PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Phil. Trans. R. Soc. Lond. B 2000 **355**, 1395-1403
doi: 10.1098/rstb.2000.0701

THE ROYAL SOCIETY

doi 10.1098/rstb.2000.0701

Genetic manipulation of carotenoid biosynthesis and photoprotection

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There are multiple complementary and redundant mechanisms to provide protection against photo-oxidative damage, including non-photochemical quenching (NPQ). NPQ dissipates excess excitation energy as heat by using xanthophylls in combination with changes to the light-harvesting complex (LHC) antenna. The xanthophylls are oxygenated carotenoids that in addition to contributing to NPQ can quench singlet or triplet chlorophyll and are necessary for the assembly and stability of the antenna. We have genetically manipulated the expression of the ε-cyclase and β-carotene hydroxylase carotenoid biosynthetic enzymes in *Arabidopsis thaliana*. The ε-cyclase overexpression confirmed that *lut2* (lutein deficient) is a mutation in the ε-cyclase gene and demonstrated that lutein content can be altered at the level of mRNA abundance with levels ranging from 0 to 180% of wild-type. Also, it is clear that lutein affects the induction and extent of NPQ. The deleterious effects of lutein deficiency on NPQ in *Arabidopsis* and *Chlamydomonas* are additive, no matter what the genetic background, whether *npq1* (zeaxanthin deficient), *aba1* or antisense β-hydroxylase (xanthophyll cycle pool decreased). Additionally, increasing lutein content causes a marginal, but significant, increase in the rate of induction of NPQ despite a reduction in the xanthophyll cycle pool size.

Keywords: carotenoid; lutein; xanthophyll cycle; photoprotection; non-photochemical quenching (NPQ)

1. INTRODUCTION

(a) *Carotenoid biosynthesis and function*

Carotenes and their oxygenated derivatives, the xanthophylls, are integral components of the photosynthetic apparatus and consequently are essential to plant viability, contributing to numerous processes—including assembly and stability of pigment–protein complexes, light harvesting and photoprotection. Four carotenoids, lutein, β-carotene, violaxanthin and neoxanthin, are nearly ubiquitous among higher plants (figure 1) (Young 1993*a*,*b*) with one of the few exceptions being the parasitic angiosperm *Cuscuta reflexa* where neoxanthin is replaced by lutein-5,6-epoxide (Bungard *et al*. 1999).

The critical roles of carotenoids are reflected in the tight regulation of carotenoid biosynthesis during plant development and adaptation to environmental stress. Their biosynthesis in plants has been reviewed elsewhere (Sandmann 1994; Hirschberg *et al*. 1997; Cunningham & Gantt 1998). In brief, lycopene is either cyclized twice by the β-cyclase to produce β-carotene or once each by the β-cyclase and ε-cyclase (*lut2*) to produce α-carotene (figure 1). The α-carotene rings are hydroxylated by the ε-hydroxylase (*lut1*) and β-hydroxylase to make lutein. β-carotene is hydroxylated twice by the β-hydroxylase to make zeaxanthin. The hydroxyl groups are added to the ends of each ring resulting in the xanthophylls being more polar than β-carotene. Zeaxanthin is epoxidated twice to make violaxanthin, which is subsequently modified to make neoxanthin. Mutations have been identified in a number of enzymes in the pathway and in a range of species; those that disrupt xanthophyll biosynthesis are listed in table 1.

(i) *Cyclization reactions*

The cyclization reactions occur at the branch point in the pathway resulting in β,β-carotenoids such as violaxanthin or β,ε-carotenoids such as lutein. Insight into the enzymes has come from isotope labelling, the use of herbicides that inhibit cyclization and genetic studies (Chunaev *et al*. 1991; Bramley 1993; Bishop 1996; Pogson *et al*. 1996). Lesions in the β-cyclase are lethal, resulting in the accumulation of the monocyclic ε,Ψ-carotene in *Arabidopsis* (B. J. Pogson & D. DellaPenna, unpublished data), indicating a requirement for bicyclic carotenoids for plant viability. The accumulation of ε,Ψ-carotene indicates that the *Arabidopsis* ε-cyclase is incapable of cyclizing both ends of the linear lycopene in contrast to all β-cyclases and the lettuce ε-cyclase (Phillip & Young 1995), which is in agreement with the activity of the cyclases expressed in *Escherichia coli* (Cunningham *et al*. 1996). Mutations have been identified that apparently disrupt the activity of the ε-cyclase enzyme in *Arabidopsis* (*lut2*), *Scenedesmus* (C-2A-34) and *Chlamydomonas* (*lor1*) (see table 1).

The ε-cyclase is believed to be a key rate-limiting step, based on *in vivo* and *in vitro* studies (Cunningham *et al*. 1996; Pogson *et al*. 1996). *In vivo*, the *lut2* mutation results in lutein being replaced by an equimolar increase in β,β xanthophylls, specifically violaxanthin and antheraxanthin.

