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

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Abstract

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Keywords

implications, phenomics, ecophysiology, dynamics, vitro, situ, thylakoids, acclimation, sun, shade, photoprotection

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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

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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 (NPQ) in the antenna of PSII in thylakoid membranes in vitro.

Keywords:

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].

Our interest in genome-environment interactions (real world phenomics in modern parlance) stems from pioneering research on ecotypic differentiation of shade-sun acclimation by Olle Björkman and his 1971 sabbatical in Canberra [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 her colleagues [6, 7]. It both initiated and sustained a long-standing association driven by her enduring enthusiasm and inspiring leadership of research in Canberra on the light reactions of photosynthesis.

The focus then was the stability and dynamics of 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 observations of light dependent changes in the violaxanthin pool [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 dynamics in plants that continues to challenge our understanding of these processes. 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 [12]. Advances in understanding thylakoid dynamics from 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.

In order to underline the importance of light environment in determining thylakoid dynamics Anderson & Osmond [5] drew attention to Terashima & Inoue [15] who showed gradients of light in leaves of *Camellia japonica* determined 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

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One contribution of XX to a Discussion meeting Issue " ……."

chloroplasts from spongy mesophyll on the shaded underside compared to 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 (~7 d) were observed for reconstitution of thylakoid membrane and other chloroplast components of shade-grown peas transferred to high light [7] and for change in 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.* [5] 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 to those grown in 5 µmol photons m^{-2} s^{-1} [18], and thylakoids / granum declined from ~100 to ~10, Sims & Pearcy [19] found that photosynthesis in fully developed shade leaves (1% sunlight) did not acclimate to 20% sunlight; acclimation was only observed in the second leaf to expand in 20% sunlight. Confocal images show smaller chloroplasts in sun compared to shade leaves of *A. macrorrhiza* and *Anthurium* spp., but also emphasize just how difficult it is to quantify thylakoid dynamics from 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 in dynamic thylakoid membranes.

2. MATERIALS and METHODS

Seedlings of "Hass" avocado (*Persea americana* Mill. cv Edranol) were sourced and grown in a deeply shaded (95-98%) portion of a temperature-controlled greenhouse (28 ºC day/18 ºC night) in Canberra as described previously [24]. Peak noon incident PFD in the un-shaded portion of the greenhouse (700-1,300 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 2009-10. Pigments were measured by 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 Q_A (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 ~450 µmol photons m^{-2} s^{-1} during shade to sun acclimation and from 0 to ~1,800 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 220s after assay. The initial kinetics of dark relaxation were obtained by normalizing NPQ data and fitting the first exponential $y = y_o \cdot e^{-kx}$ (until $y_o = 1.0 \pm 0.03$ and $R^2 > 0.9$), and deriving an estimate of $t_{1/2}$ from $t_{1/2} = 0.693 / k$. We used the 'Vaseline™ patch' test [25] to confirm that stomata were closed in attached leaves pre-dawn and opened in ~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 from in pre-dawn assays, or from Al-foil shaded areas of exposed leaves, in the absence of de-epoxidation of Lx or V were considered to represent ΔpH-dependent processes (or *qE*) [26] and were designated $NPQ_{\Delta pH}$. Changes in the kinetic properties of NPQ in response to actinic light treatments that led to xanthophyll de-epoxidation and epoxidation in the dark, presumably represent different forms of *qN* [26] in vivo and were designated as NPQ_{AZ}, $NPQ_{\Delta LAZ}$ or $NPQ_{\Delta L}$. The persistently high, slowly relaxing NPQ after sun exposure associated with residual high [L] and [A+Z] and/or PSII reaction centre photoinactivation of PSII, was designated as NPQ_{PI} (*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. It remains a large task to evaluate the importance of these 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.

(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 or A and Z during long-term acclimation to high irradiance,

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 ± SE).

