Review

From ecophysiology to phenomics: some implications of photoprotection and shade–sun acclimation in situ for dynamics of thylakoids in vitro

Shizue Matsubara¹, Britta Förster², Melinda Waterman⁴, Sharon A. Robinson⁴, Barry J. Pogson²,³, Brian Gunning² and Barry Osmond²,⁴,*

¹IBG-2: Pflanzenwissenschaften, Forschungszentrum Jülich, Jülich 52425, Germany
²Division of Plant Sciences, Research School of Biology (RSB), and
³ARC Centre of Excellence in Plant Energy Biology, RSB, Australian National University, Canberra, Australian Capital Territory 0200, Australia
⁴Institute for Conservation Biology and Environmental Management, School of Biological Sciences, University of Wollongong, Wollongong, New South Wales 2522, Australia

Half a century of research into the physiology and biochemistry of sun–shade acclimation in diverse plants has provided reality checks for contemporary understanding of thylakoid membrane dynamics. This paper reviews recent insights into photosynthetic efficiency and photoprotection from studies of two xanthophyll cycles in old shade leaves from the inner canopy of the tropical trees Inga sapindoides and Persea americana (avocado). It then presents new physiological data from avocado on the time frames of the slow coordinated photosynthetic development of sink leaves in sunlight and on the slow renovation of photosynthetic properties in old leaves during sun to shade and shade to sun acclimation. In so doing, it grapples with issues in vivo that seem relevant to our increasingly sophisticated understanding of ΔpH-dependent, xanthophyll-pigment-stabilized non-photochemical quenching in the antenna of PSII in thylakoid membranes in vitro.

Keywords: lutein epoxide cycle; xanthophyll cycle; photoinactivation

1. INTRODUCTION

Our present understanding of intra-chloroplast membrane systems depends as much on observations from five or so generations of plant biologists who have ranged widely and insightfully throughout the plant kingdom as it does on the last two decades of creative mutagenesis of Arabidopsis and Chlamydomonas. One legacy of these observations on diverse organisms is the nomenclature in use today: grana [1] and thylakoids [2]. Another is the challenge of how well contemporary understanding measures up against the extraordinary array of chloroplast morphologies and dynamics in the Kingdom Plantae [3].

From personal perspectives, our interests in genome–environment interactions (real-world phenomenics in modern parlance) stemmed from pioneering research on ecotypic differentiation of shade–sun acclimation by Olle Björkman and stimulated by his Canberra sabbatical in 1971 [4]. Collaboration [5] on an overview of shade–sun acclimation, published under the series editorship of Jim Barber after a photoinhibition workshop, provided a further stimulus. This synthesis drew heavily on the work of Jan Anderson and co-workers [6,7]. It both initiated and sustained a long-standing association driven by her enduring enthusiasm and inspiring leadership of research in photosynthetic light reactions, acknowledged here by several of her colleagues.

The focus then was the stability and dynamics of photosystem II (PSII) reaction centre functions, and its overall control in relation to Jan’s findings of lateral heterogeneity [8] and the central role of the D1 protein repair cycle in which, on an average day in average sunlight, the entire population of this protein in the thylakoid membrane turned over at least once [9]. However, ecophysiological research was about to shift this focus to photoprotection involving reversible regulation of light-harvesting efficiency in the antenna and its stabilization by xanthophyll pigment inter-conversions [10]. Interestingly, Jan had also participated in one of the earliest studies of light-dependent changes in the pool of violaxanthin (V) [11].

In this concluding contribution to the discussion meeting, our message is that, with time on its hands,
natural selection and environment have conspired to present a huge array of distinctive thylakoid membrane systems in plants and that continues to challenge our understanding of dynamic processes at this level. There are few better reminders of this challenge, or of the rich resource available in diverse plants, than the images of thylakoids and videos of chloroplast dynamics assembled by Gunning [12]. Advances in mechanistic understanding of thylakoid dynamics achieved through studies using targeted mutants of Arabidopsis [13,14] are but an ice cube on the tip of the iceberg that is the diversity of thylakoid structure, dynamics and function in the plant kingdom.

The ecophysiological studies of Terashima & Inoue [15] showed that light environment and time are key determinants of thylakoid dynamics in vivo; gradients of light in leaves of Camellia japonica produce sun-adapted photosynthetic properties in chloroplasts on the upper surface and shade-adapted properties on the lower. The organization of thylakoids in grana of chloroplasts on sun and shade sides of Glycine max cv. Mikawajima was dynamic during development [16]. After 8 days illumination with 400 μmol photons m\(^{-2}\) s\(^{-1}\), the proportion of thylakoids: granum was 6.9 ± 1.09 in chloroplasts from spongy mesophyll on the shaded underside compared with 4.6 ± 0.5 in upper palisade cells. When comparable leaves were treated with the same illumination from below, the thylakoids: granum ratio was essentially reversed (4.9 ± 0.77 in lower spongy mesophyll to 7.3 ± 0.87 in palisade cells). Similar times (approx. 7 days) were observed for reconstitution of thylakoid membrane and other chloroplast components of shade-grown peas transferred to high light [7] and for change in chlorophyll (Chl) fluorescence parameters in leaves of woody eucalypts [17].

