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Biochimica et Biophysica Acta 1553 (2002) 309–319

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Xanthophyll biosynthetic mutants of *Arabidopsis thaliana*: altered nonphotochemical quenching of chlorophyll fluorescence is due to changes in Photosystem II antenna size and stability

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Received 11 October 2001; received in revised form 21 December 2001; accepted 9 January 2002

Abstract

Xanthophylls (oxygen derivatives of carotenes) are essential components of the plant photosynthetic apparatus. Lutein, the most abundant xanthophyll, is attached primarily to the bulk antenna complex, light-harvesting complex (LHC) II. We have used mutations in *Arabidopsis thaliana* that selectively eliminate (and substitute) specific xanthophylls in order to study their function(s) in vivo. These include two lutein-deficient mutants, *lut1* and *lut2*, the epoxy xanthophyll-deficient *aba1* mutant and the *lut2aba1* double mutant. Photosystem stoichiometry, antenna sizes and xanthophyll cycle activity have been related to alterations in nonphotochemical quenching of chlorophyll fluorescence (NPQ). Nondenaturing polyacrylamide gel electrophoresis indicates reduced stability of trimeric LHC II in the absence of lutein (and/or epoxy xanthophylls). Photosystem (antenna) size and stoichiometry is altered in all mutants relative to wild type (WT). Maximal ΔpH-dependent NPQ (qE) is reduced in the following order: WT > *aba1* > *lut1* ≈ *lut2* > *lut2aba1*, paralleling reduction in Photosystem (PS) II antenna size. Finally, light-activation of NPQ shows that zeaxanthin and antheraxanthin present constitutively in *lut* mutants are not qE active, and hence, the same can be inferred of the lutein they replace. Thus, a direct involvement of lutein in the mechanism of qE is unlikely. Rather, altered NPQ in xanthophyll biosynthetic mutants is explained by disturbed macro-organization of LHC II and reduced PS II-antenna size in the absence of the optimal, wild-type xanthophyll composition. These data suggest the evolutionary conservation of lutein content in plants was selected for due to its unique ability to optimize antenna structure, stability and macro-organization for efficient regulation of light-harvesting under natural environmental conditions.

Keywords: Antenna size; Carotenoid; Lutein; Nonphotochemical quenching of chlorophyll fluorescence; Photoprotection; Xanthophyll; *Arabidopsis thaliana*

Abbreviations: A, antheraxanthin; Chl, chlorophyll; LHC II, bulk light-harvesting complex; F_0, F_m, F_v, minimal, maximal and variable Chl fluorescence levels in the dark-adapted state, respectively; F_0', F_m', F_v', minimal, maximal and variable Chl fluorescence levels in a light-adapted state, respectively; NPQ, nonphotochemical quenching of Chl fluorescence; PFD, photon flux density; PS I, PS II, Photosystem I, II; qE, ΔpH- (energy-) dependent NPQ; V, violaxanthin; WT, wild type; Z, zeaxanthin

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0005-2728/02/$ – see front matter
PII: S0005-2728(02)00184-6

1. Introduction

Carotenoids (carotenes and their oxygenated derivatives, the xanthophylls) are essential components of the photosynthetic apparatus in higher plants and their composition comprising lutein, β-carotene, violaxanthin (V) as well as V de-epoxidation products and neoxanthin, is highly conserved throughout the plant kingdom. Lutein is the most abundant carotenoid and is bound primarily to light-harvesting complex (LHC) II, the major light-harvesting chlorophyll (Chl) *a*/*b*-binding complex of Photosystem II (PS II). LHC II is composed of mixed trimers of the closely related *Lhcb1–3* gene products [1]. A 3.4 Å resolution structural model for trimeric LHC II reveals that in addition to 12 Chls, two xanthophylls (assumed to be luteins) are centrally located between two membrane-spanning α-helices in each monomeric subunit [2]. Biochemical analyses further indicate the presence of one neoxanthin and up to one V molecule per LHC II monomer [3,4]. The minor LHCs of PS II (comprising the *Lhcb4–6* gene products, also termed CP29, CP26 and CP24, respectively) as well as LHC I proteins also bind xanthophylls to a varying extent [1,4–6]. The various xanthophylls play pivotal, synergistic roles in the photosynthetic apparatus [7–9]. Xanthophylls are required for stable assembly of pigment–protein complexes in vitro and in vivo (reviewed in [8]), act as accessory light-harvesting pigments [7], and along with β-carotene are of paramount importance for photoprotection (for a recent review see [9]).

The primary photoprotective process in plants is the nonradiative dissipation of energy absorbed in excess of what can be utilized in photosynthesis (reviewed in [9]). The phenomenon can be readily visualized as nonphotochemical quenching of Chl fluorescence (NPQ). NPQ is a cooperative phenomenon which, despite intensive study, is not yet fully understood at the mechanistic level. Several processes contribute to NPQ. The major component (often termed qE, for energization-dependent quenching) is rapidly reversible and associated with acidification of the thylakoid lumen, or trans-thylakoidal ΔpH (for a review see [10]). Studies of plants with altered antenna composition and isolated LHCs in vitro indicate that qE arises in the PS II antenna system [11–14].