Photosynthetic parameters	Goodwood, QLD 4660 (25° 08' S; 152° 22' E) Elevation 62 m		Alstonville, NSW 2427 (28° 51' S; 153° 26' E) Elevation 163 m	
	Inner (n = 10)	Outer (n = 10)	Inner (n = 18)	Outer (n = 19)
Photon flux density (PFD), µmol photons m^{-2} s^{-1}	2.9 ± 0	1483 ± 136	5 ± 1	1337 ± 55
Photosynthetic efficiency, ($\Delta F/F_m'$ at prevailing PFD)	0.83 ± 0.00	0.19 ± 0.03	0.81 ± 0.00	0.35 ± 0.02
Photosynthetic electron transport (ETR), µmol electrons m^{-2} s^{-1}	1 ± 0	114 ± 23	2 ± 0	195 ± 11
Pigments, mmol mol^{-1} Chl	Inner (n = 3)	Outer (n = 5)	Inner (n = 3)	Outer (n = 6)
Lutein-epoxide (Lx)	29 ±5	11 ± 1	50 ± 11	18. ± 2
Lutein (L)	159 ± 8	279 ± 13	144 ±11	214 ± 19
Violaxanthin (V)	29 ± 13	32 ± 2	39 ± 2	69 ± 11
Antheraxanthin (A)	1 ± 0	54 ± 8	8 ± 1	30 ± 3
Zeaxanthin (Z)	1 ± 0	70 ± 11	5 ± 1	40 ± 9
α / β-carotene	1.4 ±0.2	0.2 ± 0	1.4 ± 0.1	0.5 ± 0
Chlorophyll *a* / *b*	2.1 ± 0.1	2.4 ± 0.1	1.9 ± 0	2.4 ± 0.1

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 internal xanthophyll binding sites termed L1 and L2; [30, 31]) compared to substitution with another xanthophyll is consistent with this [32]. As light energy absorbed can be reemitted 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].

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 LHCII 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 ΔpH 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 (C_2S_2, $C_2S_2M_{1\text{-}2}$, $C_2S_2M_2L_{1\text{-}2}$) as well as dynamic alterations in the alignment and distance of neighboring 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 samples 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 plants and has fundamentally important structural roles when bound to light-harvesting complexes and other potential roles in protection against reactive O_2 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 L is a stronger quencher of hydroxyl and superoxide radicals than of singlet O_2 ($^1O_2$). In vivo, and consistent with this, found that *chy1chy2lut5* mutants of *Arabidopsis* with L as the only xanthophyll are extremely sensitive to $^1O_2$ [38]. Studies with other mutant constructs show 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 due to similar mass) by $^1O_2$, in leaves of *Arabidopsis* plants grown under low light [41]. However, compared to 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 β-C endoperoxides more than

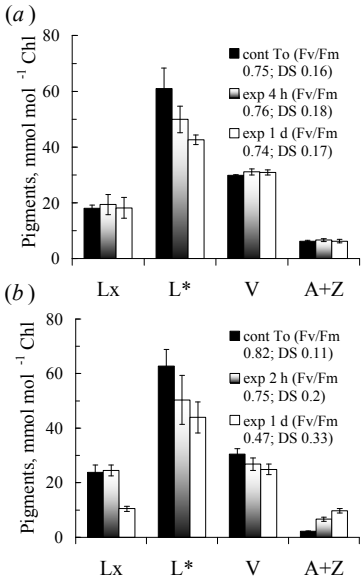

Figure 1. Decline in L before de-epoxidation of Lx in avocado shade leaves in the field following two pruning techniques used in East Australian orchards; control (T_o) before pruning: (a) the least intrusive, selective removal of lower branches and (b) more intrusive V-shaped removal of centre canopy (means ± SE; n = 3-5; note that scale for [L*] corresponds to 100-180 mmol mol^{-1} Chl to facilitate stoichiometric comparison with other xanthophylls).

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 faster relaxing NPQ in avocado shade leaves

