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Lutein Accumulation in the Absence of Zeaxanthin Restores Nonphotochemical Quenching in the *Arabidopsis thaliana npq1* Mutant ^{W|OA}

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Plants protect themselves from excess absorbed light energy through thermal dissipation, which is measured as nonphotochemical quenching of chlorophyll fluorescence (NPQ). The major component of NPQ, qE, is induced by high transthylakoid Δ pH in excess light and depends on the xanthophyll cycle, in which violaxanthin and antheraxanthin are deepoxidized to form zeaxanthin. To investigate the xanthophyll dependence of qE, we identified *suppressor of zeaxanthin-less1* (*szl1*) as a suppressor of the *Arabidopsis thaliana npq1* mutant, which lacks zeaxanthin. *szl1 npq1* plants have a partially restored qE but lack zeaxanthin and have low levels of violaxanthin, antheraxanthin, and neoxanthin. However, they accumulate more lutein and α -carotene than the wild type. *szl1* contains a point mutation in the lycopene β -cyclase (*LCYB*) gene. Based on the pigment analysis, *LCYB* appears to be the major lycopene β -cyclase and is not involved in neoxanthin synthesis. The Lhcb4 (CP29) and Lhcb5 (CP26) protein levels are reduced by 50% in *szl1 npq1* relative to the wild type, whereas other Lhcb proteins are present at wild-type levels. Analysis of carotenoid radical cation formation and leaf absorbance changes strongly suggest that the higher amount of lutein substitutes for zeaxanthin in qE, implying a direct role in qE, as well as a mechanism that is weakly sensitive to carotenoid structural properties.

INTRODUCTION

Light is required for photosynthesis in plants, but the quantity of light in natural environments is highly variable. Within a certain range of relatively low incident light intensities, photosynthetic carbon fixation increases linearly with increases in photon flux density. However, above a certain threshold, photosynthetic capacity is saturated, and a plant absorbs more light than it can actually use. Absorption of excess light can lead to overexcitation of chlorophyll and overreduction of the electron transport chain, which result in increased generation of reactive intermediates and harmful byproducts of photosynthesis (Niyogi, 1999). For example, overexcitation of chlorophyll would result in an increase in the lifetime of singlet-excited chlorophyll ($^1\text{Chl}^*$), which consequently increases the production of triplet-excited Chl ($^3\text{Chl}^*$) via intersystem crossing. $^3\text{Chl}^*$ interacts with molec-

ular oxygen to generate singlet O_2 ($^1\text{O}_2^*$), which can damage proteins, pigments, and lipids in the photosynthetic apparatus (Niyogi, 1999; Asada, 2006).

Photosynthetic organisms have evolved a suite of short-term and long-term photoprotective mechanisms to cope with the absorption of excessive light and its consequences. Among these mechanisms, the thermal dissipation of excess absorbed light energy in photosystem II (PSII), which is commonly measured and referred to as nonphotochemical quenching (NPQ), is believed to play a key role in regulating light harvesting and preventing photooxidative damage to the photosynthetic apparatus. NPQ can be induced or disengaged in response to changes in light intensity on a time scale of seconds to minutes. Although there are several components of NPQ in higher plants, pH-dependent energy dissipation (also called qE) accounts for the major part of NPQ and results in deexcitation of $^1\text{Chl}^*$ and the thermal dissipation of excess absorbed light energy in the light-harvesting antenna of PSII (Müller et al., 2001). Because it involves the deexcitation of $^1\text{Chl}^*$, qE can be easily measured as a decrease in the maximum yield of chlorophyll fluorescence in intact leaves or isolated chloroplast membranes (Müller et al., 2001).

qE is induced by a low thylakoid lumen pH (i.e., a high Δ pH) during illumination with excess light (Demmig-Adams and Adams, 1992; Horton et al., 1996; Müller et al., 2001). The low

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thylakoid lumen pH plays dual roles, one of which is to activate the violaxanthin deepoxidase (VDE) enzyme, which converts violaxanthin into antheraxanthin and then zeaxanthin as part of a xanthophyll cycle (Figure 1) (Yamamoto et al., 1999; Jahns et al., 2009). The other role of the low thylakoid lumen pH is to protonate one or more PSII proteins that are involved in qE (Horton and Ruban, 1992). A light-induced absorbance change at 535 nm (ΔA_{535}) is linearly correlated with qE (Ruban et al., 1993; Bilger and Björkman, 1994; Li et al., 2004). ΔA_{535} depends on both zeaxanthin and protonation and is thought to be due to a change in the absorption spectrum of zeaxanthin (Ruban et al., 2002).

