se, n = 3; error bars appear whenever se exceeds the symbol size. a.u., Arbitrary units.
of V in the LHCI and LHCII complexes in the thylakoid membrane (Wehner et al., 2004, 2006). In these studies, using tomato (Solanum lycopersicum), it was concluded that in both Lhca proteins (LHCI) and Lhcb proteins (LHCII), V was more accessible and deepoxidized faster when bound to the V1 or N1 site, while V bound to the L2 site (and the L1 site in some Lhcb proteins) was very slowly converted to Z or inaccessible for deepoxidation.

Consistent with these findings in higher plants, multiple pools of deepoxidation products have also been demonstrated recently in diatoms (Lepetit et al., 2010). If we assume similar distributions of V to different LHC-binding sites in avocado, this suggests that the inconvertible V pool in the light represents the fraction of V bound to the L2 and L1 sites.

De novo synthesis of L and Z dominated changes in xanthophyll composition after cessation of deepoxidation, as observed previously (Förster et al., 2009), and introduced complexity in the search for the role of xanthophylls in the stabilization of NPQ in these leaves. Light-saturated NPQ measured by standardized RLRC in leaves predawn (i.e. with closed stomata) and in shade-induced leaves (open stomata) before deepoxidation of Lx or V provides baseline estimates of NPQ_{\text{dph}} (Förster et al., 2011). It was expected that increased capacity for NPQ_{\text{dLAZ}} would be simply correlated with increased [ΔL] and [A+Z] following sunlight exposure, irrespective of whether the source was deepoxidation or de novo synthesis. This proved to be so with decline in [Lx] and increase in [ΔL] (Fig. 6A), but surprisingly, it was not the case with the V cycle. Here, the decline in [V] and increases in DESV, [A+Z], or [Z] alone showed two distinct, linearly correlated phases with a break point at the transition from deepoxidation to de novo synthesis (Fig. 6, B–D). At first glance, these correlations imply that, if [Z] was the principal determinant of continued increase in NPQ_{\text{dLAZ}} after deepoxidation of V ceased, then Z from de novo synthesis was about twice as effective as that derived from deepoxidation.

There may be several explanations for the implied apparent difference in the effectiveness of Z to enhance NPQ, depending on its source. Following from the above, differential compartmentalization of Z from de novo synthesis might occur in avocado leaves, which may promote NPQ more effectively, perhaps because of better access to key binding sites in Lhcs. Another possibility was suggested by the conclusion of Johnson et al. (2008) that DESV, rather than the absolute [Z], determined xanthophyll/NPQ kinetic relationships in Arabidopsis, thereby implying some contribution of [A] (and/or of [V]) to the stabilization of NPQ. However, in avocado shade leaves, the persistent biphasic correlation with [V], DESV, [A+Z], and [Z] alone suggests that further speculation may be unhelpful.

There is little doubt that the accumulation of [ΔL] and [A+Z] is associated with faster onset (Matsubara et al., 2012) and absolute level (Förster et al., 2011) of NPQ in these leaves. Recent fluorescence lifetime imaging studies in avocado identified a population of rapidly quenching centers associated with A and also with ΔL enhancement of ΔpH-dependent NPQ_{\text{dLAZ}} (Matsubara et al., 2011). But it is clear that [A+Z] also slows NPQ relaxation in the dark in avocado shade leaves.

Figure 7. Linear correlations of photosynthetic properties measured in experiment 1. A. The increase in light-saturated NPQ (from Fig. 3, C, F, and I) correlated negatively with the functional fraction of PSII (PSII/PSI from Fig. 2B). B. The increase in F_v/F_m correlated positively with PSII/PSI during sun exposure (from Fig. 2B). C. The reduction state of the QA pool (1−qP from Fig. 3, A, D, and G) showed similar positive correlation with PSII/PSI at approximately half-saturating PFD (123 μmol photons m^{-2} s^{-1}) and at light saturation (433 μmol photons m^{-2} s^{-1}) PSII (from Fig. 3, A, D, and G). Values are means ± se, n = 3; error bars appear when se exceeds the symbol size. a.u., Arbitrary units.
leaves (Förster et al., 2011); a threshold of DES_\(v\) of approximately 0.1 was found for the signature effect of [A+Z] in slowing the relaxation of NPQ (Matsubara et al., 2012). Much more research is needed to evaluate these pigment exchanges in Lhcs during sunlight exposure in leaves with two xanthophyll cycles. The differing kinetics of the Lx cycle in avocado, Ocotea foetans (Esteban et al., 2010), and Inga spp. may be especially useful in this context.