Transthylakoidal ΔpH induces a variety of alterations to LHC II pigments and proteins that collectively contribute to qE. A major consequence is protonation of specific LHC amino acid residues that is proposed to cause conformational changes in antenna organization facilitating qE in vivo. ΔpH-mediated quenching can be mimicked in vitro with isolated LHCs and has been used to establish the link between pH-induced LHC II aggregation and quenching [10]. In the same system, added xanthophylls, most notably zeaxanthin (Z), antheraxanthin (A) and V, have differing effects on pH-induced aggregation/quenching that are consistent with models proposed for qE. Zeaxanthin production by the xanthophyll cycle in response to high-light stress has been correlated with NPQ (for reviews see [15,16]). Violaxanthin de-epoxidase (VDE) is activated by low pH and converts V (di-epoxide), via A (mono-epoxide) into Z (epoxide-free) (see [16]). Z epoxidase catalyzes the reverse reactions (in the dark or under low light) and is inhibited by low pH [16]. Thus, a second consequence of the ΔpH generated in high light is the net formation of Z (+A) by the xanthophyll cycle. The combined protonation of LHCs and production/interaction of Z (+A) with LHCs are two major factors that cooperatively facilitate qE in vivo.

Genetic analyses have substantiated and further defined the in vivo roles of specific xanthophylls and other, novel components of NPQ in algae and plants [9]. Mutants defective in VDE activity (e.g., the *Arabidopsis (A.) thaliana npq1* mutant) are unable to form Z in response to high light. The *npq1* mutant retains a rapid, initial pH-dependent component of NPQ but is defective in a slower phase and has reduced maximal amplitude compared to the wild type (WT) [17,18]. Conversely, mutations disrupting Z epoxidase activity (e.g., the *A. thaliana aba1* mutant) accumulate constitutively high levels of Z, display accelerated NPQ induction and, surprisingly, have decreased NPQ amplitude. Together, *aba1* and *npq1* have confirmed and delineated the role for Z (+A) and the xanthophyll cycle in NPQ in vivo. A third mutation in *A. thaliana*, *npq4*, eliminates the qE component of NPQ despite having an antenna pigment and protein composition, xanthophyll cycle activity and photosynthetic rate indistinguishable from WT. The *NPQ4* locus encodes the

psbS (CP22) protein and defines this protein as essential for NPQ [19].

Another class of mutations shown to affect NPQ, the *lut* mutants, are defective in specific aspects of lutein synthesis [20]. Lutein levels are reduced by approximately 80% in *lut1* due to a disruption in ε-ring hydroxylation and as a result zeinoxanthin, the immediate biosynthetic precursor of lutein, accumulates. The *lut2* mutant is defective in ε-ring cyclization and devoid of lutein and all other α-carotene-derived xanthophylls. In addition to their lutein deficiency, both *lut* mutants also accumulate elevated levels of β-carotene-derived xanthophyll cycle pigments (V, A and Z). Similar to *npq1*, NPQ induction is retarded in both *lut* mutants and maximal amplitude is decreased, suggesting a previously unsuspected role for lutein in NPQ [21].

As a group, the *A. thaliana* xanthophyll biosynthetic mutants are particularly well suited for probing the function(s) of individual xanthophylls in vivo and for providing insight into the mechanistic contribution of specific xanthophylls to NPQ. The current study was performed to provide a more detailed understanding of the various functions of xanthophylls within the light-harvesting system, the ability and limits of various xanthophylls to functionally complement for each other and the mechanistic basis for altered NPQ in the mutants. The common theme emerging from this work is that altered NPQ in xanthophyll biosynthetic mutants is due primarily to significant changes in LHC II-stability, PS II antenna size and superstructure

2. Materials and methods

2.1. Plant materials and pigment analysis

Wild-type *A. thaliana* and the *lut1*, *lut2*, *aba1* and *lut2aba1* mutants [21] were grown in soil/vermiculite (3:1) at 100 μmol photons $m^{-2} s^{-1}$ under a 10:14-h light/dark cycle (21:19°C) in growth chambers. Leaves from 6–8-week-old plants (prior to bolting) were used for all experiments. Pigment isolation and analyses were performed as described [20] except that tissue was frozen in liquid nitrogen immediately following treatments and stored at −80°C until extraction.

2.2. Photosystem stoichiometry and antenna size measurements

Isolation of thylakoid membranes and nondenaturing polyacrylamide gel electrophoresis (PAGE) ('green gels') were performed as described [22]. PS II and PS I concentrations were assessed from the amplitude of light minus dark absorption changes of freshly prepared thylakoid membrane suspensions at 320 nm (Q_A) and 700 nm (P_{700}) [23]. Functional Chl *a*/*b*-antenna sizes of PS I and PS II were obtained from analyses of the kinetics of P_{700} photooxidation and Q_A-photoreduction, respectively [24].

2.3. Chlorophyll fluorescence measurements

In vivo Chl fluorescence was determined using a pulse amplitude modulation fluorometer (FMS 2, PP Systems, Haverhill, MA, USA) from attached leaves as previously described [12]. Fluorescence parameters are according to [25]. $F_v/F_m = (F_m - F_0)/F_m$ is the maximum photochemical efficiency of PS II, in the dark-adapted state. NPQ was quantified as $(F_m/F_m') - 1$. Photon flux densities (PFDs) were measured with a quantum sensor (Li-Cor, LI-189A; Lincoln, NE, USA).