Analysis of *Arabidopsis thaliana* mutants that lack qE has been a very useful approach to define factors that are necessary for qE, including xanthophylls, the PsbS protein, and light-harvesting complex (LHC) proteins (Niyogi, 2000). qE-deficient mutants were identified in forward genetics screens by video imaging of chlorophyll fluorescence yield during exposure of mutagenized *Arabidopsis* seedlings to excess light (Niyogi et al.,

1998; Li et al., 2000). The *nonphotochemical quenching1* (*npq1*) and *lutein-deficient2* (*lut2*) mutants exhibit diminished levels of qE (Niyogi et al., 1998; Pogson et al., 1998), whereas the *npq4* mutant is completely defective in qE and ΔA_{535} (Li et al., 2000; Peterson and Havir, 2000). The *npq1* mutant is defective in VDE and therefore lacks zeaxanthin. Characterization of the *npq1* mutant showed that zeaxanthin is necessary for most of the qE in vivo in *Arabidopsis* leaves (Niyogi et al., 1998). The *lut2* mutant affects lycopene ϵ -cyclase (LCYE) activity (Pogson et al., 1998), so it is unable to synthesize either lutein or α -carotene (Figure 1). The *npq1 lut2* double mutant is totally devoid of any qE, suggesting a possible role for lutein in qE (Niyogi et al., 2001). Complementary evidence was reported with LCYE-overexpressing transgenic *Arabidopsis* plants, which have elevated lutein and an increase in qE (Pogson and Rissler, 2000). It has been proposed that lutein might have a direct role in qE or, alternatively, that the change of lutein content could indirectly affect qE by disturbing the assembly and structure of the PSII antenna (Niyogi et al., 1997, 2001; Pogson et al., 1998; Lokstein et al., 2002).

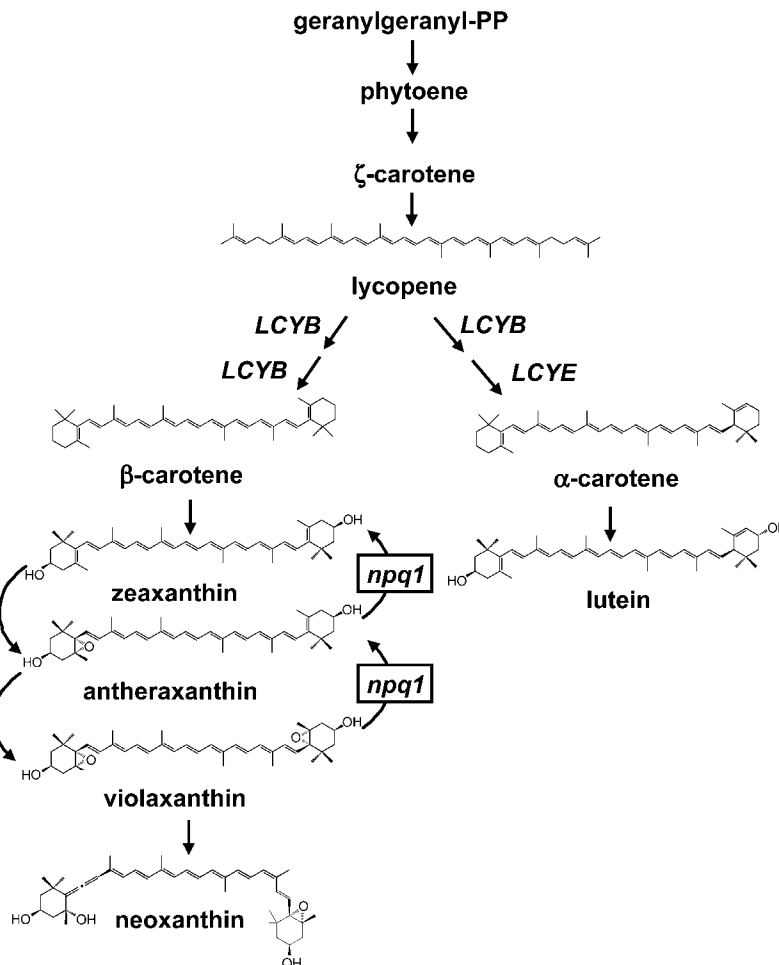


Figure 1. Carotenoid Biosynthetic Pathway in Plants.