Relationships between Lx and V cycle pigments and enhanced NPQ in sunlight may arise from the same pigment-replacement interactions detected in Lx-rich shade leaves exposed to modest light intensities (Matsubara et al., 2008; Förster et al., 2011). In avocado, [AL] from deepoxidation of Lx was retained for 48 h or more in the dark after epoxidation of A and Z to V (i.e. NPQ_{deep} < NPQ_{ALAZ} \approx NPQ_{AL}). Matsubara et al. (2007) examined thylakoids of I. sapindoides with both Lx and V cycles and demonstrated that ΔL functionally replaced Lx in the V1 and L2 binding sites of antenna Lhcs in much the same way as Z replaces V in these two xanthophyll binding sites to stabilize and enhance the capacity for NPQ. That a similar augmentation might have occurred during sunlight exposure was suggested by the transformation of the biphasic correlation between NPQ and [ΔZ] to a monophasic correlation between NPQ as a function of the sum ΔL + ΔZ, with a slope similar to that found for [Z] alone during deepoxidation (Fig. 6D). Further insight into mechanisms may be gained from Arabidopsis mutants that overexpress L in the absence of Z, which reportedly confers higher NPQ and is associated with the generation of a cation radical attributed to actively quenching L similar to the signals obtained from Z (Li et al., 2009a). Similar studies with avocado thylakoids may be revealing.

On the other hand, the biphasic correlation of NPQ_{ALAZ} with DES_\(v\) [A+Z] or [Z] after deepoxidation of V ceased may not be related to Z or ΔL at all but to other factors that continued to accelerate NPQ in general. For example, the continued decline in \(F_{v} / F_{m}\) and in PSII/PSI may indicate an increase in NPQ_{ph} after cessation of deepoxidation that adds to NPQ_{ALAZ} giving the impression of an acceleration of NPQ expressed on the basis of the concentration of any of the deepoxidized xanthophylls. We were unable to further partition these processes on the basis of data from experiment 1, but note that both \(F_{v} / F_{m}\) and PSII/PSI recovered to a large extent overnight and in the shade the next day.

Deepoxidation of Lx and V Does Not Fully Mitigate Photoinactivation of PSII Electron Transfer during Sunlight Exposure

Evidently, the photosynthetic apparatus of avocado shade leaves responds to sunlight exposure in a highly coordinated and holistic manner and seems to dynamically balance xanthophyll-dependent NPQ photoprotection against photoinactivation. The above complex interactions between NPQ and xanthophyll pigment composition in experiment 1 are indicative of a well-coordinated dissipation of excess excitation in the antenna that mitigates potential photoinactivation in the PSII reaction center with photoprotection. The strong negative correlation between the increase in NPQ and the decrease in \(F_{v} / F_{m}\) is consistent with this and with the bulk of contemporary evidence in support of the position stated by Björkman and Demmig-Adams (1994). However, we are left to explain how a decrease in the functional fraction of PSII centers, indicated by the approximately 40% decline in the arbitrary ratio of PSII/PSI, was also highly correlated with an increase in light-saturated NPQ_{ALAZ} (Fig. 7A). One possibility is that antenna photoprotection was also accompanied by partial reaction center photoinactivation.

As far as we know, the saturating single-turnover flash method used to determine PSII/PSI delivered saturating excitation to PSII centers, irrespective of the extent of antenna photoprotection. Previously, a robust linear correlation was demonstrated between the integrated transient electron flow to P700 after a fully saturating flash and PSII reaction center function assayed by the relative amount of oxygen per single-turnover flash evolved in leaves of herbaceous and woody species, wild-type and chlorophyll b-less barley (Hordeum vulgare), monocots and dicots, and C3 and C4 species (Loscialie et al., 2008). Importantly, this relationship held in plants subjected to varying extents of photoinactivation of PSII by inhibiting chloroplast D1 protein synthesis (Loscialie et al., 2008). This method has emerged as the preferred assay for PSII activity in vivo (Chow et al., 2012), and in avocado sun and shade leaves it delivered the same PSII/PSI ratio whether measured from the upper or lower epidermis.