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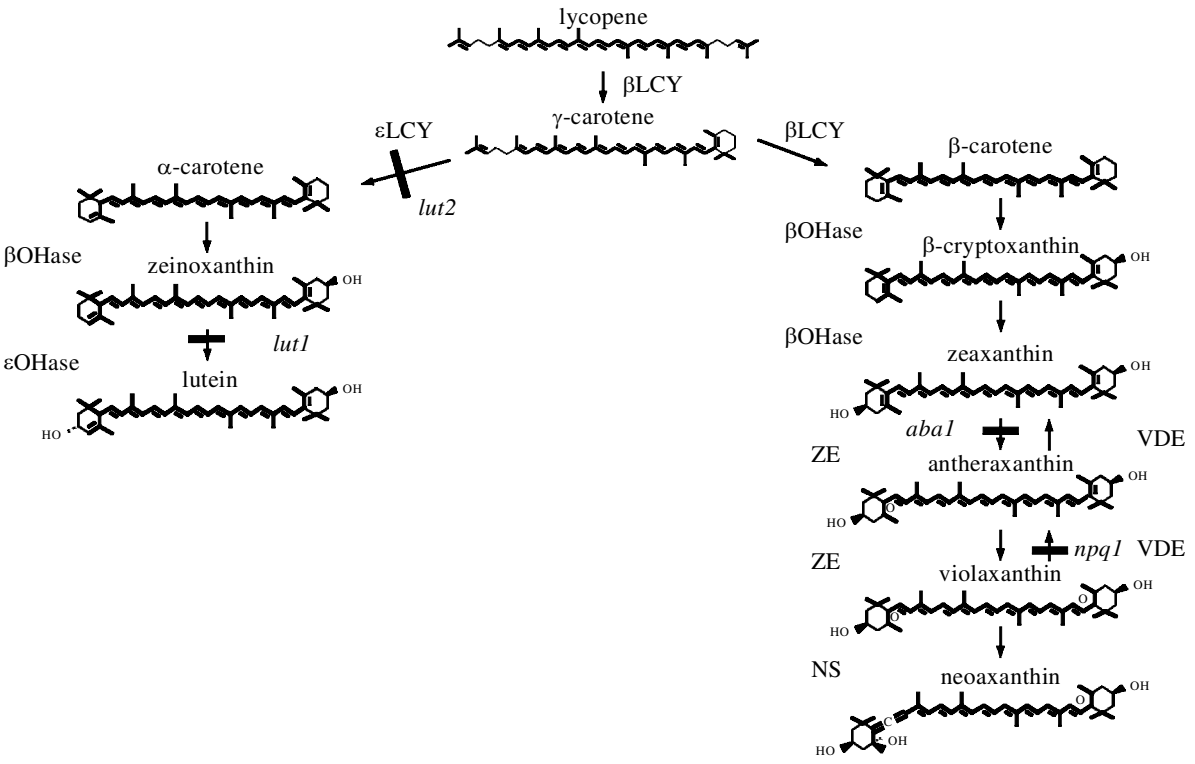

Figure 1. Carotenoid biosynthetic pathway in higher plants. Biosynthetic mutations, *lut1*, *lut2*, *aba1* and *npq1* are shown. βLCY, β-cyclase; εLCY, ε-cyclase; βOHase, β-hydroxylase; εOHase, ε-hydroxylase; ZE, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NS, neoxanthin synthase.

Heterozygotes are not completely recessive, that is lutein is slightly reduced, which is consistent with limiting levels of the ε-cyclase (Pogson *et al*. 1996). Sustained changes in light intensity can alter flux down the two branches, which reflects both changes in photosystem composition (Anderson & Osmond 1987; Senger *et al*. 1993; Young 1993*a*; Sonoike 1998) and possibly regulation of ε-cyclase enzyme mRNA (Hirschberg *et al*. 1997). If the latter is the case, then genetic manipulation of ε-cyclase mRNA levels should alter the proportion of flux down the two branches.

(ii) *Hydroxylation reactions*

It is presumed that there are two hydroxylase enzymes, an ε-hydroxylase and a β-hydroxylase based on the *lut1* mutation and isotope labelling studies, although only the β-hydroxylase has been cloned (Milborrow *et al*. 1982; Pogson *et al*. 1996; Sun *et al*. 1996). There have been no reports of herbicides specific for the hydroxylation reactions and only one mutation has been reported that clearly disrupts one of the hydroxylation reactions—the *lut1* mutation of *Arabidopsis*, which blocks ε-ring hydroxylation (Pogson *et al*. 1996).

One possible explanation for the lack of β-hydroxylase mutations is that there are multiple β-hydroxylase genes, which appears to be the case in *Arabidopsis* and *Capsicum annum* (Bouvier *et al*. 1996; Sun *et al*. 1996; Tian *et al*. 1999). The alternative explanation is that the ε-hydroxylase retains some β-hydroxylase activity providing functional redundancy. A number of the carotenoid enzymes theoretically arose from gene duplications and divergence and some still retain the enzymatic activity of the ancestral protein (Bouvier *et al*. 1994). We expressed an antisense β-hydroxylase transgene in *A. thaliana* with the result of effectively reducing the levels of β-carotene-derived xanthophylls, neoxanthin and violaxanthin (Rissler & Pogson 2000). However, this strategy did not eliminate the β-hydroxylation reactions, which lends support to the thesis of functional redundancy by the ε-hydroxylase. This is being further investigated by using the *lut1* and *lut2* mutations in combination with the antisense-β-hydroxylase.