3. Results

3.1. Photochemical efficiency and nonphotochemical chlorophyll fluorescence quenching

Maximum photochemical efficiency of PS II (measured as F_v/F_m) was similar in WT and the *lut1* and *lut2* mutants, slightly lower in *aba1* and significantly lowered in *lut2aba1* (Table 1). The lower F_v/F_m in *aba1* and *lut2aba1* most likely arise from a higher PS I contribution to F_0 and/or the presence of Z as a constitutive quencher of PS II fluorescence (refer also to later sections). NPQ induction kinetics for all genotypes at 500 μmol photons $m^{-2} s^{-1}$ actinic light are shown in Fig. 1A. As has been previously observed [21], when compared to WT (half-rise time, $t_{1/2} = 73$ s), NPQ induction is retarded in *lut1* ($t_{1/2} = 87$ s) and *lut2* ($t_{1/2} = 105$ s), and considerably accelerated in *aba1* ($t_{1/2} = 11$ s) and *lut2aba1* ($t_{1/2} = 14$ s). Maximal NPQ levels attained at 500

μmol photons $m^{-2} s^{-1}$ are reduced in the order: WT > *aba1* > *lut1* ≈ *lut2* > *lut2aba1*. With the exception of *lut2aba1*, NPQ induction in all genotypes can be described by a single exponential rise, indicating that only one process, qE, contributes to NPQ at this actinic light level. However, at 500 μmol photons $m^{-2} s^{-1}$ the *lut2aba1* curve shows, in addition to the fast initial rise, a second slower component. This second component is associated with a significant increase in F_0 (relative to the initial dark-adapted F_0 value) after relaxation of qE (data not shown) and hence is attributable to photoinhibition. This photoinhibitory component is not observed in the other genotypes following illumination with 500 μmol photons $m^{-2} s^{-1}$.

Fig. 1B displays the maximal NPQ levels reached after 6 min of illumination as a function of incident PFD for all genotypes. Wild-type, *lut1* and *lut2* curves start to saturate at ~500 μmol photons $m^{-2} s^{-1}$ while the *aba1* and *lut2aba1* curves continuously increase and are not saturated even at 1000 μmol photons $m^{-2} s^{-1}$. The maximal NPQ levels reached at 1000 μmol photons $m^{-2} s^{-1}$ decrease in the order: WT ≈ *aba1* > *lut2* > *lut1* > *lut2aba1*. This altered order (relative to 500 μmol photons $m^{-2} s^{-1}$) is due to a genotype-dependent, differential increase in the photoinhibitory component of NPQ (increase in F_0) at higher light levels as the capacity of qE build-up is exhausted (Fig. 1B and data not shown). At higher PFDs this additional slower inducible NPQ-component, indicative of susceptibility to photoinhibition, increases in the order: WT < *lut1* < *lut2* < *aba1* < *lut2aba1*.

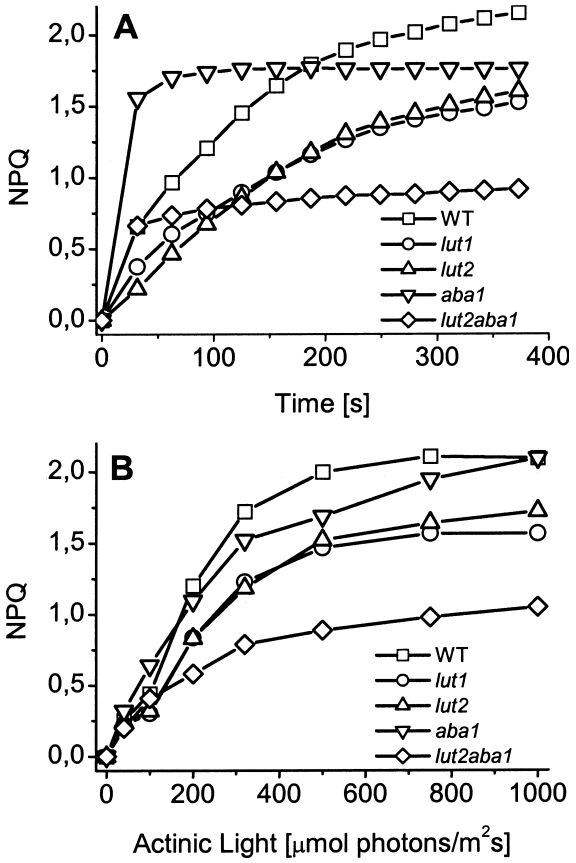

Fig. 1. (A) Nonphotochemical fluorescence quenching (NPQ) in leaves of *A. thaliana* WT and xanthophyll biosynthesis mutants, as a function of time of exposure to 500 μmol photons $m^{-2} s^{-1}$. (B) NPQ as a function of actinic light intensity. All plants were dark-adapted for 30 min prior to light exposure. Each data point is the mean of at least three separate experiments. S.D. are within the symbol size.