Thylakoid dynamics in long-lived leaves of shrubs and trees from naturally shaded habitats may be rather different from those in herbaceous plants. For example, Goodchild et al. [4] noted that the chloroplasts of shade-grown Alocasia macrorrhiza had grana stacks that, like leaves of the plant itself, ‘reach prodigious proportions’. This species soon became the archetype, extraordinarily shade-tolerant plant. Although photosynthetic O\(_2\) evolution increased about 10-fold in long-lived leaves of A. macrorrhiza grown with 800 μmol photons m\(^{-2}\) s\(^{-1}\) compared with those grown in 5 μmol photons m\(^{-2}\) s\(^{-1}\) [18], and thylakoids: granum declined from approximately 100 to approximately 10, Sims & Pearcy [19] found that photosynthesis in fully developed shade leaves (1% sunlight) did not acclimate to 20 per cent sunlight. Acclimation was observed only in the second leaf to expand in 20 per cent sunlight. Confocal images show smaller chloroplasts in sun compared with shade leaves of A. macrorrhiza and Anthurium spp., but also emphasize just how difficult it is to quantify thylakoid dynamics from transmission electron microscopy (TEM) of these huge grana [12]. Retention of old leaves in deeply shaded canopies presents but one special case of sun–shade acclimation. Although discussion of these phenomena is usually focused on cost–benefit analyses and structural considerations, we have argued that there may be additional distinctive attributes of photosynthesis in these leaves that involve rather more than repayment of their construction costs [20]. For example, the distinctive pigment composition and de-epoxidation/epoxidation kinetics in two xanthophyll cycles in woody plants of Mediterranean and tropical forests have attracted attention in this context [21–24].

This paper briefly reviews some properties of pigment dynamics in relation to photoprotection in two species, Inga sapindoides and Persea americana, especially those aspects encouraging further attention to the role of lutein (L). At the same time, it also presents new physiological data on time frames for photosynthetic development in sink leaves and on photosynthetic renovation of old leaves during shade to sun (and sun to shade) acclimation in the canopies of woody plants. It grapples with issues in vivo that may be relevant to our increasingly sophisticated understanding of ΔpH-dependent xanthophyll pigment-stabilized non-photochemical quenching (NPQ) in the antennae of PSII.

2. MATERIAL AND METHODS

Seedlings of ‘Hass’ avocado (P. americana Mill. cv EdranoL) were sourced and grown in a deeply shaded (95%–98%) portion of a temperature-controlled greenhouse (28°C day/18°C night) in Canberra, as described previously [24]. Peak noon incident photon flux density (PFD) in the un-shaded portion of the greenhouse (700–1300 μmol photons m\(^{-2}\) s\(^{-1}\)) was comparable to that measured outdoors during field studies in orchards at Summerland House Farm, Alstonville NSW, and at the Peirson Memorial Trust, Goodwood, Queensland in 2009–2010. Pigments were measured by high-performance liquid chromatography (HPLC) [24], and photosynthetic parameters were measured by Chl fluorescence using the portable MINI-PAM system (http://www.walz.com), with protocols optimized for shade leaves and designed to minimize de-epoxidation during assay [24].

The automated rapid light response curves (RLRC) used here are tantamount to naturally occurring sun flecks and provide ‘snapshots’ of changes in redox state of QA (1–qP), photosynthetic electron transport (ETR) and NPQ of Chl fluorescence at intervals during acclimation over days and weeks. The PFD profiles in RLRC usually ranged from 0 to approximately 450 μmol photons m\(^{-2}\) s\(^{-1}\) during shade to sun acclimation and from 0 to approximately 1800 μmol photons m\(^{-2}\) s\(^{-1}\) during sun to shade acclimation, with 30 s dwell time at each PFD, followed by relaxation in the dark for 220 s after assay. The initial kinetics of dark relaxation were obtained by normalizing NPQ data and fitting the first exponential \(y = y_0 \cdot e^{-kt}\) (until \(y_0 = 1.0 \pm 0.03\) and \(R^2 > 0.9\), and deriving an estimate of \(t_{1/2} = 0.693/k\).

We used the ‘Vaseline patch’ test [25] to confirm that stomata were closed in attached leaves pre-dawn and opened in approximately 30 min during induction in the shade (5–20 μmol photons m\(^{-2}\) s\(^{-1}\)).

Designation of different forms of NPQ on the basis of kinetic responses in vivo follows the subscript conventions proposed previously [20,25]. Measurements of NPQ in pre-dawn assays, or from aluminium-foil shaded areas of exposed leaves, in the absence of de-epoxidation of lutein epoxide (Lx) or V were considered to represent ΔpH-dependent processes (or qE) [26] and
were designated NPQΔH. Changes in the kinetic properties of NPQ in response to actinic light treatments that led to xanthophyll de-epoxidation and subsequent epoxidation in the dark were designated as NPQAZ, NPQMLAZ or NPQΔMLAZ, according to the de-epoxidized xanthophylls (A, antheraxanthin; Z, zeaxanthin; L, lutein) retained in leaves. While they are termed differently in our attempt to separate the effects of two xanthophyll cycles on NPQ kinetics, these NPQ components and also NPQΔH may or may not have a common mechanistic basis. The persistently high, slowly relaxing NPQ after sun exposure associated with residual high [L] and [A + Z], as well as PSI reaction centre photoinactivation that involves distinctly different mechanisms, was designated as NPQPI (qI) [26].

3. RESULTS AND DISCUSSION

Old leaves in the deeply shaded inner canopy of avocado contain high concentrations of Lx on a Chl basis, [Lx], comparable to or greater than [V], and sun leaves contain nearly twice the [L] of shade leaves, substantially exceeding the sum of [V + A + Z] at noon (table 1). This distinctive pigment composition, arising from the co-occurrence of two xanthophyll cycles (the Lx- and V- cycles) that differ markedly in epoxidation kinetics, is now known to be quite widespread in plants [27–29]. Although in vivo de-epoxidation of Lx is sometimes slower and requires higher light intensity than V, the de-epoxidation products L and A + Z now are recognized to have two main functions: amplification of energy-dependent photoprotection and protection against photo-oxidation. Concerning the former function, the other side of the coin is suppression of strong ΔpH-induced quenching in the presence of Lx and V. It remains a large task to evaluate the importance of these light-dependent xanthophyll interconversion processes in functional biodiversity under natural conditions, but perhaps is less of a challenge to accept the insights they provide for research using model systems.