(b) *Photosystem assembly and photoprotection*

Photosystem II consists of a core complex including CP43, CP47, D1/D2 encircled by the antenna, comprising an inner ring of minor light-harvesting complexes (LHCs) surrounded by trimers of the major LHC IIb (Lhcb1/2) (Dreyfuss & Thornber 1994; Jansson 1994). In brief, β-carotene is bound to D1/D2 and the xanthophylls to the antenna proteins. The lethal photobleaching in the absence of cyclic carotenoids in plants was thought to reflect the critical role of β-carotene in the reaction centre. This is based on the fundamental photoprotective role of β-carotene in triplet quenching in bacteria, which requires the carotenoid to be in Van der Waals contact (*ca*. 0.4 Å) to enable the electron exchange mechanism of triplet quenching (Krinsky 1971). However, in plants the oxidative potential of the chlorophyll special pair, P_{680}, is such that it would oxidize any carotenoids in close proximity. In fact, instead of triplet quenching, a cationic β-carotene may donate an electron to the oxidized special pair, P^{+}_{680}, and thereby provide a limited form of photoprotection perhaps via electron transport involving chl_z and Cyt b_{559} (Hanley *et al*. 1999; Vrettos *et al*. 1999).

Table 1. *Xanthophyll and NPQ mutations*

(References are Koornneef *et al*. (1982); Bishop (1996), Marin *et al*. (1996), Pogson *et al*. (1996), Niyogi *et al*. (1997, 1998), Li *et al*. (2000).)

mutation	species	protein
aba1 (*npq2*)	*Arabidopsis thaliana*	zeaxanthin epoxidase
aba2	*Nicotiana tabacum*	zeaxanthin epoxidase
npq1	*A. thaliana*	violaxanthin de-epoxidase
npq1	*Chlamydomonas reinhardtii*	violaxanthin de-epoxidase
lut1	*A. thaliana*	ε-hydroxylase[a]
lut2	*A. thaliana*	ε-cyclase
lor1	*C. reinhardtii*	ε-cyclase[a]
C-2A′-34	*Scenedesmus obliquus*	ε-cyclase[a]
npq4	*A. thaliana*	PsbS (CP22)

[a]Indicates mutations whose genetic identifications are not confirmed. Alternative names are given in parentheses.

If β-carotene is not involved in quenching of singlet or triplet excited chlorophyll, there is the prospect of a more fundamental role for the xanthophylls in these processes. However, there are no xanthophylls proximal to P_{680}. Instead, they are located in the LHCs and the inner antenna proteins, CP43 and CP47, where they could provide protection by quenching singlet and triplet excited states of chlorophyll. As yet, no one has had access to an oxygenic photosynthetic organism lacking all xanthophylls so it has not been possible to define the respective functions of xanthophylls and β-carotene in plants.

In vitro reconstitution studies have shown that xanthophylls are required for folding and stability of the LHCs including Lhcb1/2, Lhcb4 (CP29) and Lhcb5 (CP26) (Plumley & Schmidt 1987; Giuffra *et al*. 1996; Ros *et al*. 1998). The binding site for lutein that is essential for folding and a second site that can bind either violaxanthin or neoxanthin have recently been identified (Bassi *et al*. 1999). In the green alga *Scenedesmus*, the absence of lutein results in reduced LHCs and, as a consequence, an increase in the chlorophyll *a*/*b* ratio (Heinze *et al*. 1997). A similar, but less pronounced, trend is observed in the *Chlamydomonas* orthologue, *lor1*, supporting the view that lutein has been evolutionarily conserved in all green plants due to a fundamental role in LHC assembly (Chunaev *et al*. 1991). However, in higher plants, elimination of lutein has little, if any, effect on growth and development (Pogson *et al*. 1996, 1998). Thus, despite evidence that lutein is more effective than other xanthophylls in reconstitution studies, its implied identification in the crystal structure of the LHC and its evolutionary conservation (Plumley & Schmidt 1987; Kuhlbrandt *et al*. 1994), it is not essential *in vivo* (Pogson *et al*. 1996). This does not show that lutein is dispensable but rather that it is replaceable by xanthophylls with similar chemical composition and structure. The replacement of the wild-type xanthophylls (lutein, violaxanthin and neoxanthin) by zeaxanthin in the double mutant, *lut2 aba1*, caused delayed greening with high (about one-third) seedling mortality and reduced NPQ (Pogson *et al*. 1998). Yet, the plants are still viable and the photosynthetic rate and chlorophyll *a*/*b* ratio approach wild-type levels in mature leaves. Apparently, there is a substantial degree of functional complementarity between structurally similar xanthophylls (Pogson *et al*. 1998). The question remains as to what are the limits of this capacity for carotenoids to substitute for each other?

(c) *Non-photochemical quenching*

In addition to their roles in assembly, some xanthophylls are involved in photoprotection by quenching chlorophyll fluorescence via non-radiative dissipation in a process referred to as non-photochemical quenching (NPQ) (Demmig-Adams & Adams 1992; Frank *et al*. 1994; Gruszecki *et al*. 1999). In fact, the distinct roles of individual xanthophylls are evidenced in the high light-induced accumulation of 'quenching' carotenoids, zeaxanthin and antheraxanthin, by the de-epoxidation of violaxanthin via the xanthophyll cycle (Yamamoto 1962; Niyogi 1999). Exposure to high photon flux densities results in an acidification of the thylakoid lumen, which induces the xanthophyll cycle and the protonation of two glutamate residues of Lhcb1/2 (Walters *et al*. 1996; Gilmore *et al*. 1998; Kramer *et al*. 1999). Collectively, NPQ leads to non-radiative quenching of singlet chlorophyll fluorescence, thereby protecting against the generation of chlorophyll triplets and thus oxidative damage from singlet oxygen and other reactive oxygen species (Niyogi 1999).