Table 1
Chl *a*/*b*-ratios, photosystem stoichiometries, antenna sizes and maximum PS II efficiencies (F_v/F_m) in *A. thaliana* WT and xanthophyll mutants

	WT	*lut1*	*lut2*	*aba1*	*lut2aba1*
Chl *a*/*b*-ratio	2.72	2.88*	2.86*	3.04	3.23
F_v/F_m	0.840	0.843	0.846	0.805	0.783
Chl *a*+*b*/P_{700}	774	616	548	663	627
Chl *a*+*b*/Q_A	527	409	351	466	319
PS II/I (Q_A/P_{700})	1.47	1.51	1.56	1.42	1.97
Chl *a*+*b*/PS II	377*	275	213	373*	203
Chl *a*+*b*/PS I	220**	202*	202*	289	229**

Unless otherwise indicated all values are significantly different between genotypes (*t*-test, significance level = 0.05). $n > 30$ for Chl *a*/*b*-ratio and F_v/F_m. $n = 3$–9 for all other measurements. *,**Not significantly different.

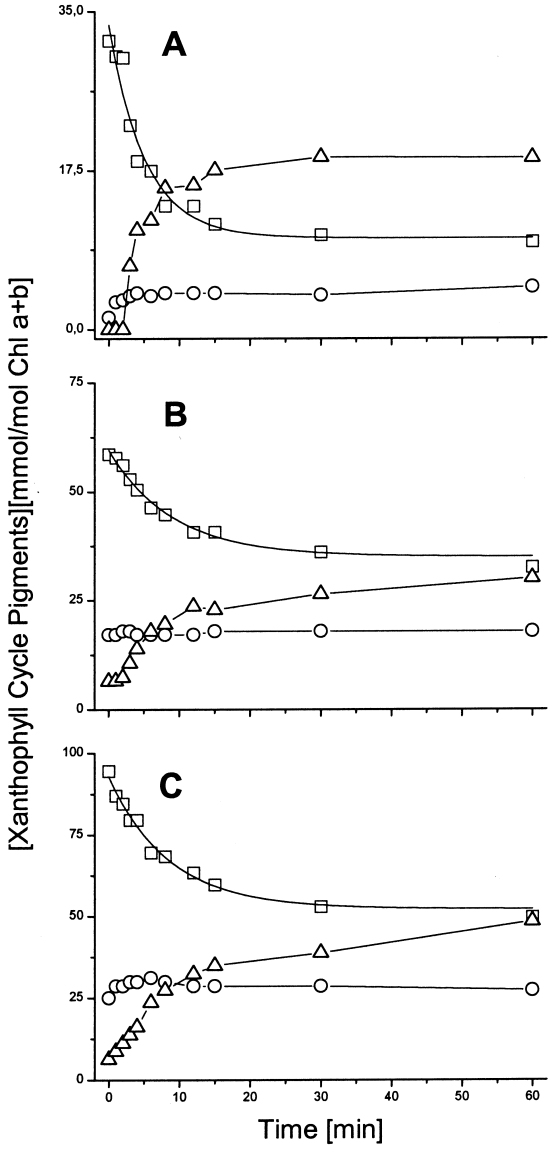

Fig. 2. Kinetics of V de-epoxidation via A to Z in leaves of *A. thaliana* WT (A) and the *lut1* (B) and *lut2* (C) mutants exposed to a PFD of 1000 μmol photons $m^{-2} s^{-1}$. All plants were dark-adapted for 24 h prior to light exposure. □, violaxanthin; △, zeaxanthin; ○, antheraxanthin. Each data point represents the mean of at least five separate experiments. S.D. are within the symbol size. Note the different abscissa scales.

3.2. Kinetics of violaxanthin de-epoxidation in WT, lut1 and lut2

A pivotal role for Z (and A) in NPQ is well-established [9,10,14–16]. Therefore, we first investigated whether the compromised NPQ development in *lut* mutants might be due to altered xanthophyll cycle activity and/or V+A+Z pool sizes. Time-dependent changes in the relative amounts of V, A and Z in leaves of plants dark-adapted for 24 h and then exposed to high light (1000 μmol photons $m^{-2} s^{-1}$) are shown in Fig. 2. All data are expressed as mmol of the respective xanthophyll mol^{-1} Chl *a*+*b* instead of the more conventional % of the V+A+Z pool, as the V+A+Z pools in *lut1* and *lut2* were 2.5- and 3.8-fold larger, respectively, than in WT (Table 2 and Fig. 2).

In dark-adapted WT plants (Fig. 2A), Z was absent and only traces of A were detectable. The 60-min light treatment resulted in de-epoxidation of 21.5 mmol V to 18.2 and 3.3 mmol Z and A, respectively, with 11.6 mmol V (35%) being recalcitrant to de-epoxidation. Both *lut* mutants (Fig. 2B,C) have larger V+A+Z pools than WT and contained high levels of A and Z in the dark-adapted state (17.1 and 24.9 mmol A and 6.5 and 6.2 mmol Z for *lut1* and *lut2*, respectively). The 60-min light treatment had little effect on A levels but caused de-epoxidation of 26 and 44.8 mmol V (mainly to Z) with 36.6 and 40.7 mmol V being recalcitrant to de-epoxidation in *lut1* and *lut2*, respectively. While these de-epoxidation levels as attained after 60 min illumination are much higher than in WT, when one considers the time dependent product formation (Z+A) and that NPQ is near maximal after 6 min of light treatment in all genotypes (Fig. 1), the xanthophyll cycle activity that may be relevant to NPQ is remarkably similar in all genotypes. During the 6-min treatment leading to maximal NPQ 14.4, 11.1 and 22.1 mmol Z+A are formed with 50% product formation times of 2.5, 3.1 and 2.7 min, for WT, *lut1* and *lut2*, respectively. In the light of these minor differences in rates and product formation, it seems unlikely that altered xanthophyll cycle activity is the primary cause of compromised NPQ in *lut* mutants.