De-epoxidation in the Lx-cycle in avocado, in Mediterranean oak (*Quercus rubra*), in *I. sapindoides* and several other species is only very slowly reversible [23], in marked contrast to that in parasitic *Cuscuta reflexa* [43], *Amyema miquelii* [44] and in *Ocotea foetans* [45]. Although Lx is readily converted to L in the light by violaxanthin de-epoxidase (VDE), restoration of [Lx] in the dark, supposedly catalyzed by zeaxanthin epoxidase (ZE), is 1 to 2 orders of magnitude slower than the restoration of V from A and Z in avocado [24]. 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 ([ΔL] from de-epoxidation of Lx) that "locks-in" a high capacity for $NPQ_{\Delta L}$ for prolonged periods in the dark, after epoxidation of A+Z in three species *Quercus* [21], *Inga* [22] 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 $NPQ_{\Delta LAZ}$ compared to $NPQ_{\Delta pH}$ measured pre-dawn. However the maximum rates of $NPQ_{\Delta pH}$ and $NPQ_{\Delta LAZ}$ were similar. Enhancement of NPQ associated with xanthophyll de-epoxidation under these assay conditions was presumably mitigated by the induction of ETR that increased by 60% in the actinic light used to transform the pigments. Whereas A and Z were largely epoxidized overnight, ΔL was not. With ETR unchanged, persistent ΔL sustained higher $NPQ_{\Delta L}$ that relaxed after assay with kinetics similar to $NPQ_{\Delta pH}$, in marked contrast to the slower relaxation of $NPQ_{\Delta LAZ}$. The light-saturated capacity for $NPQ_{\Delta L}$ in similar experiments was ~80% of that of $NPQ_{\Delta LAZ}$, and ~35% greater than $NPQ_{\Delta pH}$ (*qE*) 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 $NPQ_{\Delta LAZ}$ for example, substitute for $NPQ_{\Delta pH}$ or is it simply additive?

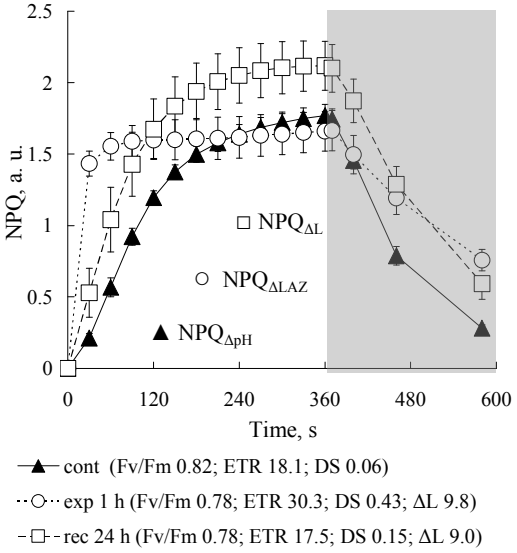

Figure 2. Three forms of NPQ assayed by photosynthetic induction curves (300 μmol photons m^{-2} s^{-1}) in avocado shade leaves. $NPQ_{\Delta pH}$ was obtained with leaves kept in the dark overnight. $NPQ_{\Delta LAZ}$ was obtained with discs exposed to 220 μmol photons m^{-2} s^{-1} for 1 h on water at 21ºC and dark pretreated for 30 min before assay. $NPQ_{\Delta L}$ was obtained with another subset of the treated discs after 24 h recovery on water in the dark.

However, dark relaxation of $NPQ_{\Delta pH}$ was faster ($t_{1/2}$ = 83 s) after induction of ETR in the shade than in pre-dawn assays ($t_{1/2}$ = 108 s). Modest actinic light treatments that resulted in de-epoxidation of both Lx and V caused noticeably slower relaxation of $NPQ_{\Delta LAZ}$, but importantly, retention of ΔL after epoxidation of A+Z sustained elevated $NPQ_{\Delta L}$ that relaxed with kinetics similar to $NPQ_{\Delta pH}$ (table 2). Stomata closed during sunlight exposure of detached leaves with petioles in water and illumination at the CO_2 compensation point led to much higher NPQ_{PI} that neither recovered fully overnight nor relaxed rapidly in the dark (table 2), and was associated with high [ΔL] and [A+Z] and depressed PSII activity (Jia & Chow, unpublished).

NPQ category	NPQ	$t_{1/2}$, s*	ΔL **	DS***
Experiments with attached leaves; n = 4				
Induced $NPQ_{\Delta pH}$	1.2 ± 0	85 ± 6	nil	0.02 ± 0.01
Exposed 1h $NPQ_{\Delta LAZ}$	1.5 ± 0.1	143 ± 6	10.7 ± 2.2	0.31 ± 0.03
Control 24 h $NPQ_{\Delta pH}$	1.3 ± 0.1	94 ± 12	nil	0.03 ± 0.01
Recovery 24h $NPQ_{\Delta L}$	1.4 ± 0.1	89 ± 11	11.1 ±2.0	0.10 ± 0.02
An experiment with detached leaves				
$NPQ_{\Delta pH}$	0.9	165	nil	0.10
$NPQ_{\Delta LAZ}$	3.9	886	25	0.78
NPQ_{PI}	2.7	578	23	0.59

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

Table 2: Properties of different categories of NPQ during exposure of attached avocado shade leaves to ~200 µmol photons m^{-2} s^{-1} for 1 h and of detached leaves exposed to ~800 µmol photons m^{-2} s^{-1} for 5 h.