This interpretation is also supported by the studies of Oja and Laisk (2000) using different single-turnover flash/oxygen yield methods to analyze PSII function in relation to different NPQ components in sunflower (Helianthus annuus) leaves grown at 450 μmol photons m\(^{-2}\) s\(^{-1}\). When pretreated with strong actinic light (1,700 μmol photons m\(^{-2}\) s\(^{-1}\)) to engage qL, oxygen yield in saturating single-turnover flash assays decreased, consistent with partial photoinactivation of populations of PSII reaction centers. When qE associated with antenna quenching (and presumably with photoprotection) was induced, oxygen yield did not decrease in saturating flashes.

If concurrent PSII reaction center inactivation occurred during sun exposure of attached leaves in experiment 1, it was largely reversible after 24 h in the dark and shade the next day, perhaps even more reversible than NPQ_{ALAZ} (compare Figs. 2B and 3J). Yet other experiments reported here clearly show that shade-grown avocado leaves experience increasing photoinactivation with increasing light stress, in spite of the full engagement of photoprotection stabilized by two xanthophyll cycles. Although the attached leaves used in a second longer, stronger sun exposure experiment achieved higher ETR, attained lower...
NPQ$_{\Delta LAZ}$ than in experiment 1, and experienced only approximately 20% reduction in $F_v/F_m$ and PSII/PSI, the latter parameters did not recover (Table I). Detached leaves in experiment 3 had similar sun exposure as in experiment 1, had closely similar concentrations of xanthophylls, and attained the same levels of NPQ$_{\Delta LAZ}$, but stomatal closure limited metabolism to recycling of photorespiratory CO$_2$ and restricted ETR in sunlight by 30% (to predawn levels). Under these conditions, $F_v/F_m$ and PSII/PSI declined by approximately 60% and showed little recovery (Fig. 4D).

Most importantly, the light-response curves for ETR in experiment 3 (Fig. 5B) showed responses reminiscent of many early “photoinhibition” experiments (Osmond and Chow, 1988; Osmond, 1994; Murchie and Niyyogi, 2011), characterized first by inhibition of the initial slope without any effect on the maximum rate at saturating PFD (diagnostic of photoprotection, but not ruling out photoinactivation of PSII to an extent short of limiting whole-chain electron transport) followed by further depression of the initial slope and maximum rate with incomplete recovery in the dark and shade (diagnostic of photoinactivation). A second detached leaf experiment at 50% higher PFD for only 90 min (experiment 4) led to much more drastic declines in both properties of ETR, in $F_v/F_m$ and in PSII/PSI, despite record levels of NPQ$_{\Delta LAZ}$, DES$_{AV}$ and DES$_{V}$, none of which showed much recovery (Table II).

It is difficult to escape the conclusion that, in these detached leaf experiments, photoinactivation was rampant.

At this stage, biochemical analyses of damage and repair in the D1 reaction center protein of PSII (Russell et al., 1995; Förster et al., 2005) would greatly assist further attempts to resolve the relative impact of photoprotection versus photoinactivation in avocado shade leaves. Anderson et al. (1998) estimated that on an average day in an average leaf, all of the D1 protein is damaged and repaired at least once, and it is likely that these processes are accelerated in sun-exposed shade leaves. For example, transfer of the shade plant *Monstera deliciosa* to sun led to the accumulation of damaged D1 protein that was “labeled” for repair by phosphorylation (i.e. D1*), despite retention of high [A+Z] (Ebbert et al., 2001). A fraction of D1* was retained during dark recovery, when dephosphorylation of D1* protein would normally initiate the D1 repair cycle (Chow and Aro, 2005). Perhaps D1* centers constitute the population of more rapidly quenching centers that correlate with the decline in the functional fraction of PSII in photoinactivated pepper (*Capsicum annum*; Matsubara and Chow, 2004). Weak light is required for the D1 repair cycle, and perhaps the much slower recovery of $F_v/F_m$ and PSII/PSI in the dark in experiment 2 reflects this requirement. The hypothesis that the accumulation of these reaction center proteins following light stress “favors maximal energy dissipation over photochemistry” (Ebbert et al., 2001) seems compatible with the protective function proposed for this population of rapidly quenching centers (Matsubara and Chow, 2004). Furthermore, as first reported by Depka et al. (1998) and recently explored by Beisel et al. (2010), there also may be links between PSII reaction center turnover, release of β-carotene and de novo syntheses, and retention of Z following extreme light stress (Cazzonelli and Pogson, 2010).