Table 1. Midday spot measurements of photosynthetic parameters and leaf pigment compositions in fully expanded inner and outer canopy leaves of avocado in two orchards in eastern Australia (mean ± s.e.).

<table>
<thead>
<tr>
<th>photosynthetic parameters</th>
<th>Goodwood, QLD 4660 (25°08'S; 152°22'E) elevation 62 m</th>
<th>Alstonville, NSW 2427 (28°51'S; 153°26'E) elevation 163 m</th>
</tr>
</thead>
<tbody>
<tr>
<td>photon flux density (PFD), μmol photons m⁻² s⁻¹</td>
<td>2.9 ± 0 inner (n = 10)</td>
<td>5 ± 1 inner (n = 18)</td>
</tr>
<tr>
<td>photosynthetic efficiency, (ΔF/F°m at prevailing PFD)</td>
<td>0.83 ± 0.00 outer (n = 10)</td>
<td>0.81 ± 0.00 outer (n = 19)</td>
</tr>
<tr>
<td>photosynthetic electron transport (ETR), μmol electrons m⁻² s⁻¹</td>
<td>1 ± 0 inner (n = 3)</td>
<td>2 ± 0 inner (n = 3)</td>
</tr>
<tr>
<td>pigments, mmol mol⁻¹ Chl</td>
<td>inner (n = 10)</td>
<td>outer (n = 10)</td>
</tr>
<tr>
<td>lutein epoxide (Lx)</td>
<td>29 ± 5</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>lutein (L)</td>
<td>159 ± 8</td>
<td>279 ± 13</td>
</tr>
<tr>
<td>violaxanthin (V)</td>
<td>29 ± 13</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>antheraxanthin (A)</td>
<td>1 ± 0</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>zeaxanthin (Z)</td>
<td>1 ± 0</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>α/β-carotene</td>
<td>1.4 ± 0.2</td>
<td>0.2 ± 0</td>
</tr>
<tr>
<td>chlorophyll a/b ratio</td>
<td>2.1 ± 0.1</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>

(a) Does accumulation of Lx in shade leaves enhance light-harvesting efficiency?

Pronounced accumulation of Lx in shade and its conversion to L following short illumination, and the substitution of L for A and Z during long-term acclimation to high irradiance, may imply unique dual functions for Lx under shade conditions. Evidence that Lx might potentially enhance light-harvesting efficiency in the shade emerged from experiments in vitro with model systems. The higher fluorescence yield in recombinant Arabidopsis Lhcb5 reconstituted with Lx as the only xanthophyll (i.e. with Lx presumably bound in the two xanthophyll binding sites termed L1 and L2; [30,31]) compared with reconstitution with L is consistent with this [32]. As light energy absorbed can be re-emitted as fluorescence or dissipated as heat in isolated antenna complexes, high fluorescence yield of the recombinant Lhcb5 with Lx, despite the presence of traces of A in the Lx used in reconstitution, indicates that less energy was lost via thermal dissipation, despite traces of A potentially exerting an opposite, dissipative effect [33]. In vivo replacement of Lx by L and vice versa in these binding sites during xanthophyll cycle operation or sun–shade acclimation could serve to modulate light harvesting in leaves: enhancement of light harvesting (or inhibition of strong NPQ) by Lx and promotion of NPQ capacity by L.

Clearly, much more work is needed to elucidate the function of Lx in shade leaves. For example, unlike the above observation in Lhcb5, Lx-L substitution did not alter energy transfer within recombinant Lhcb1 from Hordeum vulgare (barley) or native trimeric LHCCI isolated from I. sapindoides [32]. Moreover, Lx and L did not alter the susceptibility of the recombinant Arabidopsis Lhcb5 or barley Lhcb1 to photobleaching. As no recombinant Lhc protein has been available from Lx-cycle species for in vitro reconstitution with Lx or L, the question remains open as to whether Lx-L exchange brings about the same protein conformational change and thermal dissipation in antenna.
complexes of plants having the Lx- and V-cycles as those having only the V-cycle. Further experiments with recombinant or isolated antenna complexes may show changes in intrinsic properties of the antenna complexes caused by binding of different xanthophylls but in the absence of ApH and PSII macroorganization. Remarkable flexibility and variability in the higher organization of the photosynthetic apparatus is underscored by the finding of a variety of PSII–LHCII supercomplex organizations (C2S2M1–2, C2S2M2L1–2) as well as dynamic alterations in the alignment and distance of neighbouring PSII complexes in thylakoid preparations from Arabidopsis and Spinacia oleracea [34]. The molecular function of Lx needs to be investigated in the context of protein macroorganization by using preparations from Lx-cycle plants.

(b) An initial small decline in [L] prior to de-epoxidation of Lx in shade leaves transferred to weak sunlight—the possibility of chemical photo-oxidation?

Lutein is the most abundant xanthophyll in higher plant leaves and has fundamentally important structural roles when bound to light-harvesting complexes (LHCs) and other potential roles in protection against reactive oxygen species (ROS) [35]. Transfer of avocado shade leaves to moderate sunlight in the greenhouse [24], or when inner canopy leaves are exposed to stronger light following pruning in the field (figure 1), commonly leads to a small decline in [L] before detectable de-epoxidation of Lx and de novo synthesis of L. This hitherto unnoticed response is not associated with increase in pools of either its precursor α-carotene (α-C) or its product Lx, and is easily obscured in experiments with overzealous ‘light-shocks’ that initiate immediate de-epoxidation of Lx followed by de novo synthesis of L.