Consistent with these observations, 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 LHCII aggregation [50]. Also with regards to the site(s) of action, ΔL substitutes for Lx in the peripheral V1 site of trimeric Lhcs, and the internal L2 site in both monomeric and trimeric Lhcs in leaves of *I. sapindoides* [31], 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 szl1npq1* 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 LHCII [53] is in line with the higher Chl fluorescence yield measured in Lhcb5 reconstituted with Lx but not in Lhcb1 and trimeric LHCII [31]. 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 $NPQ_{\Delta L}$ has attributes similar to the ΔpH- and Z-dependent component of NPQ. However, the distinctive, and therefore probably important, features of $NPQ_{\Delta L}$ lie in its rapid induction upon illumination (albeit less than $NPQ_{\Delta LAZ}$) which sustains an enhanced NPQ capacity compared with $NPQ_{\Delta pH}$ (figure 2), combined with its dark relaxation, which parallels $NPQ_{\Delta pH}$ and is more rapid than $NPQ_{\Delta LAZ}$ (table 2).

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

The 'end-members' of the potential photosynthetic activity in sun and shade leaves of avocado were found by in-situ spot-measurements of outer and inner canopy ETR in the field under prevailing light conditions (table 1). A range of 100-fold in ETR was found under operating conditions, in which there was a four-fold range in photosynthetic efficiency in situ ($\Delta F/F_m'$). The light-saturated capacity of in vivo ETR achievable in the laboratory and the field is an order of magnitude less, ranging from ~35 in shade leaves to ~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 predawn [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 [23].

Developing avocado leaves are 'sink leaves' and do not achieve net CO_2 assimilation until ~17 d after emergence [54] and development of the photosynthetic apparatus may occur with closed stomata and photorespiratory CO_2 cycling providing the principal ETR sink. The Q_A pool was more oxidized, and whereas 1-qP was insensitive to PFD in

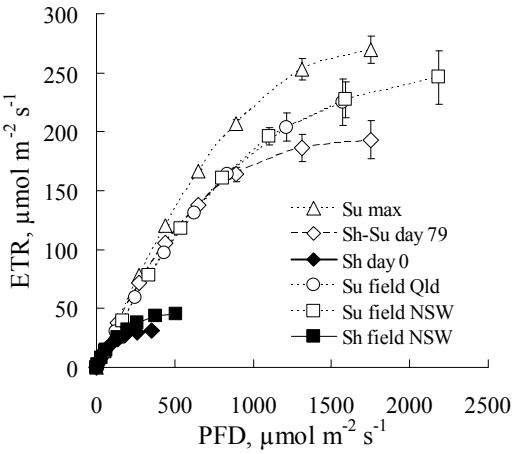

Figure 3: Photosynthetic electron transport (ETR) as a function of light intensity in avocado leaves grown in the sun in the greenhouse (Su max), after transfer from the shade (Sh-Su day 79) and in the shade (Sh day0). Measurements in the canopies of sun (Su) and shade (Sh) leaves in orchards in Eastern Australia are shown for comparison.

youngest leaves it became increasingly responsive to PFD in older leaves (figure 4 *a*). Young leaves had lower light-saturated ETR than fully expanded leaves. In younger leaves ETR declined above 1,000 µmol photons m^{-2} s^{-1} (figure 4 *b*). The PFD profiles of NPQ were the inverse of the profiles of ETR, with highest NPQ in the youngest leaves (figure 4 *c*) despite lowest DS (0.47in Y1 vs 0.64 in Y2 and 0.80 in FE). Unexpectedly, NPQ relaxed with equal rapidity ($t_{1/2}$ = 23 s) irrespective of DS (figure 4 *d*).

In the most sun-exposed young leaves, it seems that chloroplasts developed and function in a strongly oxidizing environment from the outset, even at low PFD. With 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 Q_A 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 d 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 Q_A pool, that resembled the PFD profile of very young leaves (c.f. figures 4 *a*, *e*), and showed rapid acceleration of ETR to very high levels that was reflected in an initially slower engagement of NPQ (c.f. figures 4 *b, f* and *c, g*).