None of this is unexpected, but the new data reported here are relevant to the early events in long-held hypotheses of photoinhibition and acclimation (Anderson and Osmond, 1987). Shade leaves in general have low rates of photosynthesis, low concentrations of Rubisco, low capacities for photosynthetic ETR, and, importantly, low pools of xanthophyll pigments for photoprotection (Demmig-Adams, 1996). Avocado shade leaves were no exception, with maximum capacities of light-saturated ETR ranging from 10 to 25 μmol m$^{-2}$ s$^{-1}$ in detached or attached leaves with closed stomata to 30 to 45 μmol m$^{-2}$ s$^{-1}$ when fully induced, compared with more than 200 μmol m$^{-2}$ s$^{-1}$ in sun leaves, with [Lx+L] and [V+A+Z] about one-third to one-half those of sun leaves (Matsubara et al., 2012). So sun exposure at PFD five to 10 times above light saturation represents severe light stress, and it is not surprising that, in the end, photoinactivation occurs. Clearly, the capacity of shade leaves to recycle photorespiratory CO$_2$ under these conditions was limited and did little to protect against photoinactivation in sunlight, as proposed earlier (Osmond,
demonstrated to also afford photoprotection (Matsubara et al., 2012). Longer term shade-to-sun acclimation experiments in the laboratory and selective pruning experiments in the field show the hallmarks of the processes discussed above. Sun exposure of inner canopy leaves on day 1 after pruning in the field (C.B. Osmond and B. Förster, unpublished data) or after transfer of shade-grown canopies to sun in the greenhouse (Matsubara et al., 2012) showed evidence for photoinactivation. In the latter experiments, 10 d or more were required to enlarge L and V+A+Z pools (Förster et al., 2009) and to begin building the higher ETR capacity observed in sun leaves. Remarkably, after approximately 10 weeks of sun exposure, these mature shade leaves reconstructed a photosynthetic apparatus with all the attributes of outer canopy leaves developed in sunlight (Matsubara et al., 2012).

**Photoprotection in the Wider Context: The Complementarity of Opposite Concepts**

A holistic, schematic summary of interactions between reversible photoprotection and photoinactivation with irradiance and time, based on ancient Chinese insights into the complementarity of opposite concepts, was proposed previously (Osmond, 1994). A contemporary version, adapted to the twin xanthophyll cycles in avocado and other species, is offered in Figure 8. Some artistic license has been exercised with respect to yin-yang symbolism in the expansive shape of green leaf photosynthetic physiology that is transformed from high to low ØPSII through rapidly engaged and reversible biophysical and biochemical processes (NPQ<sub>4ph</sub>) at low irradiance and short times. With increasing irradiance and time, more rapidly engaged but more slowly reversible dissipation of excess excitation as heat, through various forms of xanthophyll-dependent photoprotection in the antenna (NPQ<sub>L</sub>, NPQ<sub>LAZ</sub>, and NPQ<sub>AL</sub>), is shown in yellow-green through amber. Even more slowly reversible dissipation of excess excitation, involving the accumulation of photoinactivated PSII centers (NPQ<sub>pH</sub>) due to imbalance in the rates of damage and repair of PSII reaction center components such as the D1 protein, is shown in red. The scheme harmonizes the well-established but opposing concepts of universal antenna photoprotection (Demmig-Adams and Adams, 1992; Demmig-Adams et al., 2012) and of the consequences of reaction center photoinactivation, presciently described by Horton (1987) as “the final defence mechanism used when high irradiance is both prolonged and at a level in excess of that which can be controlled by other dissipative processes,” a process that has since been demonstrated to also afford photoprotection (Matsubara and Chow, 2004).