The function of the xanthophyll cycle is consistent with the predicted excited singlet state energy levels (S_1) derived from the energy gap law for each of the carotenoids. Neoxanthin and violaxanthin have predicted S_1 energy levels higher than that for chlorophyll (Q_X and Q_Y) and, accordingly, these molecules can effectively transfer excitation energy to chlorophyll (Gruszecki *et al*. 1999). Recently, the S_1 fluorescence spectra for violaxanthin and zeaxanthin were determined and the S_1 energy levels calculated by this method support the role of violaxanthin in resonance energy transfer to chlorophyll (Frank *et al*. 2000). Conversely, the predicted S_1 state of zeaxanthin is lower than that of chlorophyll, theoretically promoting fluorescence quenching (Frank *et al*. 1994, 2000). The photophysical properties of lutein and antheraxanthin would theoretically enable either light harvesting or quenching (Frank *et al*. 1994). In fact, the recent direct measurement of the xanthophyll S_1 energy states and their

corresponding spectral overlap with the excited singlet state of chlorophyll would suggest that either violaxanthin or zeaxanthin can harvest or quench (Frank *et al*. 2000). However, this is clearly not the case *in vivo*, where violaxanthin and zeaxanthin have defined roles in impeding and facilitating quenching, respectively. Factors other than just the photophysical properties of the carotenoids must be involved (Horton *et al*. 1996). The roles of lutein and antheraxanthin *in vivo* are still being clarified (Gilmore 2000; Gilmore *et al*. 1998; Pogson *et al*. 1998).

One of the ways that the mechanisms of NPQ is being elucidated is by analysing quenching in carotenoid biosynthetic and NPQ mutants of *Chlamydomonas* and *Arabidopsis* (table 1). *npq1* is a lesion in the violaxanthin de-epoxidase and exhibits markedly reduced NPQ, presumably due to the lack of antheraxanthin and zeaxanthin, whereas *npq4*, which recently has been identified as a lesion in PsbS (CP22), has almost no NPQ yet still accumulates antheraxanthin and zeaxanthin (Niyogi *et al*. 1998; Li *et al*. 2000). The *npq* mutants are discussed in detail elsewhere (Baroli & Niyogi, this issue). In brief, insight from such studies supports a major role for zeaxanthin and PsbS in the quenching process.

Significantly, *npq1* mutants still had some reversible NPQ, which could be eliminated by preventing lutein biosynthesis (Niyogi *et al*. 2000). This supports our earlier observations that the lutein-deficient lines of *A. thaliana*, *lut1* and *lut2*, exhibit a delayed induction of NPQ kinetics as well as a lower maximal NPQ (Pogson *et al*. 1998). The induction kinetics of NPQ can be accelerated in constitutive zeaxanthin-accumulating mutants, *aba1* and *lut2 aba1*, despite a reduced level of maximum NPQ and a loss of LHC IIb trimers (Tardy & Havaux 1996; Hurry *et al*. 1997; Pogson *et al*. 1998). Analyses of carotenoid and NPQ mutants, and the effects of LHC IIb protonation on NPQ, suggest that antenna structure, a functional PsbS protein and xanthophyll molecules all influence quenching kinetics. The mechanisms for these interactions, however, remain unclear. Is there a direct involvement of 'quenching' carotenoids with PsbS and does this include zeaxanthin, antheraxanthin and lutein? Alternatively, does lutein facilitate changes in antenna structure that promote quenching and if so how do antenna proteins and PsbS interact?

The carotenoids aid in the capture of light, provide photoprotection against excess light and promote antenna assembly. Recent work has helped to define how the most abundant carotenoid, lutein, might contribute to these processes. However, much remains to be learned. The dual role of xanthophylls in LHC assembly and photoprotection makes it difficult to design definitive experiments because changes in LHC structure may alter photoprotection. Therefore, we are taking a new approach to further elucidate the roles of lutein and the xanthophyll cycle pigments by under- and overexpressing key genes in the carotenoid biosynthesis pathway. Our *in vivo* approach, which replaces one or more xanthophylls with their physiological homologue, minimizes problems with perturbations to the antenna. These transgenic plants are being used to study the regulation of the ε-cyclase and β-hydroxylase *in vivo* and to understand the effects of altered xanthophyll content on LHC structure and photoprotection.

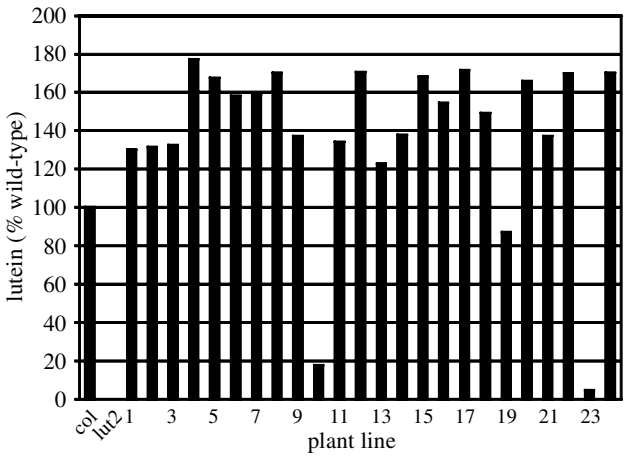

Figure 2. Lutein levels in wild-type (col), *lut2* and 24 T_2 plants overexpressing the ε-cyclase transgene in a wild-type background. Pigments were extracted from leaves of two- to five-week-old plants and quantified by HPLC. Values are presented as per cent of wild-type levels for which lutein content was 168 mmol mol^{-1} chl *a*.