The data in Fig. 2 suggest that the large differences in de-epoxidized and recalcitrant V between *lut* mutants and WT originate from the existence of at least two functionally distinct sub-pools of V (and A+Z for the mutants) with differential accessibility by VDE and Z epoxidase. One sub-pool is bound to the more peripherally located xanthophyll (V, A, Z) binding sites of LHC-proteins and hence, is accessible by the xanthophyll cycle enzymes. A second 'protected' sub-pool (11.6 mmol V in WT and 60.2 and 71.8 mmol V+A+Z in *lut1* and *lut2,* respectively) is

Table 2
Carotenoid composition of *A. thaliana* WT and xanthophyll mutant leaves

	WT	*lut1*	*lut2*	*aba1*	*lut2aba1*
Lutein[a]	139	26	–	117	–
Neoxanthin	38	35[b]	35[b]	–	–
β–Carotene	81	91	114[b]	98	112[b]
Zeinoxanthin	–	50	–	–	–
V+A+Z[c]	33	81	124[b]	122[b]	191
Σ Carotenoids	291[b]	284[b]	273	337	303

Unless otherwise indicated all values are significantly different between genotypes (*t*-test, significance level = 0.05). $n > 30$ for all measurements.
[a]All carotenoid contents are expressed as mmol carotenoid/mol Chl *a*+*b*.
[b]Not significantly different.
[d]Pool size of the xanthophyll-cycle pigments V, A and Z.

bound to sites within LHC II and inaccessible by the xanthophyll cycle enzymes. In the case of the *lut* mutants, a significant proportion of this recalcitrant V+A+Z sub-pool is apparently substituting for lutein in the internally located lutein-binding sites of LHC II [2] and unavailable to the xanthophyll cycle during light stress. Pigment compositional analysis of isolated LHCs from WT and the mutants support this conclusion (refer to later sections and Table 3). Finally and most significantly, the large amount of protected, internally located Z+A (23.6 and 31.1 mmol *lut1* and *lut2*, respectively) appears to not be NPQ-active. To further test this hypothesis we performed NPQ light-activation experiments in WT and the *lut* mutants.

3.3. Light-activation of nonphotochemical quenching in WT, lut1 and lut2

Light-activation of NPQ and its correlation with xanthophyll cycle activity is a well-established phenomenon [11]. Leaves of dark-adapted WT and *lut* mutants were subjected to 500 μmol photons $m^{-2}\,s^{-1}$ for 6 min (inducing V de-epoxidation and NPQ build-up), subsequently dark-adapted for 2 min (sufficient to relax the qE component of NPQ but not to re-epoxidate the newly formed Z+A) and then subjected to a second high-light treatment (Fig. 3).

NPQ induction in *lut1* and *lut2* during the initial illumination period is delayed compared to WT, consistent with the data in Fig. 1A. However, during the second illumination period, NPQ induction in both *lut* mutants is similar to WT and is complete in all three genotypes in less than 30 s. This observation reveals the functional significance of the different pools of xanthophyll cycle pigments (V+A+Z) in the *lut* mutants. Only Z (and possibly A) formed by xanthophyll cycle activity during the first illumination period is NPQ-active. This xanthophyll cycle (and NPQ-) active V+A+Z is bound most likely to peripheral pigment binding sites of the LHCs. The large amounts of constitutively present Z (and A) in

Table 3
Pigment composition of *A. thaliana* WT and xanthophyll mutant light-harvesting complexes as resolved by nondenaturing electrophoresis as displayed in Fig. 4

	Chl *b*[a]	Lutein	Neoxanthin	VAZ	Σ Carotenoids
WT-LHC II_3	5.4	1.8	1.2	0.2	3.2
WT-LHC_{mono}	5.4	1.7	1.1	0.2	3.0
lut1-LHC_{mono}	5.3	0.3	1.0	1.5	2.8
lut2-LHC_{mono}	5.5	n.d.[b]	1.3	2.0	3.3
aba1-LHC_{mono}	5.8	1.5	n.d.	1.5[c]	3.0
lut2aba1-LHC_{mono}	5.9	n.d.	n.d.	3.5[c]	3.5

[a]All pigments are expressed as per 7 molecules Chl *a*; $n = 3$, S.D. are about 10%.
[b]Not detectable.
[c]Only zeaxanthin.