We speculated that some photo-oxidative destruction of L may be an early response of shade leaves acclimating to sunlight [24]. Such a fate for L and Z is recognized as a minor chemical component (as distinct from biophysical quenching) of protection against ROS in mammalian retinas [36] but little attention seems to have been given to these possibilities in plants. In vitro experiments [37] indicated that L is a stronger quencher of hydroxyl and superoxide radicals than of singlet O2 (1O2). In vivo, and consistent with this, it was found that chy1chy2lut5 mutants of Arabidopsis with L as the only xanthophyll are extremely sensitive to 1O2 [38]. Studies with other mutant constructs show that L and other xanthophylls could not substitute for the protection against photo-oxidation that can be attributed to a pool of Z that is bound [39] or not bound to antenna LHCS [40].

Notably, a recent HPLC–MS study has detected carotenoid endoperoxides, the major oxidative cleavage products of β-C and xanthophylls (Z and L, indistinguishable owing to similar mass) by 1O2, in leaves of Arabidopsis plants grown under low light [41]. However, compared with β-C that undergoes continuous turnover in the light together with Chl a [42], accumulation of L and/or Z endoperoxides in leaves appears to be much less and their levels change little during exposure of plants to high light and low temperature, while the concentrations of β-C endoperoxides are more than double under the same condition. The β-C molecules giving rise to these oxidation products are most likely bound in the PSII core complex, but the location of the small pool of L and/or Z photo-oxidized in leaves is unknown. Identification of the avocado thylakoid membrane fraction(s) in which photo-oxidative L depletion occurs will be rewarding. Interestingly, the loss of L in these experiments was quantitatively similar to that subsequently recovered by de-epoxidation of Lx.

(c) A small addition to the lutein pool (ΔL from de-epoxidation of Lx) replaces A + Z to ‘lock-in’ enhanced capacity for fast relaxing NPQ in avocado shade leaves

De-epoxidation in the Lx-cycle in avocado, Meditteranean oak (Quercus rubra), I. sapindoides and several other species is only very slowly reversible [23], in marked contrast to that in parasitic Cuscuta reflexa [43] and Amyema miquelii [44] or in a Macaronesian evergreen tree Ooetia foetens [45]. Indeed, the rapid restoration of [Lx] in O. foetens after short (3 min) artificial sun flecks is evidence for the role of Lx as a ‘perfect switch’ in this species, supporting high photosynthetic efficiency in the shade as

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discussed earlier, and its de-epoxidation providing enhanced NPQ in a sun fleck [45].

Although Lx is readily converted to L in the light by violaxanthin de-epoxidase (VDE) in avocado, restoration of [Lx] in the dark, supposedly catalysed by zeaxanthin epoxidase (ZE), is 1 to 2 orders of magnitude slower than the restoration of V from A and Z [27]. Thus, slow accumulation of Lx in avocado shade leaves over many days and weeks is presumably due to a ZE with low affinity for (or restricted access to) L [27] functioning in a shade environment in which de-epoxidation of Lx rarely occurs. These distinctive in vivo kinetic properties of the two xanthophyll cycles facilitated the demonstration that retention of a small addition to the L pool ([Lx] in the dark, supposedly catalysed by violaxanthin de-epoxidase (VDE) in avocado, restoration of [Lx] in the dark, supposedly catalysed by zeaxanthin epoxidase (ZE)) ‘locks-in’ a high capacity for NPQ₁ for prolonged periods in the dark, after epoxidation of A + Z in four plants: Quercus [21], I. sapindoides [22], I. marginata [46] and Persea [24].

It is the custom in the biochemical literature to use photosynthetic induction curves (figure 2) to relate the properties of NPQ to xanthophyll pigment composition in vivo. Weak actinic light (1 h at 200 μmol photons m⁻² s⁻¹) was used to bring about de-epoxidation of both Lx and V, and the effect on the kinetics of NPQ was assayed in induction curves. The actinic treatment initially accelerated NPQᵢ sustained higher NPQᵢ that relaxed after assay with kinetics similar to NPQᵢ in marked contrast to the slower relaxation of NPQᵢ. The light-saturated capacity for NPQᵢ in similar experiments was approximately 80 per cent of that of NPQᵢ, and approximately 35 per cent greater than NPQᵢ attained in the absence of xanthophyll de-epoxidation [25]. Although xanthophyll-dependent and independent forms of NPQ may share a common mechanism [47], it is unclear at the physiological level how these capacities relate; does NPQᵢ for example, substitute for NPQᵢ or is it simply additive?

However, dark relaxation of NPQᵢ was faster (t₁/₂ = 83 s) after induction of ETR in the shade than in pre-dawn assays (t₁/₂ = 108 s). Modest actinic light treatments that resulted in de-epoxidation of both Lx and V caused noticeably slower relaxation of NPQᵢ, but importantly, retention of ΔL after epoxidation of A + Z sustained elevated NPQᵢ that relaxed with kinetics similar to NPQᵢ (table 2). Stomata closed during sunlight exposure of detached leaves with petioles in water and illumination at the CO₂ compensation point led to much higher NPQᵢ that neither recovered fully overnight nor relaxed rapidly in the dark (table 2), and was associated with high [ΔL] and [A + Z] and depression of the functional

Table 2. Properties of different categories of NPQ during exposure of attached avocado shade leaves to approximately 200 μmol photons m⁻² s⁻¹ for 1 h and of detached leaves exposed to approximately 800 μmol photons m⁻² s⁻¹ for 5 h.