2. METHODS

(a) *Transformation of* A. thaliana *with the sense ε-cyclase and the antisense β-hydroxylase transgene and growth of plants*

The transgene pBP244 was constructed to contain the ε-cyclase cDNA in the sense orientation under the control of the cauliflower mosaic virus (CaMV) promoter in the part7/27 binary vector (Gleave 1992). The plasmid pBP254 was constructed by cloning the *Arabidopsis* β-hydroxylase cDNA (GenBank accession number U58919) in the antisense orientation in the same vector using the same promoter (Rissler & Pogson 2000). An *npt* II kanamycin resistance gene provided for antibiotic selection of the transgenic plants (Gleave 1992). Both constructs were independently transformed into *Arabidopsis thaliana*, Columbia ecotype and *lut2*, using the floral dip method followed by vacuum infiltration (Clough & Bent 1998). Seeds were harvested and transgenic generation one (T_1) seedlings were selected for kanamycin resistance on Murashige–Skoog media containing 2% sucrose and 30 μg ml^{-1} kanamycin. Plants were grown at 21 °C under continuous light (80 μmol m^{-2} s^{-1}). T_2 seeds were collected from the kanamycin resistant plants and grown in soil under an 18 h light–dark cycle (150 μmol m^{-2} s^{-1}) at 21 °C.

(b) *Pigment analysis and measuring chlorophyll fluorescence*

Pigments were extracted from leaves of two- to five-week-old plants and analysed by HPLC chromatography (Hewlett-Packard series 1100) as described previously (Pogson *et al*. 1998). Chlorophylls were quantified by HPLC and by using published extinction coefficients (Porra *et al*. 1989). Chlorophyll fluorescence measurements and NPQ calculations were performed on intact leaves as described previously (Niyogi *et al*. 1998; Pogson *et al*. 1998).

3. RESULTS AND DISCUSSION

(a) *Establishing transgenic plants with a range of carotenoid compositions*

(i) *Increased lutein by overexpressing the ε-cyclase*

We screened wild-type and *lut2* transgenics for insertion of the BP244 ε-cyclase transgene by kanamycin

Table 2. *Carotenoid levels in* A. thaliana *leaves overexpressing the ε-cyclase transgene (sense ε-cyclase BP244)*

(Values given are for carotenoid content expressed as mmol mol^{-1} chlorophyll *a*; *wt*, wild type; *lut2*, lutein deficient; N, neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; L, lutein; β, β-carotene.)

	N	V	A	Z	L	β	total
wt	50	70	0	0	170	90	380
lut2	45	150	20	5	0	110	330
wt sense ε-cyclase (BP244-C31)	50	45	0	0	250	110	460
lut2 sense ε-cyclase (BP244-Z67)	55	50	0	0	220	100	430

Table 3. *Carotenoid levels in wild-type and* lut2 A. thaliana *leaves expressing the antisense β-hydroxylase*

(Values given are for carotenoid content expressed as mmol mol^{-1} chlorophyll *a*; wt, wild type; *lut2*, lutein deficient; N, neoxanthin; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; L, lutein; β, β-carotene; βOHase, β-hydroxylase.)

	N	V	A	Z	L	β	total
wt	62 ± 2	59 ± 2	0	0	172 ± 18	91 ± 2	380
wt anti-βOHase (BP254-K1)	25 ± 2	21 ± 3	0	0	164 ± 15	111 ± 16	320
lut2	41 ± 4	124 ± 2	25 ± 2	6 ± 2	0	136 ± 2	330
lut2 anti-βOHase (BP254-B1)	34 ± 4	72 ± 2	22 ± 2	6 ± 2	0	162 ± 3	300

resistance and increased lutein content. Significantly, lutein accumulation was restored in all transformed *lut2* lines. The *lut2* mutation has a biochemical phenotype consistent with a disruption to the ε-cyclase, i.e. a complete lack of carotenoids with an epsilon ring (Pogson *et al*. 1996). Additionally, the *lut2* and ε-cyclase loci map to the same region of the genome, that is the bottom of chromosome 5. Thus, data from biochemical, genetic and functional complementation experiments all support the conclusion that *lut2* is a mutation in the ε-cyclase gene.

Figure 2 expresses lutein content as a percentage of wild-type for a range of wild-type plants transformed with the BP244 ε-cyclase transgene. This procedure identified dozens of plants with altered lutein content with levels ranging from 10 to 150% of wild-type levels in the T_1 generation, indicating a combination of co-suppression and overexpression. In the T_2 generation, most of the 25 transformed lines segregated 3:1 for kanamycin resistance, indicating one insertion, some segregated 15:1, indicating two, and lutein content ranged from 5 to 180% of wild-type, which was the same or higher than the respective parental T_1. Interestingly, in the T_3 generation the lutein content of some high accumulating lines dropped to wild-type or lower levels, perhaps suggesting a subsequent gene silencing, although this has not been confirmed.