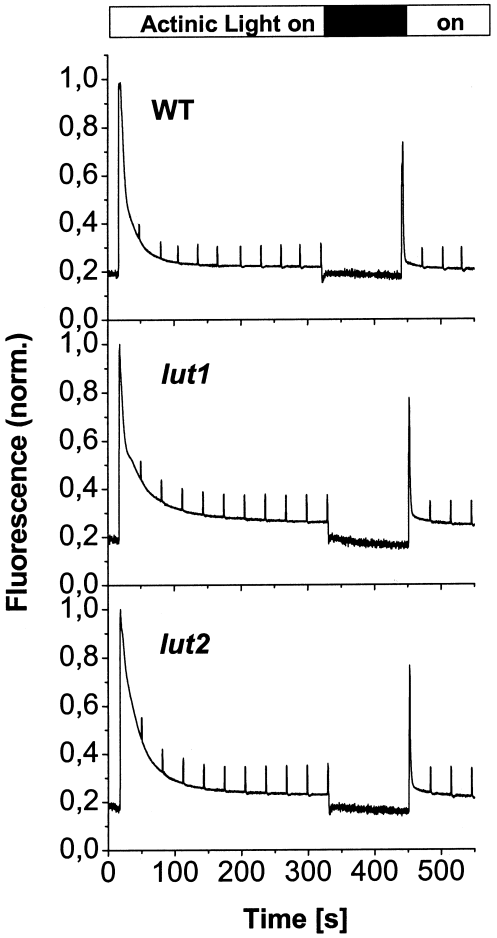

Fig. 3. Light activation of NPQ in leaves of *A. thaliana* WT, the *lut1* and *lut2* mutants. Dark-adapted leaves were illuminated with a PFD of 500 μmol photons $m^{-2} s^{-1}$ for 6 min followed by 2 min darkness and a second illumination.

dark-adapted *lut* mutants (bound to the lutein-binding sites of LHC II) do not contribute to fast NPQ induction. Moreover, the same can be logically inferred for lutein normally bound to these sites in WT. Thus, the hypothesis that lutein is directly involved in the mechanism of NPQ [17,21] is not supported. An alternative explanation for compromised NPQ in the *lut* mutants (and in xanthophyll-deficient *A. thaliana* mutants in general) would be more indirect and result from alterations in LHC assembly/stability.

3.4. Assembly, composition and stability of light-harvesting complexes in xanthophyll mutants

In vitro reconstitution studies have shown that xanthophylls are indispensable for stable assembly of monomeric LHCs [8]. In order to investigate assembly/stability of pigment–protein complexes in the various xanthophyll mutants, thylakoid membranes were solubilized in mild (nonionic) detergents and subjected to nondenaturing PAGE ('green gels', Fig. 4). Most striking is the considerably reduced stability of trimeric LHC II in all xanthophyll mutants. Whereas the *lut1* mutant retains a minor fraction of LHC II-trimers (10–20% of the WT level, corresponding approximately to its residual lutein content), only a very faint trimeric LHC II band is observed for *aba1*. The LHC II trimer band is absent in *lut2* and *lut2aba1*, both of which are completely deficient in lutein. Disappearance of trimeric LHC II is accompanied by a concomitant increase of a band attributable to LHC II monomers (including also monomeric LHC I and minor LHCs II, i.e., CP24, CP26 and CP29) in all mutant genotypes. The free pigment band (almost exclusively carotenoids, V+A+Z as well as zeinoxanthin in *lut1*) is least pronounced in WT, markedly stronger in all xanthophyll mutants and most prominent in the *lut2aba1* double mutant. Attempts to improve the yield of mutant trimeric complexes in green gels through the use of alternate gel systems [26] and/or by altering detergent concentrations and/or compositions (e.g., using sodium dodecyl sulfate, *n*-decyl-β-D-maltoside, *n*-undecyl-β-D-maltoside, *n*-dodecyl-β-D-maltoside or *n*-octyl-β-D-glucopyranoside and various combinations

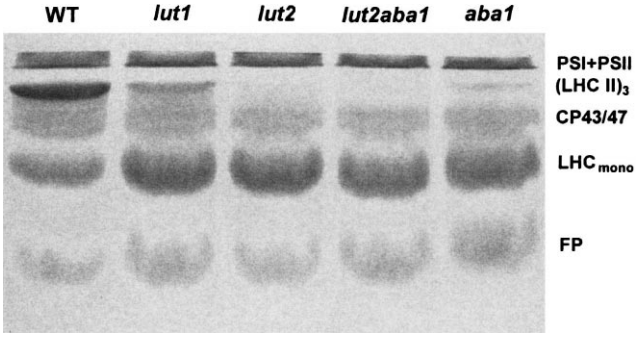

Fig. 4. Nondenaturing ('green') gel electrophoretic separation of pigment–protein complexes from *A. thaliana* thylakoid membranes of the indicated genotypes. Note that the LHC_{mono} band also contains LHC I and minor LHC II complexes; FP designates free pigment. The pigment composition of the LHC bands is given in Table 3.

thereof) were unsuccessful (not shown). Moreover, the isolation of pure (trimeric) LHC II with standard procedures [27,28] also varying solubilization conditions (e.g., 0.2 to 0.7% Triton X-100 or 0.05 to 1.0% *n*-dodecyl-β-D-maltoside) proved to be impossible, too.

The bands corresponding to trimeric LHC II (WT) and monomeric LHCs (all genotypes; note that the latter contains also the minor LHCs II and LHC I) were excised and their pigment content analyzed by high-performance liquid chromatography. The pigment composition of the bands is given in Table 3. WT trimeric LHC II, WT monomeric LHC and *aba1* monomeric LHC pigment compositions were similar to those previously reported [29]. In the *lut1* and *lut2* monomeric LHCs lutein is replaced on a nearly equimolar basis by xanthophyll cycle pigments (V+A+Z). In *lut2aba1* lutein, V and neoxanthin are replaced by Z.