Marked changes in these parameters were already evident after 4 d in the shade, with Q_A becoming more reduced, and with a dramatic reduction in ETR (figures 4 *a*, *b* and *e*, *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 $NPQ_{\Delta pH}$ (DS< 0.1) within a few days of transfer to the shade. Again, the increase in NPQ during assay reflected the decline in ETR. Dark relaxation of NPQ was rapid, with $t_{1/2}$ only slowing from 19 to 29 s and consistent with sustained low de-epoxidation status of the V-cycle 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. Important other changes in pigment composition include the increase in [Lx] and small decline in [V+A+Z] that were complete within 24 d. The much slower change from sun to shade leaf signature in the ratio of α-C / β-C, from 0.37 to 1.87 after 97 d in this experiment, was initially associated with an increase in α-C and a subsequent decrease in β-C (data not shown).

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

Recent studies of shade-sun acclimation in avocado shade leaves has focused on short-term issues, similar to those examined in wildtype *Arabidopsis* [55] and other plants to obtain insights into photoinactivation and photoprotection that are relevant to 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_v/F_m measured on induced leaves in the shade enclosure (~20 µmol photons m^{-2} s^{-1}) and then 1 h, 1 day and 1 month after transfer to sun (800-1,100 µmol photons m^{-2} s^{-1}). Characteristically, Q_A was more oxidized after 1 h in sun but recovered the next day (figure 4 *i*) whereas depression of ETR became more pronounced the next day (figure 4 *j*). The decline in the initial slope and maximum rate of ETR was consistent with photoinactivation of PSII centres [57].

Non-photochemical quenching increased markedly after 1 h sun exposure and increased further the next day and, compared to shade controls, but relaxed much more slowly in the dark after assay. Whereas 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 d. In this, as in other experiments discussed above, 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 ΔL 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_{PI} due to photoinactivation. After a month in sunlight, Q_A remained oxidized throughout the PFD profile (figure 4 *i*), ETR increased approximately two-fold at ~450 µmol photons m^{-2} s^{-1} (figure 4 *j*) and although NPQ at this light intensity was similar to that after 1 h and 1 d of exposure (figure 4 *l*), 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% of the initial shade control, [L] increased to 127% and [V+A+Z] increased to 327% of the initial shade control. The ratio α-C / β-C had declined from 2.28 to 0.63 as acclimation proceeded. The substantially lower reduction state of Q_A 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 4 *l*). Another, longer shade-sun acclimation experiment explored the above changes in more detail during the first experiment from July-October 2009 averaged 16.9 ± 0.6 MJ m^{-2} (65% more daily irradiance than in figure 4 *i* - *l*). In this experiment ETR doubled after 6 d, doubled again after 17 d and again after 78 d.

Taken together, these and other in vivo experiments show that the photosynthetic properties of old avocado shade leaves are capable of 'renovation', which for the most part is of a biochemical rather than structural nature, to resemble those of sun leaves within a month, with the most important transformations largely complete between 10 and 17 d.