The block in xanthophyll metabolism in the *npq1* mutant, which lacks zeaxanthin due to a defect in the violaxanthin deepoxidase gene, is indicated by the symbol "*npq1*."

Despite these advances in identifying components of qE, the actual biophysical mechanism of ^1Chl deexcitation in qE remains controversial. Two mechanisms, which are not mutually exclusive, have been proposed recently based on ultrafast transient absorption (TA) spectroscopy experiments (Holt et al., 2005; Ruban et al., 2007). Both mechanisms include a role for PsbS as a sensor of lumen pH that triggers conformational changes in the PSII antenna that result in efficient deexcitation of $^1\text{Chl}^*$ (Ahn et al., 2008; Avenson et al., 2008; Horton et al., 2008). In one model, zeaxanthin has a direct role in quenching $^1\text{Chl}^*$ through a charge-transfer (CT) mechanism (Holt et al., 2005) in the minor LHCS associated with PSII, CP29 (Lhcb4), CP26 (Lhcb5), and CP24 (Lhcb6) (Ahn et al., 2008; Avenson et al., 2008, 2009). The CT quenching mechanism involves energy transfer from chlorophyll to closely coupled chlorophyll and zeaxanthin molecules, followed by charge separation that transiently produces a zeaxanthin radical cation and chlorophyll radical anion. Subsequent charge recombination dissipates the excitation energy as heat (Holt et al., 2005). The second model proposes that qE occurs in the peripheral, trimeric antenna of PSII called LHCII (Pascal et al., 2005), and its molecular mechanism involves energy transfer from $^1\text{Chl}^*$ to a low-lying S1 excited state of a carotenoid (lutein 1) (Ruban et al., 2007). According to this model, zeaxanthin is not required for qE but functions indirectly as an allosteric regulator (Crouchman et al., 2006) by increasing the pH sensitivity of qE (Noctor et al., 1991).

To investigate the xanthophyll dependence of qE and, possibly at the same time, to identify new components of qE, we performed a screen for suppressors of the *npq1* mutation that exhibit higher qE despite the absence of zeaxanthin. We isolated a suppressor that accumulates more lutein but has a very small xanthophyll cycle pool size. Molecular, physiological, and biophysical analysis of this mutant suggest that the higher amount of lutein can substitute for zeaxanthin to act directly as a quencher in qE.

RESULTS

Isolation of Suppressors of *npq1*

We used a chlorophyll fluorescence video imaging system (Niyogi et al., 1997) to isolate suppressors of the *npq1* mutant. Ten potential suppressors that showed much higher NPQ level compared with that of the *npq1* parent were isolated out of 15,000 M2 seedlings (Figure 2), and six suppressors were shown to lack zeaxanthin during the secondary screen by pigment analysis. One of these six suppressors with partially restored NPQ had very low levels of violaxanthin and neoxanthin but accumulated more lutein (Figure 3A). The suppressor was named *suppressor of zeaxanthin-less1 (szl1)*, and the *szl1 npq1* plant was backcrossed to the *npq1* parent three times and then crossed to the wild type to isolate an *szl1* single mutant, which was able to synthesize zeaxanthin upon exposure to high light (Figure 3B).

Pigment Content and NPQ in the Suppressor

The wild-type, *npq1*, *szl1*, and *szl1 npq1* plants were grown under low light (LL) conditions ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). In

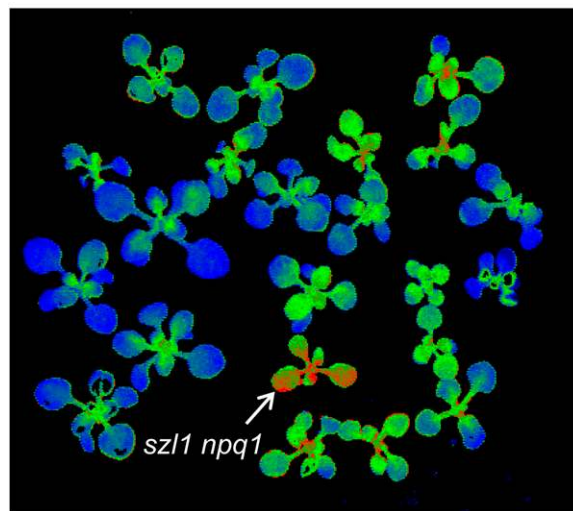
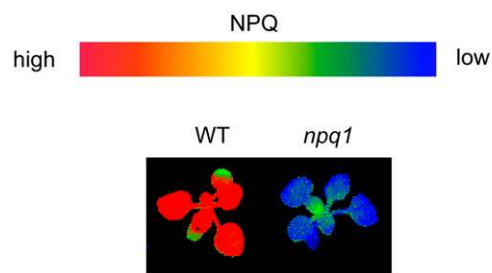


Figure 2. Screening for Suppressors of *npq1* by Video Imaging of Chlorophyll Fluorescence Quenching.