**CONCLUSION**

Sun exposure of avocado shade leaves revealed four major cooccurring changes in pigment composition and photosynthetic activities. (1) Deepoxidation in both the Lx and V cycles was incomplete and ceased after approximately 60 min in sunlight but was followed by further de novo synthesis of L and Z. (2) Whereas light-saturated NPQ was linearly correlated with decline in [Lx] and increase in [ΔL] from either source, the transition to de novo synthesis was marked by a break point in a biphasic correlation of NPQ with DES<sub>ΔV</sub>, [A+Z], or [AZ]. Restoration of monophasic correlation in plots against [ΔL] + [AZ] suggested augmented stabilization of NPQ by L and Z. (3) Photochemical efficiency of PSII (F<sub>e</sub>/F<sub>m</sub>) and the independently estimated functional fraction of PSII centers declined continuously in sunlight and were also highly correlated with NPQ, indicating a participation of photoinactivated PSII centers in photoprotection. (4) Photorespiratory CO<sub>2</sub> recycling evidently contributed little to the mitigation of light stress in detached leaves with closed stomata, presumably because these shade leaves had very low Rubisco and electron transport capacity. More research is needed to resolve the interactions between the two xanthophyll cycles as well as the relationships between various components of NPQ and the functional state of PSII centers. Avocado shade leaves transferred to sunlight present many opportunities for further detailed exploration of the underlying mechanisms.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Seedlings of avocado (Persea americana ‘Edranol’) were purchased from Vallance’s Nursery and grown in a shade enclosure in a temperature-controlled glasshouse for 24 months (18°C night/29°C day) as described earlier (Förster et al., 2009, 2011). Shade was provided by several layers of neutral beige-colored shade netting and was unrepresentative of changes in the spectral composition of natural shade due to the absorption of shorter wavelength light by other canopy leaves. Mature fully expanded leaves ranged from 20 to 35 cm in length and were thinner and deeper green in the shade than in the sun. They had numerous small stomata that are restricted to the underside, and photosynthetic parameters were routinely measured from the upper epidermis, but measurements from the lower epidermis were not significantly different. As judged by the Vaseline patch test (application of Vaseline halves ETR and doubles NPQ when stomata are open but has no effect if they are closed; Supplemental Fig. S1), stomata on attached leaves in the shade enclosure were closed predawn, opened in the course of photosynthetic induction in the shade (or in response to actinic light treatments), and closed again during recovery in the dark laboratory overnight. Using the same criteria, stomata in detached shade leaves over water opened in response to low laboratory light (approximately 5 μmol photons m<sup>-2</sup> s<sup>-1</sup>) but were closed during exposure to sunlight.

**Experimental Protocols**

Results from experiments of two types, with mature fully expanded attached and detached avocado shade leaves exposed to sunlight, are reported. In experiment 1, three attached leaves were selected on a plant in the shade enclosure and sampled predawn for pigments, measurements of F<sub>e</sub>/F<sub>m</sub> were performed using the photosynthetic efficiency analyzer (PEA) system (www.hansatech-instruments.com), and photosynthetic parameters were measured in situ using RLRC of chlorophyll fluorescence with the MINI-PAM system (www.walz.com) as described below. After 3 h of induction in the shade enclosure (maximum of 20 μmol photons m<sup>-2</sup> s<sup>-1</sup>), one disc from each leaf was taken to the dark laboratory (2–5 μmol photons m<sup>-2</sup> s<sup>-1</sup>) in moist paper
toweling and examined using the following elapsed time sequences of assays: functional fraction of PSII centers (0–5 min); RLRC in the MINI-PAM (5–20 min); $F_v/F_m$ measured with the PEA and subsampling for pigment analysis (10–25 min).

About one-half of each leaf was then covered with black cloth and aluminum foil before transferring the plant to the unshaded greenhouse. Leaves were arranged horizontally during 200 min in sunlight (750 μmol photons m$^{-2}$ s$^{-1}$, 28°C), and discs were removed from exposed areas at frequent time intervals for assay in the sequence described above. The plant was returned to the shade enclosure, and unexposed control areas were assayed after 250 min and again the next day, 28 h after commencement of the experiment. As usual, values of $F_v/F_m$ from PEA were slightly higher than from the MINI-PAM but were highly correlated ($r^2 = 0.98$; Supplemental Fig. S2A), and although $F_v$ values of most parameters increased with the duration of sun exposure, these were not a product of the sampling/protocol measurement.