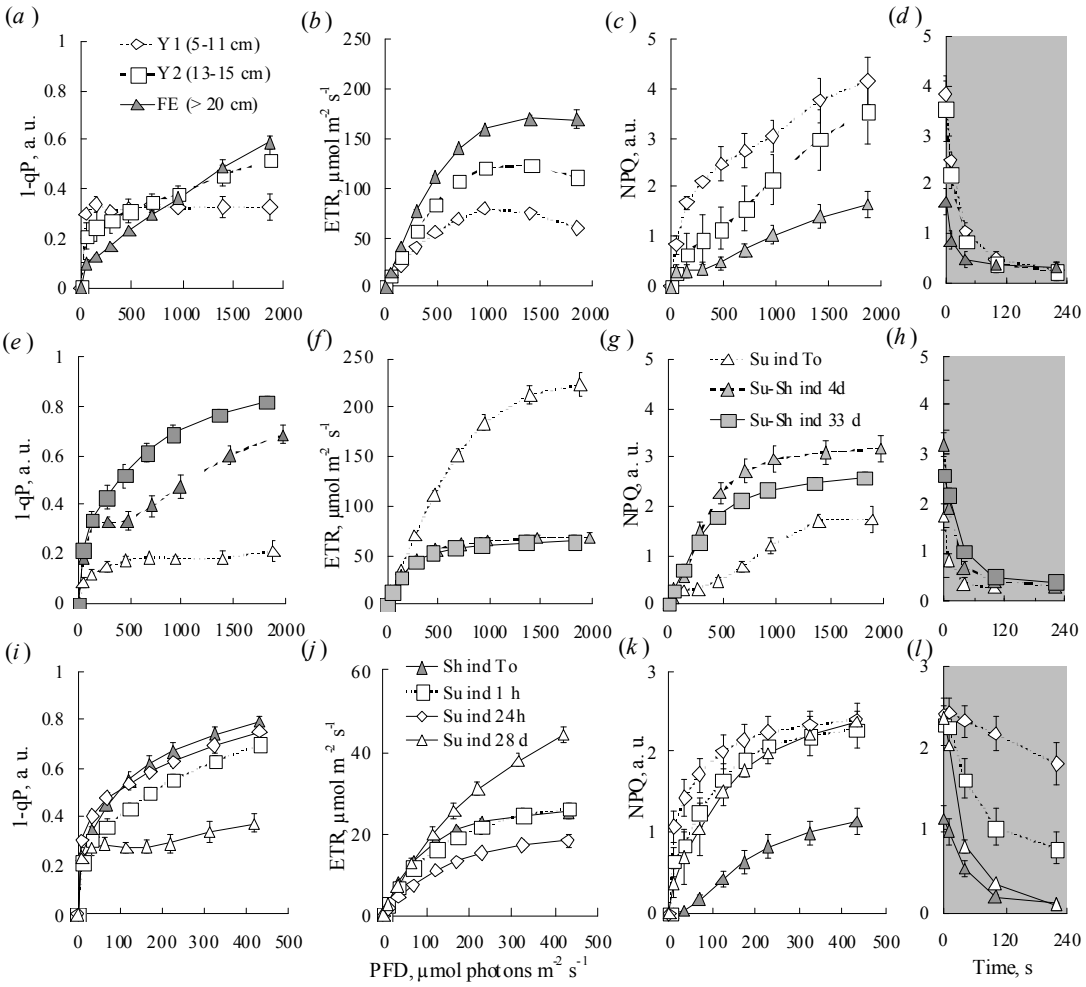

Figure 4. Rapid light response curves of photosynthetic parameters in avocado leaves during acclimation. All data were collected with photosynthetic activities fully induced (ind) at midday (11:00 to 14:00 h). In the developing canopy (*a* - *d*) data for youngest (Y1), young (Y2) and fully expanded (FE) leaves are means. During sun-shade acclimation (*e* - *h*) data for the initial sun leaves (Su To) and after 4 and 33 d in the shade (Su-sh) are means ± SE, n= 4. Shade-sun acclimation data (*i* - *l*) for the initial shade leaves (Sh To) and after 1 h, 24 h and 28 d are means ± SE, n = 3. (Error bars appear when SE exceeds size of symbol)

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

It is evident from the above that the response profiles of 1-qP and ETR in dark to light induction curves (figure 2) and in RLRC (figure 4) are indispensible to 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 pretreatments or close during assay. On darkening, we can expect relaxation of trans-thylakoid ΔpH, 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 [57]. Thus, relaxation of NPQ in the dark has the potential to uncover ΔpH/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 ΔpH 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 capacity (DS = 0.47). They function with highly oxidized Q_A (figure 4 *a*). In contrast, fully expanded leaves lower in the canopy had higher capacity for ETR, smaller xanthophyll pools, and operated with only slightly more reduced Q_A, but engage 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 development has not. The kinetics of dark relaxation of $NPQ_{\Delta LAZ}$ 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 ~50% increase in $t_{1/2}$ of dark relaxation of what is reasonably assumed to be $NPQ_{\Delta pH}$.

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 (c.f., figure 3) before readjusting to that of fully expanded sun leaves with high DS after 28 d. 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 photoinactivation of PSII centres remains to be determined. Using a Chl fluorescence-independent assay of the functional fraction of PSII centers compared to PSI (H-S. Jia & W. S. Chow, unpublished) found the decline in the arbitrary ratio PSII / PSI was highly correlated with independently measured F_v/F_m and NPQ. They concluded that photoinactivation 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_{PI} associated with persistently high xanthophyll concentrations and with photoinactivation remains a daunting task.