<table>
<thead>
<tr>
<th>NPQ category</th>
<th>NPQ</th>
<th>t₁/₂(s)</th>
<th>ΔL</th>
<th>DS</th>
</tr>
</thead>
<tbody>
<tr>
<td>experiments with attached leaves; n = 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>induced NPQᵢ</td>
<td>1.2 ± 0</td>
<td>85 ± 6</td>
<td>nil</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>exposed 1 h NPQᵢ ─ LAZ</td>
<td>1.5 ± 0.1</td>
<td>143 ± 6</td>
<td>10.7 ± 2.2</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>control 24 h NPQᵢ ─ L</td>
<td>1.3 ± 0.1</td>
<td>94 ± 12</td>
<td>nil</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>recovery 24 h NPQᵢ ─ M</td>
<td>1.4 ± 0.1</td>
<td>89 ± 11</td>
<td>11.1 ± 2.0</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>an experiment with detached leaves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPQᵢ ─ L</td>
<td>0.9</td>
<td>165</td>
<td>nil</td>
<td>0.10</td>
</tr>
<tr>
<td>NPQᵢ ─ LAZ</td>
<td>3.9</td>
<td>886</td>
<td>25</td>
<td>0.78</td>
</tr>
<tr>
<td>NPQᵢ ─ P</td>
<td>2.7</td>
<td>578</td>
<td>23</td>
<td>0.59</td>
</tr>
</tbody>
</table>

*Half time for relaxation of NPQ in the dark.
*Change in [L] mmol mol⁻¹ Chl.
*De-epoxidation status [A + Z]/[V + A + Z].

Consistent with these observations, an in vivo Chl fluorescence lifetime imaging analysis revealed rapid formation of a short (0.5 ns) lifetime component of PSII fluorescence upon illumination of avocado shade leaves retaining A and ΔL in the dark [48]. The fluorescence lifetime of this component is comparable with the values reported for NPQ-related lifetime components, such as the 0.3 ns component associated with PsbS and [A + Z] [49] or the 0.4 ns component associated with LHClI aggregation [50]. When linear ETR and ΔpH formation were inhibited in the avocado leaves, the 0.5 ns lifetime component was not detected despite the presence of A and ΔL, suggesting interaction between ΔpH and NPQΔLA. With regard to the site(s) of action, ΔL substitutes for Lx in the peripheral V1 site of trimeric LHChs, and the internal L2 site in both monomeric and trimeric LHChs in leaves of I. sapindoides [32], in much the same way as Z replaces V in these two xanthophyll binding sites to stabilize and enhance NPQ [51]. These and other data from Lx-cycle studies show that ΔL supports enhanced NPQ and presaged evidence that Arabidopsis si1npq1 mutants over-expressing L in the absence of Z partially recovered NPQ and displayed a Z-like radical cation ascribed to Chl–carotenoid (in this case due to L) charge transfer quenching [52].

The fact that Chl-Z charge transfer quenching predominantly occurs in minor antenna complexes (Lhcb4, 5 and 6) and not in LHClI [53] is in line with the higher Chl fluorescence yield measured in Lhcb5 reconstituted with Lx but not in Lhcb1 and trimeric LHClI [32]. Interestingly, recombinant Arabidopsis Lhcb5 exhibits carotenoid radical cations in both L1 (with L) and L2 (with Z) sites although binding of Z in L2 seems to be a prerequisite for Chl-L charge transfer quenching in L1 [54]. The bottom line is that NPQΔL has attributes similar to the ΔpH- and Z-dependent component of NPQ. However, the distinctive, and therefore probably important, features of NPQΔL lie in its rapid induction upon illumination (albeit less than NPQΔLA) which sustains an enhanced NPQ capacity compared with NPQΔpH (figure 2), combined with its fast dark relaxation, which parallels NPQΔpH and is more rapid than NPQΔLA (table 2).

**d) Photosynthetic acclimation during leaf development in an avocado canopy**

In situ spot measurements of outer and inner canopy ETR under prevailing light conditions in the field showed a 100-fold range in photosynthetic activity in sun and shade leaves of avocado with a fourfold range in photosynthetic efficiency in situ (ΔF/Fm′). The light-saturated capacity of in vivo ETR achievable in the laboratory and the field is an order of magnitude less, ranging from approximately 35 in shade leaves to approximately 260 μmol electrons m⁻² s⁻¹ in sun leaves (figure 3). Although some shade leaves emerge and develop within the shade canopy, most form in sunlight and acclimate to shade as they are overshadowed by ‘flushes’ of new sun leaves.

Pigment composition and photosynthetic parameters were examined in leaves of different ages in a small canopy that developed on a single cutting in full sunlight in the greenhouse [20]. Youngest leaves had only traces of Lx but highest [L] and highest [V + A + Z] similar to sun leaves in the field (table 1). A decline in [L] with leaf age was associated with increase in [Lx] and [α–C], but [Lx], [L] and [α–C] changed little between dark (pre-dawn) and full sunlight at midday at any stage of development. In the two youngest leaf categories, the increase in [A + Z] at midday greatly exceeded the decline in [V], indicating that de-epoxidation was augmented by de novo synthesis and moreover, epoxidation was incomplete overnight. Diurnal de novo synthesis of A + Z was not at the expense of [β–C] at any stage of development. Self-shading after leaf expansion had ceased in the developing canopy led to diminished de-epoxidation of V, de novo synthesis of A + Z and residual pre-dawn [A + Z]. These changes in xanthophyll pigment composition during avocado leaf and canopy development closely resemble those observed previously as mature shade and sun leaves acclimated after transfer to sun and shade, respectively [24].