3.5. Photosystem stoichiometry and antenna size in xanthophyll mutants

It has been shown previously that the extent of NPQ can be correlated with PS II antenna size [14]. The xanthophyll mutants showed an increasing Chl *a*/*b* ratio in the order: WT < *lut1* ~ *lut2* < *aba1* < *lut2aba1* (Table 1). Since Chl *b* is associated exclusively with LHCs (mainly LHC II), the Chl *a*/*b* ratios are consistent with a reduction in LHC II levels. However, alterations in photosystem stoichiometry alone or in combination with altered antenna sizes could bring about similar changes in Chl *a*/*b* ratios. To further address these possibilities, photosystem stoichiometry and antenna size were analyzed in all genotypes (Table 1).

Light-induced absorption difference spectra of thylakoid membrane preparations were used to determine the concentrations of Q_A and P_{700} as a measure of PS II and PS I reaction centers, respectively [23]. The corresponding PS antenna sizes were derived from the kinetics of Q_A-photoreduction and P_{700}-photooxidation [24].

Lack of lutein leads to an increase in the ratio of PS II to PS I in the order: *lut2aba1* > *lut2* > *lut1* > WT. Conversely, the lack of epoxidized xanthophylls (neoxanthin and V) in *aba1* leads to a decrease in the ratio of PS II to PS I relative to the WT. With regard to PS I antenna size (Chl *a*+*b*/PS I), no clear tendency is observed in relation to a specific xanthophyll deficiency. However, the lack of lutein is strongly correlated with a reduction in PS II antenna size (Chl *a*+*b*/PS II) in the following order: WT ~ *aba1* > *lut1* > *lut2* > *lut2aba1*. Remarkably, this reduction in PS II antenna size parallels the decrease in maximum attainable NPQ (as measured at 500 μmol photons $m^{-2} s^{-1}$ actinic illumination (cf. Fig. 1A) to avoid interference by photoinhibition).

4. Discussion

Xanthophylls are indispensable, integral components of plant photosynthetic pigment–protein complexes. In addition to their accessory light-harvesting function, they are required for stable assembly of both antenna and reaction center complexes as well as for effective photoprotection (see [8]). The ability of plants to dissipate excess excitation energy (measurable as NPQ) is of paramount importance for photoprotection and has been correlated with light-induced trans-thylakoidal ΔpH and Z formation [9,11,14,15]. It has also been proposed that the xanthophyll cycle intermediate, A, may be involved in NPQ [30].

Mutations in *A. thaliana* have been useful in advancing our understanding of NPQ in plants and fall into two general classes: those that affect NPQ without altering the pigment compositions of unstressed leaves (e.g., *npq1* and *npq4*) and those that affect NPQ but also alter pigment compositions in unstressed leaves (e.g., *lut1*, *lut2*, *aba1* and their double mutants). In *aba1*, NPQ induction is more rapid but attains lower amplitude than in WT, while in *lut1* and *lut2* both the induction and amplitude of NPQ are negatively impacted (Fig. 1A and [21]). These effects are additive in *lut1aba1* and *lut2aba1* double mutants indicating the *aba1* and *lut* mutations affect independent processes related to NPQ (Fig. 1A and [21]). These general observations can be extended to green algae: analogous mutations in *Chlamydomonas reinhardtii* show similarly compromised NPQ [17]. Such studies have led to the proposal that in addition to light-induced trans-thylakoidal ΔpH and Z formation, lutein is also required for efficient NPQ, either

directly or indirectly, by an unknown mechanism [17,21]. In the current study we have compared and contrasted aspects of photosystem structure and function across four *A. thaliana* xanthophyll mutant lines (*lut1*, *lut2*, *aba1* and *lut2aba1*) and WT in an attempt to identify a common mechanism(s) whereby xanthophyll modifications differing from WT, in particular lutein deficiency, influence NPQ.

Given the importance and effects of the xanthophyll cycle (in particular Z formation) in NPQ build-up, it would seem logical that any alterations in xanthophyll cycle pigment pool size (V+A+Z), especially the levels of Z and A prior to light stress, would have an impact on NPQ induction. This is indeed the case; however, instead of positively affecting NPQ as one might expect, the high constitutive levels of Z (and A) and ~3-fold increase in V+A+Z pools in *lut* mutants coincide with compromised NPQ induction kinetics and amplitudes. Perhaps the compromised NPQ in the mutants is due to an alteration in xanthophyll cycle activity/kinetics, rather than absolute xanthophyll cycle pigment levels. This appears not to be the case either, as xanthophyll cycle activity (V to Z conversion rates and amounts) during the timeframe of NPQ induction was remarkably similar in *lut* mutants and WT.