The increase in lutein of up to 180% preferentially resulted in a reduction in violaxanthin content and, in some lines, an increase in the total carotenoid pool (table 2). We did not detect a marked change in β-carotene or neoxanthin and neither α-carotene nor α-cryptoxanthin were detected when flux down either branch of the pathway was increased, which indicates that the hydroxylation reactions are not limiting. The change in the total pool size requires more detailed study to determine if there is a feedback effect due to the transgene. Many studies that have genetically altered xanthophyll accumulation have demonstrated that carotenoid accumulation is tightly regulated (Chunaev *et al*. 1991; Rock & Zeevaart 1991; Bishop 1996; Pogson *et al*. 1996, 1998; Rissler & Pogson 2000). That is, whenever possible, the alteration in levels of one xanthophyll results in a converse change in levels of a structurally or functionally similar carotenoid in preference to a more global affect on all carotenoids.

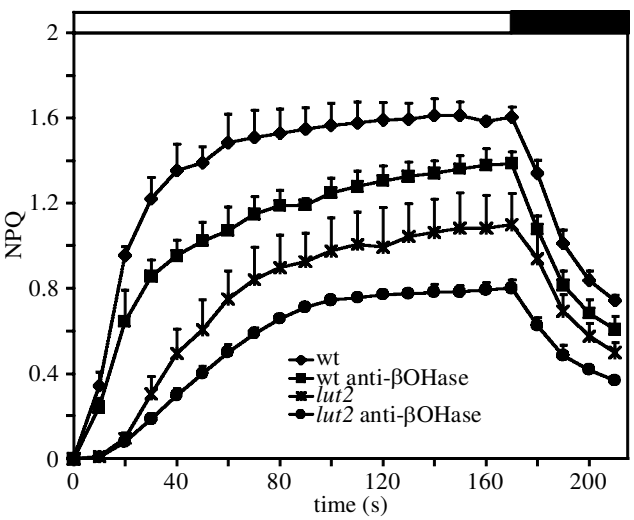

Figure 3. Non-photochemical quenching of chlorophyll fluorescence (NPQ) in wild-type, *lut2* and plants expressing the antisense β-hydroxylase transgene (anti-βOHase). Leaves from five-week-old plants were exposed to actinic light (1000 μmol m^{-2} s^{-1}, high light) from 0 to 170 s with 10 000 μmol m^{-2} s^{-1} pulses every 10 s. Actinic light was switched off (dark) at 170 s. Open bar, actinic light on; closed bar, actinic light off. NPQ was calculated as $(F_m - F'_m)/F'_m$ at 10 s intervals.

(ii) *Antisense inhibition of the β-hydroxylase: biosynthesis and LHC assembly*

An antisense β-carotene hydroxylase transgene, BP254, in wild-type plants resulted in a maximum reduction in violaxanthin of *ca.* 64% and in neoxanthin of 41% for both T_1 and T_2 generations (Rissler & Pogson 2000). In addition, there was a 5% reduction in lutein, a 22%

Table 4. *Summary of xanthophyll and NPQ levels in carotenoid biosynthetic mutants and transgenics*

(The measure for NPQ incorporates both the rapidly reversible ('q_E') and slowly reversible (q_I) components. We have estimated q_E as maximal NPQ minus NPQ after 30 s of dark relaxation ('q_E'). This illustrates that the rapidly reversible component is nearly eliminated in the double mutants, *npq1 lut2* and *npq1 lor1*. V + A + Z (violaxanthin + antheroxanthin + zeaxanthin); βOHase, β-hydroxylase.)

species	line or mutation	lutein (% wt)	V + A + Z (% wt)	NPQ at 30 s (% wt)	max. NPQ (% wt)	'q_E' (% wt)
Chlamydomonas reinhardti	*npq1*[a]	100	102	100[a]	80	60
	lor1[a]	0	306	88[a]	63	50
	npq1 lor1[a]	0	304	38[a]	28	15
Arabidopsis thaliana	*npq1*[a]	100	116	53[a]	30	14
	lut2[a]	0	262	24[a]	67	62
	npq1 lut2[a]	0	350	13[a]	24	6
	anti-βOHase[a]	95	35	69	84	n.c.[b]
	lut2 anti-βOHase	0	169	14	50	n.c.
	130% lutein	130	69	130	104	n.c.

[a] Data derived from Niyogi *et al.* (1997, 2000), Pogson *et al.* (1998) and Rissler & Pogson (2000).
[b] Not calculated.

increase in β-carotene and a 16% reduction in total carotenoid levels (table 3). The disruption of one step in the pathway resulting in a reduction in the total pool of carotenoids is something not observed for *lut1*, *lut2* or the *aba1* mutations. In *lut1* there was a marked increase in violaxanthin and antheraxanthin, despite the fact that the disruption was after the branch point in the pathway, implying feedback or sink-driven regulation (Pogson *et al.* 1996). However, the antisense hydroxylase plants cannot have a corresponding increase in lutein to compensate for lost violaxanthin and neoxanthin as lutein also requires the β-hydroxylase for its synthesis—instead the only option is for an increase in β-carotene. Given that carotenes are rarely, if ever, found in LHC complexes, turnover of unincorporated β-carotene could be reflected in the reduction in the total carotenoid pool.