Measurements in the field confirmed ~50% slower $t_{1/2}$ for dark relaxation and 50% higher NPQ in naturally acclimated shade leaves with lower DS and ETR, compared with sun leaves. The former may be predominately ascribed to $NPQ_{\Delta pH}$ and the latter predominately to $NPQ_{\Delta LAZ}$. 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 [58].

4. SUMMARY AND CONCLUSIONS

In general, shade leaves seem to test the limits of thylakoid dynamics in vivo, from huge grana in *A. macrorrhiza* that have limited potential for acclimation to sunlight, to the novel possibility of Lx-enhanced light harvesting efficiency in Lhcs of *I. sapindoides* shade leaves. In species such as *Ocotea foetans* with a fully reversible Lx-cycle the comparatively rapid restoration of [Lx] after short (3 min) artificial sun flecks is suggestive evidence for such a role of Lx in vivo [45]. The de-epoxidation of Lx in sun flecks may offer the ultimate "photoprotective molecular switch in the PSII antenna" [59].

Studies with avocado shade leaves showed the enhanced capacity for $NPQ_{\Delta L}$ was retained in the dark for as long as ΔL remained and that metabolism of ΔL in prolonged darkness did not involve epoxidation to Lx [25]. However, based on studies in plants having V or Z but few L in V1, Jahns & Holzwarth [35] concluded that L is unlikely to be involved in aggregation-dependent quenching associated with Z. Lutein has a primary function in photoprotection through its role as the most effective xanthophyll quencher of triplet Chl (^{3}Chl) when occupying the L1 and L2 positions in LHCII [60]. There seems little likelihood that L substitutes for Z in protection against $^1O_2$ [39, 40], so the causes of, and the significance of, an initial net loss of L in avocado shade leaves on exposure to sunlight remain enigmatic.

Physiological assessments of these processes in shade leaves of avocado raised many issues and underlined the principle that during acclimation, both cycles are engaged and regulated by concerted interactions with all components of photosynthetic processes. Chlorophyll fluorescence analysis suggested that 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 Q_A redox status and capacity for ETR interacted with the expression of NPQ, and in turn modulated interactions with specific xanthophyll pigments. For example, initial relaxation of NPQ in the dark always followed a simple exponential, but its $t_{1/2}$ was sensitive to pigment composition and modulated by ΔpH inferred from ETR capacity. There seems little doubt that de-epoxidation of Lx, and de-novo synthesis of L, potentially have roles in photoprotection of shade leaves during sun flecks [20].

Reconstruction of shade chloroplasts for service in sunlight involves at least five major transformations other than those associated with de-epoxidation of L and V that tends 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 $NPQ_{\Delta Z}$ [24]. Initially (i.e., within hours) de-novo synthesis in itself is inadequate to prevent slowly reversible photoinactivation of PSII reaction centres in avocado shade leaves in sunlight (Jia & Chow; unpublished).

The second is the photoprotective capacity associated with photoinactivated PSII reaction centres and regulation of the D1 repair cycle [61]. Matsubara & Chow [62] demonstrated that these inactive centres are highly dissipative and potentially protective. Identification of these inactivated but protective centers with particular pigment containing intermediates of the D1 repair cycle is a challenge, but this component of NPQ_{PI}, with its particularly slow relaxation in the dark, seems to persist for one to two weeks in avocado leaves.

Further astonishing technical advances [63] show that photoinhibition, presumably some of it involving photoinactivation 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 in vivo. Acclimation was particularly slow in photoinhibition images printed on shade leaves of *Cissus* that remained clearly visible by Chl fluorescence imaging for at least 10 d recovery in laboratory light [64].

A third and slower component, involves amplification of ETR capacity in thylakoids. In agreement with [8 ,9] our data suggest that the redox status of Q_A 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 a terminal energy sink for the products of photochemistry and capacity builds slowly over several days and is dependent on N availability [65] and reallocation [66]. Pathways leading to alternative electron sinks and cyclic electron transport also contribute to up-regulation of ETR during shade-sun acclimation [67, 68].

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 [69] has been examined in detail by Oguchi *et al.* [70] 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 [71].

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 surfaces to apposed inter-disc surfaces. As we return to ponder the perplexing questions of plausible functional advantages of gigantic grana in *A. macrorrhiza* in deep shade might we allow that there may none? Shade plants in general have low capacity for ETR and low ATPase content, demanding little space in stromal thylakoids. Could the stacking simply be a consequence of the limited demand for this primary level spatial constraint?

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