This experiment was repeated (experiment 2; Table I) with four attached leaves on another plant in the shade enclosure that were assayed in situ as above. Leaf clips were applied to provide dark controls later in the experiment before the plant was transferred to an unshaded area of the glasshouse. Leaves were arranged horizontally for exposure to winter sunlight (100 μmol photons m$^{-2}$ s$^{-1}$, declining 400 μmol photons m$^{-2}$ s$^{-1}$ in experiment 4), with a saturating light transition (Chow et al., 1991). Pigments and photosynthetic parameters were assayed again after 1 and 5 h of sunlight exposure. Leaf clips were again applied to 5-h exposed areas of the leaves before returning the plant for 24 h of recovery in the dark.

Two experiments were also done with detached leaves. These experiments facilitated longer exposure to stronger sunlight (5 h, approximately 700 μmol photons m$^{-2}$ s$^{-1}$ in experiment 3; 90 min, 1,200 μmol photons m$^{-2}$ s$^{-1}$ in experiment 4), were easier to manipulate, and avoided the exposure of all leaves in the canopy to variable sunlight, thereby conserving plant material for other studies. Leaves were assayed in the shade enclosure before being detached (petiole cut under water and kept in transparent containers with the lamina supported in air at 28°C) and partly covered with aluminum foil to serve as dark controls, then arranged and exposed to sunlight as in the above attached leaf experiments. Experiment 3 was a close approximation to experiment 1, but with assays 1 and 5 h after transfer to sunlight and recovery in the dark 24 h after commencement of the experiment. Detached leaves in experiment 4 (Table II) experienced the strongest sunlight exposure (1,200 μmol photons m$^{-2}$ s$^{-1}$), with leaf discs removed for pigment analysis after 15, 30, 60, and 90 min and after recovery overnight. Photosynthetic parameters were measured in discs from exposed areas after 60 and 90 min at 1,200 μmol photons m$^{-2}$ s$^{-1}$ and then again after 23 h of recovery in the dark laboratory overnight. Controls were measured before exposure, then after 90 min, and again after 24 h. Measurements of Photosynthetic Properties

Photosynthetic parameters were conveniently and reproducibly measured in air using the Photosynthesis Yield Analyzer MINI-PAM fitted with leaf clip holder 2030-B (www.walz.com). Chlorophyll fluorescence was measured randomly from a spot of about 12 mm diameter in control and exposed areas on attached or detached leaves or on discs cut from these leaves and supported on a moist glass fiber filter during measurement. Assay protocols were optimized with respect to saturating flash intensity and actinic light treatments to minimize pigment deepoxidation and artifacts in photosynthetic parameters during measurements on the extremely light-sensitive shade leaves (Forstner et al., 2011). RLRC had a dwell time of 30 s at each of eight steps from darkness to approximately 400 μmol photons m$^{-2}$ s$^{-1}$ (to approximate remote control of 600 μmol photons m$^{-2}$ s$^{-1}$, with a saturating flash at the end of each step. Dark relaxation of NPQ was usually followed for 220 s at the end of RLRC, but the short time intervals between sampling in experiment 1 made this impossible, so only a few representative dark relaxation profiles were collected. Light intensities during the experiments were measured with the light sensor fitted to the leaf clip of the MINI-PAM system.

Calculation of Photosynthetic Parameters

The MINI-PAM apparatus measured intrinsic chlorophyll fluorescence ($F_v$) from a spot on a leaf during a saturating flash ($F_m$) to give the quantum yield of photochemical energy conversion $\Phi_{pv}$ or $F_v/F_m = (F_m - F_v)/F_m$. Photosynthetic electron transport was calculated from fluorescence yield in subsequent saturating flashes under actinic light ($F_{m_{act}}$) using the quantum yield of photochemical energy conversion ($\Delta F/F_m = F_m - F/F_{m_{act}}$) and the PFD measured at that spot (adjusted for absorbance of 0.85 and assuming equal light absorption in PSII and PSI). Sunlight exposure of shade-grown leaves led to large, slowly relaxing (hours to days) decreases in $F_{m_{act}}$ as well as a marked decline (~20%) in $F$ within 100 s after actinic light was extinguished (presumably due to the absence of background far-red light in the MINI-PAM) that produced misleading results from automated calculation of NPQ and qP. These problems were addressed by recalculating all NPQ data using $F_{m_{act}}$ measured on the leaf kept in the dark overnight before light treatment and/or $F_{m_{act}}$ from aluminum foil–shaded areas of the leaf during and after treatment. Likewise, $1 - q_P$ data were recalcualted using the minimum value of $F$ obtained within 100 s after actinic light was switched off. This latter procedure may have been responsible for the unusual kinetic responses of $1 - q_P$ at low PFD.