The limit of 60% reduction in violaxanthin in one-third of the lines could either be the result of post-transcriptional regulation, inefficient inhibition of the second putative β-hydroxylase gene product, or a degree of functional redundancy due to the ε-hydroxylase (Rissler & Pogson 2000). Considering the latter, the *Arabidopsis* β-hydroxylase enzyme does exhibit some ε-hydroxylase activity in *E. coli* and *lut1* accumulates some lutein (Pogson *et al.* 1996; Sun *et al.* 1996). If the converse occurs, then the ε-hydroxylase could be hydroxylating β-carotene in the antisense plants. Currently, we are testing the three hypotheses by crossing the most severe antisense lines into the *lut1* background to determine whether or not a concomitant decrease in the β- and ε-hydroxylases will result in a further reduction in violaxanthin and neoxanthin. Clearly, many questions remain about the regulation of the pathway *in vivo*.

Chlorophyll content, Western blots of major LHCs and non-denaturing 'green gels' all consistently indicated that little alteration in antenna composition had occurred in the antisense β-hydroxylase plants (Rissler & Pogson 2000). This preservation of LHCs is in agreement with *in vitro* reconstitution data that show violaxanthin is preferred for optimal stability, but is not essential for folding except for Lhcb5 (Ros *et al.* 1998). Interestingly, in the most severe antisense β-hydroxylase plants the chlorophyll *a*/*b* ratio was 2.33 ± 0.1, which was significantly lower than wild-type (2.85 ± 0.28). A possible explanation for the lower ratio is that there may be an increase in Lhcb1/2 trimers (Rissler & Pogson 2000), which have a chlorophyll *a*/*b* ratio of 1.3 (Peter & Thornber 1991; Bassi *et al.* 1993). Additionally, exposure of wild-type and antisense β-hydroxylase plants to high light showed that the pool of violaxanthin is tightly controlled to optimize LHC structure at the expense of availability of violaxanthin for de-epoxidation.

(iii) *Photoprotection*

The xanthophyll cycle has been proposed to play a role in photoprotection in conjunction with LHC protonation and a functional PsbS protein (Walters *et al.* 1996; Niyogi 1999; Li *et al.* 2000). In the absence of zeaxanthin, quenching is greatly reduced (Niyogi *et al.* 1998). However, when zeaxanthin was reduced by 75% there was only a slight 16% reduction in the extent of NPQ (Rissler & Pogson 2000) (figure 3). These results demonstrate that only a limited fraction of the total zeaxanthin in wild-type plants is required for optimal chlorophyll fluorescence quenching *in vivo* and are in agreement with studies on isolated thylakoids that suggested only a few zeaxanthin molecules are required (Gilmore *et al.* 1998). This would imply that in wild-type plants, more zeaxanthin is synthesized than is actually required for achieving maximal NPQ. The 'excess' zeaxanthin that is not directly involved in chlorophyll fluorescence quenching may contribute to other processes such as protection against lipid peroxidation (Havaux & Niyogi 1999).

Whenever zeaxanthin accumulation has been prevented, either by dithiothreitol treatment or a de-epoxidase mutation, reversible quenching was reduced but never eliminated (Gilmore & Yamamoto 1991; Niyogi *et al.* 1997, 1998). This residual component of the rapidly reversible quenching (q_E) is *ca.* 60% in *Chlamydomonas* and 14% in *Arabidopsis* (table 4). The two lutein deficient mutations of *Arabidopsis* and *Chlamydomonas*, *lut2* and *lor1*, respectively, have delayed and reduced NPQ (Niyogi *et al.* 1997; Pogson *et al.* 1998). When they are crossed into the

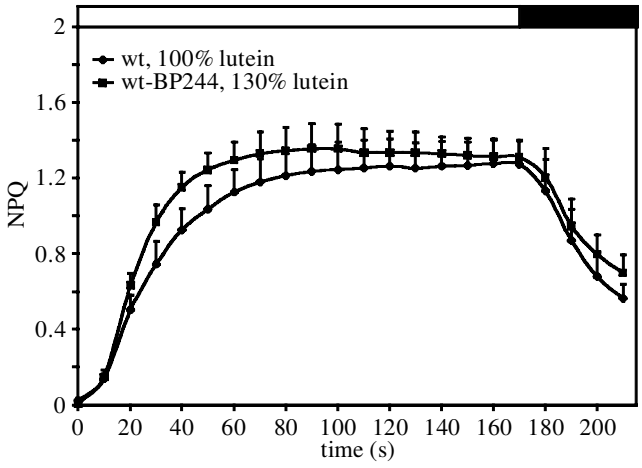

Figure 4. Non-photochemical quenching of chlorophyll fluorescence (NPQ) in wild-type (wt) and a plant overexpressing the ε-cyclase transgene (wt-BP244, T_3 generation), in which lutein levels were 130% of wild-type. See figure 3 for details.

npq1 background, the remaining rapidly reversible NPQ is essentially eliminated (table 4) (Niyogi *et al*. 1997, 2000). Additionally, when the antisense β-hydroxylase line that has a 16% reduction in NPQ is crossed into the *lut2* background, there is a further 34% reduction in NPQ (figure 3). Conversely, constitutively increasing the proportion of lutein in BP244 (figure 4) and zeaxanthin in *aba1* (Pogson *et al*. 1998) increases the rate of induction of NPQ. Likewise, the delayed quench in *lut2* can be restored by constitutively accumulating either lutein or zeaxanthin.