Developing avocado leaves are ‘sink leaves’ and do not achieve net CO₂ assimilation until approximately 17 days after emergence [55], and development of the photosynthetic apparatus may occur with closed stomata and photorespiratory CO₂ cycling providing the principal ETR sink. The Qₐ pool was more oxidized, and although 1-qP was insensitive to PFD in youngest leaves, it became increasingly responsive to PFD in older leaves (figure 4a). Young leaves had lower light-saturated ETR than fully expanded leaves. In younger leaves, ETR declined above 1000 μmol photons m⁻² s⁻¹ (figure 4b). High capacity for ETR clearly modulated expression of NPQ, which was lowest in fully expanded leaves despite the higher de-epoxidation status of the V cycle (DS) in these leaves (0.47 in Y1 versus 0.64 in Y2 and 0.80 in FE). Unexpectedly, NPQ relaxed with equal rapidity (t₁/₂ = 23 s) irrespective of DS (figure 4d).
In the most sun-exposed young leaves, it seems that chloroplasts develop and function in a strongly oxidizing environment from the outset, even at low PFD. With a limited capacity for ETR, young leaves evidently make full use of both the protection against photo-oxidation, and the disposal of excitation as heat that is potentially afforded by the constitutively high [L] and diurnally regenerated high [A + Z]. Amplification of the capacity for ETR with leaf age seems associated with modulation of QA redox state in response to PFD, and with an increasing role for ETR and a lesser role for NPQ, in dissipation of excitation.

(e) Sun to shade acclimation in fully expanded mature leaves of avocado
Avocado leaves that develop in sunlight in the field may become overshadowed by subsequent canopy expansion and spend the next 300–500 days in the shade, continuing to function and to maintain photosynthetic integrity at extraordinarily low PFD at midday (table 1). In a glasshouse experiment, sun leaves at midday had a highly oxidized QA pool that resembled the PFD profile of very young leaves (cf. figure 4a,e), and showed rapid acceleration of ETR to very high levels that was reflected in an initially slower engagement of NPQ (cf. figure 4b,f,g).

Marked changes in these parameters were already evident after 4 days in the shade, with QA becoming more reduced, and with a dramatic reduction in ETR (figure 4a,b,c,f). The initially more slowly engaged, but rapidly relaxing NPQ in sun leaves with high [L] and [A + Z] (DS > 0.3) was replaced by an almost equivalent capacity of NPQ$_{\text{2\alphaH}}$ (DS < 0.1) within a few days of transfer to the shade. Again, the increase in NPQ during assay reflected the decline in ETR.

**Figure 4.** Rapid light response curves of photosynthetic parameters in avocado leaves during acclimation. All data were collected with photosynthetic activities fully induced (ind) at midday (11.00–14.00 h). In the developing canopy (a–d), data for youngest (Y1), young (Y2) and fully expanded (FE) leaves are means. During sun–shade acclimation (e–h) data for the initial sun leaves (Su$_{i}$) and after 4 and 33 days in the shade (Su–Sh) are means ± s.e., n = 4. Shade–sun acclimation (i–l) for the initial shade leaves (Sh$_{i}$) and after 1 h, 24 h and 28 days are means ± s.e., n = 3. (Error bars appear when standard error exceeds size of symbol. Keys in a, d and j apply to all graphs in the particular row.)
Dark relaxation of NPQ was rapid, with $t_{1/2}$ only slowing from 19 to 29 s and consistent with sustained low DS after transfer to shade, but inexplicably similar to those in the presence of high A + Z in developing leaves. From all indications in this and other experiments, it seems that acclimation of photosynthetic light reactions to shade was essentially complete within a week after transfer. Other important changes in pigment composition include the increase in [Lx] and small decline in [V + A + Z], which were complete within 24 days. The much slower change from sun to shade leaf signature in the ratio of $\alpha$-C : $\beta$-C, from 0.37 to 1.87 after 97 days in this experiment, was initially associated with an increase in $\alpha$-C and a subsequent decrease in $\beta$-C (data not shown).

(f) Acclimation to sunlight in fully expanded avocado shade leaves

Recent studies of shade–sun acclimation in avocado shade leaves have focused on short-term issues, similar to those examined in wild-type Arabidopsis [56] and other plants to obtain insights into photoinactivation and photoprotection that are relevant to concepts of thylakoid dynamics discussed at this meeting. We were surprised to discover that although photosynthetic efficiency of old shade leaves of avocado initially declines markedly immediately after transfer to sun, these leaves have a remarkable capacity to reconstruct the photosynthetic apparatus to match the performance of new fully expanded leaves that develop in the sun on the same plant.

These responses were examined in two experiments, the first commencing in early winter, when short day length constrained total daily sunlight exposure to only 10.3 ± 0.3 MJ m$^{-2}$. Photosynthetic parameters were referenced to $F_o/F_m$ measured on induced leaves in the shade enclosure (approx. 20 $\mu$mol photons m$^{-2}$ s$^{-1}$) and then 1 h, 1 day and one month after transfer to sun (800 to 1100 $\mu$mol photons m$^{-2}$ s$^{-1}$). Characteristically, QA was more oxidized after 1 h in sun but recovered the next day (figure 4f), whereas depression of ETR became more pronounced the next day (figure 4f). The decline in the initial slope and maximum rate of ETR was consistent with photoinactivation of PSII centres [57].