Measurements of the Functional Fraction of PSII Centers

The low rates of photosynthesis in shade-grown avocado leaves (gas exchange of approximately 2 μmol m$^{-2}$ s$^{-1}$ CO$_2$, chlorophyll fluorescence of approximately 10 μmol m$^{-2}$ s$^{-1}$ ETR at 100 μmol photons m$^{-2}$ s$^{-1}$) precluded frequent measurement of the concentration of active PSII centers by oxygen yield in single-turnover flashes (Chow et al., 1989, 1991). Instead, another more rapid method was used based on an estimation of the integrated electron flux $\Delta F$ after superimposing a saturating single-turnover flash on steady background far-red light (Losciiale et al., 2008; Chow et al., 2012). A dual-wavelength (810/870 nm) detector unit (ED-P700D; www.walz.com) was used in the reflectance mode, and PI700 reductions were measured in a leaf disc after 2 min of steady-state far-red light (12 μmol photons m$^{-2}$ s$^{-1}$, peak, 723 nm; half-width, 20 nm; detector/emitter 102 FR; www.walz.com) followed by single-turnover saturating xenon flashes (XST 103) controlled by a pulse/delay generator (model 555; Berkeley Nucleonics). Flashes (25) were given at 0.2 Hz, and data collected for 900 ms (Supplemental Fig. S2) were digitized and stored using a custom-built computer program. Data analysis to estimate the functional fraction of PSII centers (PSII/PSI) was as described (Losciiale et al., 2008). This rapid in vivo method for estimation of the functional fraction of PSII centers (about 2 min per measurement) was well suited for kinetic examination of the responses to sunlight in conjunction with, but independent from, other photosynthetic parameters derived from chlorophyll fluorescence ($F_v/F_{m_{act}}, \Delta F/F_{m_{act}}$). In three experiments over many months, shade leaves kept in the dark always had lower PSII/PSI (0.55 ± 0.02; $F_v/F_{m_{act}}$ of 0.80 ± 0.01) than the same leaves after 2 to 4 h of induction of photosynthesis in the shade enclosure (0.67 ± 0.01; $F_v/F_{m_{act}}$ of 0.80 ± 0.02), an observation consistent with the initially lower estimates of oxygen flash yield after dark-to-light transition (Chow et al., 1991).

Pigment Analyses

Leaf discs (1 cm diameter) were punched from treated and control areas of leaves at the times specified in each experiment, wrapped in foil, and immediately frozen in liquid nitrogen. Pigments were extracted from individual discs in ethyl acetate:acetone (60:40, v/v), displaced from the aqueous to the ethyl acetate phase, and measured by HPLC, as described earlier (Forstner et al., 2009). Pigments were identified by retention times and spectra, and carotenoid concentrations were calculated using conversion factors for $A_{440}$ obtained with pure pigments, as determined by Dr. Shizue Matsubara (personal communication). The deepoxidation status of the Lx cycle was calculated as DESLx = $[\Delta L]/(\Delta L+|\Delta L|)$, and that of the V cycle was calculated as DESVx = $|\Delta A+|\Delta Z|)/(|\Delta A||\Delta Z|)$. The L pool in avocado shade leaves was usually 3- to 5-fold greater than that of the other xanthophylls, so the values for L have been reduced by 100 mmol mol$^{-1}$ chlorophyll (marked L*) in graphical presentations to facilitate more sensitive comparative assessment of stoichiometric relationships.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The "Vaseline patch" test applied to detached avocado shade leaves during rapid light-response curve assays.

Supplemental Figure S2. Methods used for independent measurement of PSII efficiency and the arbitrary ratio of PSI/PSI.

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