Overexpressing the ε-cyclase resulted in a 31% reduction in the xanthophyll pool size but a more rapid induction of NPQ (table 4, figure 4), which is contrary to the antisense β-hydroxylase data where decreasing the xanthophyll cycle pool delayed induction (table 4, figure 3). Reducing the xanthophyll cycle pool therefore reduces quenching if lutein content is unaltered, but if the reduced xanthophyll cycle pool is replaced by a corresponding increase in lutein, the rate of induction is slightly and significantly accelerated (figure 4). This apparent paradox may be explained by the hypothesis that increasing lutein resulted in more 'free' lutein, which could substitute for zeaxanthin and participate in the initial phase of NPQ since most of the lutein would be tightly bound within the LHC (Kuhlbrandt *et al*. 1994) and probably not participate in NPQ.

4. CONCLUSIONS

Genetic manipulation of the ε-cyclase and β-hydroxylase has shown that it is possible to alter flux through the pathway and the carotenoid pool size by manipulating mRNA abundance of one gene. However, there appear to be limits to the extent that manipulating gene expression results in altered carotenoid accumulation. That is, there is a maximal increase of *ca*. 180% for lutein in the sense ε-cyclase lines and a maximal decrease of *ca*. 60% for violaxanthin in the antisense β-hydroxylase lines. How transcription is regulated and to what extent post-transcriptional processes—such as chaperone-mediated targeting, the proposed multi-enzyme complexes and incorporation into LHCs (Giuffra *et al*. 1996; Bonk *et al*. 1997; Cunningham & Gantt 1998)—affect accumulation remains to be determined.

The physiological relevance of xanthophylls and NPQ is exemplified by the bleaching phenotype that is observed in several carotenoid biosynthetic and NPQ mutants. In the absence of both zeaxanthin and lutein, *Chlamydomonas* cultures photobleach and mature *Arabidopsis* leaves senesce and photobleach under high light (Niyogi *et al*. 1997, 2000). There is a much less bleaching if only zeaxanthin is eliminated and there is residual NPQ. Also, photobleaching is not as extensive in *npq4* mutants that exhibit a loss of NPQ without a change in xanthophyll biosynthesis (Baroli & Niyogi, this issue). These differences may reflect the role of xanthophylls in other processes in addition to NPQ, including photosystem stability, quenching of chlorophyll singlets and triplets, and protection against lipid peroxidation.

It is possible and photophysically feasible that lutein might participate in the quenching process, particularly during the first 30 s of high light prior to substantial zeaxanthin synthesis. Chlorophyll singlets are formed within seconds of high light exposure and intersystem crossing to form potentially damaging triplets occurs rapidly. An increase in lutein did increase NPQ levels during the first 30 s and maximal NPQ was unaffected (figure 4). This increase in the early phase of NPQ was despite a decrease in the xanthophyll cycle pool size. Yet, a reduction in the xanthophyll cycle pool in the antisense hydroxylase lines caused a 16% reduction in maximal NPQ if lutein content was unaltered, and this decrease could be enhanced by another 34% if lutein was eliminated (figure 3). In fact, lutein deficiency has a deleteriously additive effect on NPQ in a range of *Arabidopsis* and *Chlamydomonas* genotypes, including wild-type, *npq1* (zeaxanthin deficient), antisense β-hydroxylase (xanthophyll cycle pool decreased) or *aba1* (table 4). Questions remain concerning the number of lutein molecules contributing to NPQ (Gilmore 2000) and whether lutein directly or indirectly affects chlorophyll singlet quenching and interacts with PsbS. We still do not understand the mechanism of NPQ, but recent progress has enabled more specific questions to be addressed and has shown that it is indeed a physiologically relevant process.

We are grateful to Angelica Elliot, Raymund Beirne and Carey Hendrix for assistance with screening for transgenic plants and NPQ measurements. We express our gratitude to colleagues at Arizona State University (ASU), Australian National University (ANU) and the University of Nevada. This work was supported in part by the Australian Research Council, project no. F00077; the ANU Endowment for Excellence; the National Science Foundation Graduate Research Training Grant and a Research Experience for Undergraduates grant to the Center for the Study of Early Events in Photosynthesis at ASU.

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Discussion

E. Hideg (*Institute of Plant Biology, Biological Research Centre, Szeged, Hungary*). In order to act as efficient triplet quenchers or singlet oxygen quenchers, carotenoids have to be close enough to chlorophylls or other potential sites of O_2 production. When the carotenoid composition is altered, do pigments have the same localization as in the wild-type?

B. J. Pogson. That is a good question to which the answers are still being sought. It would appear that there are not dramatic changes to chlorophyll content and antenna function in the single mutants *lut1*, *lut2* and *aba1*. Also, the antisense β-hydroxylase plants have a similar pigment content and Lhcp content as wild-type. Based on those results, I suspect there are no dramatic changes to the capacity to quench triplet chlorophylls in the mutants or transgenic lines that contain substantial levels of at least one wild-type xanthophyll. However, we require more detailed analyses to confirm this. I think the story will be more complex for xanthophyll-reduced and xanthophyll-deficient lines.