The experiments in Fig. 2 show that approximately 65% of the enlarged V+A+Z pool in *lut* mutants is 'protected' from xanthophyll cycle activity. This is most likely due to these pigments occupying binding sites in LHCs that would normally (in WT) bind lutein. Given the role of Z (and possibly A) as NPQ-active xanthophylls, one would again anticipate that any Z (or A) would increase NPQ or at least predispose the system to NPQ. However, NPQ light activation experiments clearly show that the large amounts of Z+A (occupying lutein sites) in the *lut* mutants do not contribute to NPQ indicating that, as in WT, only Z (and A) produced by the xanthophyll cycle is active in NPQ in *lut* mutants. These observations are significant for several reasons. First, they provide direct evidence that the location of a particular xanthophyll (e.g., Z and A) within the pigment–protein complexes is as important – if not more important – than the identity of the pigment for determining its role in NPQ. Second, since Z (and A) incorporated into lutein-binding sites do not directly participate in NPQ, it follows that the lutein that normally occupies these sites in WT also does not directly participate in NPQ. Still, the question remains: If lutein is not directly involved in NPQ and substituted Z (and A) is not 'NPQ-active', by what mechanism does the absence of lutein in *lut* mutants affect NPQ?

Although xanthophyll biosynthetic mutations affect many aspects of the plant photosynthetic apparatus, when taken together, our studies of LHC stability, antenna size and photosystem stoichiometry indicate that the effect of xanthophyll compositional changes on NPQ results primarily from alterations to the PS II antenna. Nondenaturing PAGE analysis of pigment–protein complexes indicates LHC II trimer stability is substantially reduced across the spectrum of *A. thaliana* xanthophyll mutants studied, though most severely in those that lack lutein (Fig. 4). This is consistent with previous reports of LHC II instability in a α-carotenoid-free mutant of the green alga *Scenedesmus obliquus* [31,32]. It is most likely that the destabilization of LHC II trimers in *A. thaliana* xanthophyll mutants is brought about by subtle structural changes in monomeric LHC II units due to the binding of V+A+Z instead of lutein.

A clear inverse correlation exists between the maximal attainable NPQ and the reduction of functional PS II antenna size across the full spectrum of mutants studied (Fig. 1 and Table 1). We propose that this is the primary, underlying lesion that affects NPQ in all xanthophyll biosynthetic mutants. The lack of a WT xanthophyll composition leads to instability of LHC II trimers. This reduction of LHC II trimer stability is likely translated into a disruption of optimal, higher-order, macro-organization of the PS II-antenna system (and probably less connectivity between PS II centers) and hence, is responsible for the compromised NPQ. This is consistent with previous findings that NPQ decreased in parallel with the reduction of PS II antenna size in a Chl *b*-less barley mutant [12–14]. Interestingly, Chl *b*-less barley mutants grown under an intermittent light regime [14] show a NPQ phenotype that closely resembles the *A. thaliana npq4* mutant [19]. Intermittent light grown Chl *b*-less barley mutants are devoid of all major and minor LHCs but retain WT levels of the psbS protein [33], while the *npq4* mutant is unaffected in PS II-antenna composition but completely lacks psbS [19]. This indicates that both psbS and an

optimally organized and functional PS II antenna system are required for maximum NPQ build-up.

The xanthophyll composition of plant photosynthetic pigment–protein complexes is remarkably conserved across evolution. This is presumably due to strong selection pressure for retention of these particular xanthophylls in order to enable specific function(s) and/or the resultant fitness of land-based photosynthetic organisms. In particular, lutein is the most abundant thylakoid carotenoid and its presence is diagnostic for land-based photosynthetic organisms. Moreover, lutein is present in the branch of algae that presumably gave rise to land plants. Thus, the initial identification of lutein-deficient *A. thaliana* mutants, which lacked an obvious phenotype, and the subsequent generation of xanthophyll double mutants that lacked all WT xanthophylls with relatively minor observable whole plant phenotypes were both surprising and puzzling [20]. If, as the single and double *A. thaliana* xanthophyll mutations imply, the plant photosynthetic apparatus is so flexible with regard to xanthophyll composition, why do all plants produce lutein, have such a highly conserved xanthophyll composition and finally, why doesn't one find plants in nature with xanthophyll profiles similar to the *A. thaliana* mutants?

The observation that all single and double *A. thaliana* xanthophyll biosynthetic mutants are defective in aspects of NPQ suggested that plants with 'non-WT' xanthophyll compositions are impaired in their ability to cope with light in excess of what is needed for photosynthesis [21]. The current study clearly shows that although other xanthophylls (in particular V, A and Z) are able, to a certain degree, to assume the structural role of lutein in the mutants, the resulting 'non-WT' xanthophyll compositions severely compromise antenna size, stability and function. These 'replacement' xanthophylls do not provide the same unique combination of antenna stability, flexibility and function that lutein does to fulfill the requirement of land plants for fast and effective regulatory and protective responses under rapidly fluctuating environmental conditions. In this light, the strong selection for maintenance of a specific xanthophyll composition in plant thylakoids during evolution is easily understood. This may also explain why only descendants of green algae with their seemingly redundant manifold of Chl *a*/*b*-binding and xanthophyll containing antenna complexes that confer unique adaptive flexibility (the xanthophyll cycle and NPQ) have ever successfully conquered land.

Acknowledgements

We would like to thank Professor Anastasios Melis (UC Berkeley) for providing access to his photosystem stoichiometry/antenna size measuring facilities. The authors also acknowledge Erica Rust and Aimee Snell for assistance with the HPLC analysis.

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