NPQ increased markedly after 1 h sun exposure and increased further the next day when compared with shade controls, but relaxed much more slowly in the dark after assay. While only traces of A + Z were present in the shade control, DS increased from 0.07 to 0.60 after 1 h, rising to 0.72 after 28 days. In this, as in other experiments discussed earlier, the transition from rapidly relaxing to slowly relaxing NPQ in the dark was a signature of the transition from NPQ$\Delta$pH ($t_{1/2}$ = 39 s) in the near absence of A + Z to NPQ$\Delta$LAZ ($t_{1/2}$ = 85 s) attributable to the presence of both $\Delta$L and A + Z after 1 h in sunlight. The highest, and most slowly dark-relaxing NPQ ($t_{1/2}$ = 533 s) after the first day of sun exposure was further indicative of a component of NPQ$\Delta$pH due to photoinactivation. After a month in sunlight, QA remained oxidized throughout the PFD profile (figure 4f), ETR increased approximately twofold at approximately 450 $\mu$mol photons m$^{-2}$ s$^{-1}$ (figure 4f), and although NPQ at this light intensity was similar to that after 1 h and 1 day of exposure (figure 4f), dark relaxation was now similar to that of NPQ$\Delta$pH in the shade control ($t_{1/2}$ = 25 s) in spite of a high DS = 0.53 ± 0.08. Not surprisingly, xanthophyll pigment composition had changed remarkably. In conformity with earlier studies [24], [Lx] declined to 29 per cent of the initial shade control, [L] increased to 127 per cent and [V + A + Z] increased to 327 per cent of the initial shade control. The ratio $\alpha$-C : $\beta$-C had declined from 2.28 to 0.63 as acclimation proceeded. The substantially lower reduction state of QA and higher capacity for ETR in these leaves evidently conspired to accelerate the dark relaxation kinetics of NPQ$\Delta$LAZ ($t_{1/2}$ = 25 s) in the presence of high [A + Z] and DS in these sun leaves (figure 4f). Another, longer shade–sun acclimation experiment explored the above changes in more detail during the first experiment from July to October 2009 under the average daily irradiance of 16.9 ± 0.6 MJ m$^{-2}$ (65% more than in figure 4i–f).

In this experiment, ETR doubled after 6 days, doubled again after 17 days and again after 78 days. Taken together, these and other in vivo experiments show that the photosynthetic properties of old avocado shade leaves are capable of ‘renovation’, which involves many biochemical changes and changes in structure at all levels, to resemble those of sun leaves within a month, with the most important transformations largely complete between 10 and 17 days.

(g) Complex changes in the capacity and kinetics of NPQ during acclimation in avocado

It is evident from the above discussions that the response profiles of 1-qP and ETR in dark to light induction curves (figure 2) and in RLRC (figure 4) are indispensable to the interpretation of the kinetics of NPQ and its response to xanthophyll pigment composition in vivo. Analyses of these interactions in vivo are highly sensitive to growth and actinic light treatments, to xanthophyll de-epoxidation during assay, and to whether stomata open during pre-treatments or close during assay. On darkening, we can expect relaxation of $\Delta$pH, which de-activates NPQ$\Delta$pH and the enzyme VDE, to occur more rapidly than epoxidation by ZE that continues for minutes to hours in the dark [58]. Thus, relaxation of NPQ in the dark has the potential to uncover $\Delta$pH/pigment interactions. For example, induction of ETR in shade leaves at low PFD has no effect on pigment composition, but is accompanied by reduction in NPQ$\Delta$pH and acceleration of dark relaxation, suggesting that modulation of $\Delta$pH by increased ETR also modulates NPQ.

Of course, these interactions are potentially compounded during shade–sun acclimation, but similar intriguing possibilities emerged from the experiments in figure 4. Leaf age made no difference to dark relaxation of NPQ$\Delta$LAZ and to the level of NPQ expressed during the assay. Youngest sink leaves at the top of the canopy had lowest light ETR and presumably confronted the highest light stress. They developed the highest NPQ but surprisingly did not fully exploit this to the maximal capacity (DS = 0.47). They function with highly oxidized QA (figure 4a). By contrast,
fully expanded leaves lower in the canopy had higher capacity for ETR, smaller xanthophyll pools and operated with only slightly more reduced QA, but engaged lower NPQ at higher DS.

Although thylakoid membrane dynamics during chloroplast development have received considerable attention [12], the acquisition and engagement of diverse mechanisms of photoprotection during leaf and canopy development has not. The kinetics of dark relaxation of NPQ\textsubscript{L\textsubscript{1/2}} remained relatively constant during development, and showed little of the sensitivity to residual A + Z observed in shade-grown leaves with much slower rates of ETR at low DS, and by approximately 50 per cent increase in \(t_{1/2}\) of dark relaxation of what is reasonably assumed to be NPQ\textsubscript{pH}.

Sun acclimation in shade-grown leaves confirmed the slowing of \(t_{1/2}\) for dark relaxation of NPQ. The \(t_{1/2}\) doubled in the first hour, and increased more than 10-fold in the first day (cf. figure 3) before readjusting to that of fully expanded sun leaves with high DS after 28 hours. How much of the initial increase in \(t_{1/2}\) can be ascribed to de-epoxidation of xanthophylls and how much can be ascribed to the concurrent photoactivation of PSII centres remains to be determined. Using a Chl fluorescence-independent assay of the functional fraction of PSII centres compared with PSI (H. Jia, B. Förster, W. S. Chow, B. J. Pogson & B. Osmond 2012, unpublished data), it was found that the decline in the arbitrary ratio PSII:PSI was highly correlated with independently measured \(F_{v}/F_{m}\) and NPQ. They concluded that photoactivation of PSII continued in sun-exposed shade leaves of avocado after de-epoxidation in both Lx- and V-cycles ceased, and in spite of continued de novo synthesis of L and A + Z. Apportioning components of NPQ\textsubscript{pH} associated with persistently high xanthophyll concentrations and with photoactivation remains a daunting task.

Measurements in the field confirmed approximately 50 per cent slower \(t_{1/2}\) for dark relaxation and 50 per cent higher NPQ in naturally acclimated shade leaves with lower DS and ETR, compared with sun leaves. The former may be predominately ascribed to NPQ\textsubscript{L1/2} and the latter predominately to NPQ\textsubscript{L1/2}. We have not been able to decide whether the capacity of NPQ, the kinetics of onset or the kinetics of relaxation in the dark are more highly correlated with [Z] than with DS [59].

4. SUMMARY AND CONCLUSIONS
Physiological assessments of shade leaves of avocado using Chl fluorescence during acclimation revealed the holistic engagement of many components of the photosynthetic apparatus. The terms of this engagement changed with leaf development in sunlight, with aging in the shade and on exposure of shade leaves to sunlight. The PFD profiles of QA redox status and capacity for ETR interacted with the expression of NPQ, and in turn modulated interactions with specific xanthophyll pigments. For example, although initial relaxation of NPQ in the dark always followed a simple exponential, its \(t_{1/2}\) was sensitive to pigment composition and modulated by \(\Delta\text{pH}\) inferred from ETR capacity.

In general, shade leaves seem to test the limits of thylakoid dynamics \textit{in vivo}, from huge grana in \textit{A. macrorrhiza} chloroplasts that have limited potential for acclimation to sunlight, to the possibility that Lx-enhanced light-harvesting efficiency in LHCs of \textit{I. sapindoides} shade leaves. These studies focus renewed attention on the roles of L in addition to its primary function as the most effective xanthophyll quencher of triplet Chl when occupying the L1 and L2 positions in LHCII [60]. Although there seems little likelihood that L substitutes for Z in protection against \(^{1}\text{O}_2\) [39,40], the causes of, and the significance of, an initial net loss of L in avocado shade leaves on exposure to sunlight remain enigmatic.

On the basis of studies in plants having V or Z but little L in V1 of LHCs, Jahns & Holzwarth [35] concluded that L is unlikely to be involved in aggregation-dependent quenching associated with Z. Yet our studies with shade leaves of \textit{Inga} spp. and avocado showed the enhanced capacity for NPQ\textsubscript{L} was retained in the dark for as long as L remained. By contrast, the rapid reversibility of the Lx cycle in \textit{O. foetens} [45] may offer the ultimate ‘photoprotective molecular switch in the PSII antenna’ [61].

Reconstruction of shade chloroplasts for service in sunlight involves at least four major transformations other than those associated with de-epoxidation of L and V that tend to be complete within an hour. The first is enhanced de novo synthesis of L and of A + Z, the latter amplifying diel capacity for reversible NPQ\textsubscript{L1/2} [24]. Initially (i.e. within hours) de novo synthesis in itself is inadequate to prevent slowly reversible photoactivation of PSII reaction centres in avocado shade leaves in sunlight (H. Jia, B. Förster, W. S. Chow, B. J. Pogson & B. Osmond 2012, unpublished data). The second is the photoprotective capacity associated with these photoinactivated PSII reaction centres and regulation of the D1 repair cycle [62]. Matsubara & Chow [63] demonstrated that these inactive centres are highly dissipative. This component of NPQ\textsubscript{pH}, with its particularly slow relaxation in the dark, seems to persist for one to two weeks in avocado leaves. Identification of these inactivated but dissipative centres with particular pigment containing intermediates of the D1 repair cycle is a challenge, but further astonishing technical advances [64] show that photoinhibition, presumably some of it involving photoactivation accompanied by protein phosphorylation, mobilizes a significant population of Chl protein complexes in the D1 repair cycle within 10 min. However, our experiments show such processes must continue for days and weeks during acclimation \textit{in vivo}; photoinhibition images printed on shade leaves of \textit{Cissus} remained clearly visible by Chl fluorescence imaging for at least 10 days in laboratory light [65].

A third and slower component involves amplification of ETR capacity in thylakoids. In agreement with earlier studies [8,9], our data suggest that the redox status of QA responds faster than the capacity of ETR, possibly because it is rate-limited by increase in Rubisco and other elements of regulated metabolism. These serve as terminal energy sinks for the products of photochemistry. Their capacity builds slowly over several days and is dependent on N availability [66] and reallocation [67]. Pathways leading
to alternative electron sinks and cyclic ETR also contribute to upregulation of ETR during shade–sun acclimation [68,69].

The fourth component of the ability for a pre-existing shade leaf to fully acclimate was not taken into account in our experiments. As noted earlier [19], fully expanded pre-existing shade leaves of A. macrorrhiza were unable to acclimate to sun, whereas the second leaf to expand in sunlight acclimated fully. The new sun leaves were thicker, whereas the fully expanded shade leaves did not thicken. This commonly observed structural constraint [70] has been examined in detail by Oguchi et al. [71], who found that although chloroplasts in mature leaves of low-light-grown Chenopodium album increased in size and occupied a higher proportion of cell walls adjacent to air spaces, leaf thickness did not increase. Moreover, leaves of deciduous woody plants showed at least three versions of these forms of anatomical constraints on the capacity to acclimate to light intensity [72]. Distinctions have been drawn previously between chloroplast and leaf level components of acclimation [73] and that in the long run, thylakoid dynamics may also be determined by these higher level constraints of space and time.

These four physiological transformations occur concomitantly with, and are all functionally linked to, the fifth and fundamental suite of structural transformations involved in remodelling of thylakoids into grana of markedly different size and shape, with different numbers of discs and different ratios of end-granal sur-}

tations involved in remodelling of thylakoids into grana of marked differences in size and shape, with different numbers of discs and different ratios of end-granal surfaces to apposed inter-disc surfaces. As we return to ponder the perplexing question of the functional advantages of gigantic grana in A. macrorrhiza in deep shade, might we allow there may be none? Shade plants in general have low capacity for ETR and low ATPase content, demanding little space in stromal thylakoids, and only a small stromal compartment overall for low levels of Rubisco and the enzymes of the carbon reduction cycle. Could stacking of grana to ‘prodigious proportions’ simply be a consequence of the limited demand for these primary level spatial constraints?

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