Mutagenized *npq1* plants (M2 generation) on agar medium were exposed to $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 1 min. In this false-color image of NPQ, the wild type appears red and an *npq1* mutant appears blue, whereas the *szl1 npq1* suppressor appears greenish red.

these growth conditions, the suppressor *szl1 npq1* and single mutant *szl1* exhibited a very similar whole-plant phenotype to that of the wild-type and *npq1* plants, with the major difference being slightly smaller sizes (Figure 4A). The pigment composition and content of the four genotypes were measured before and after a short treatment of LL-grown plants with high light (HL; $1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). All four genotypes had the same total chlorophyll content, chlorophyll *a/b* ratio, and total carotenoids under both conditions, although *szl1* and *szl1 npq1* had lower total carotenoids and correspondingly higher total xanthophylls (Table 1). Figure 4B compares the relative content of β -carotene-derived violaxanthin, antheraxanthin, and zeaxanthin and α -carotene-derived lutein in wild-type and mutant plants after HL treatment. Xanthophyll cycle pigment pool size (the sum of violaxanthin, antheraxanthin, and zeaxanthin) was the same in the wild type and *npq1*, but it was reduced nearly 75% in *szl1* and *szl1 npq1*. Because the *npq1* mutation affects the VDE gene, no zeaxanthin was detected in either *npq1* or *szl1 npq1*. The concentration of lutein in the *szl1* and *szl1 npq1* mutants was nearly two times higher than in the wild type and *npq1* mutant. The smaller xanthophyll cycle pool size and the greater lutein concentration in the suppressor *szl1 npq1* and the *szl1* single

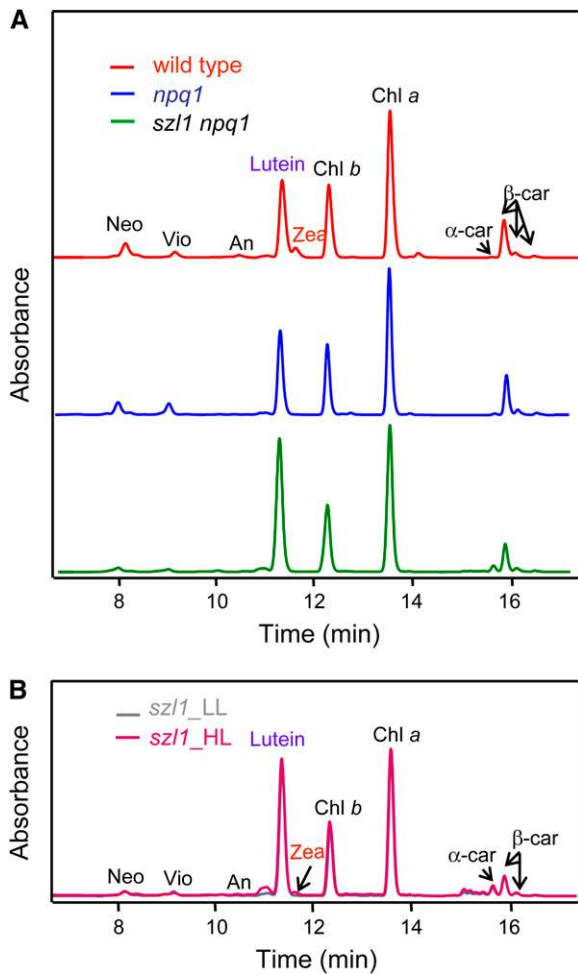


Figure 3. HPLC Analysis of Pigments in the Wild Type, *npq1*, *szl1*, and *szl1 npq1*.

(A) Comparison of pigment profile of the wild type, *npq1*, and *szl1 npq1* after exposure to high light ($1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 30 min.

(B) Overlay of HPLC traces of a *szl1* single mutant before (LL) and after (HL) treatment with high light. Neo, neoxanthin; Vio, violaxanthin; An, antheraxanthin; Zea, zeaxanthin; Chl a, chlorophyll a; Chl b, chlorophyll b; α -car, α -carotene; β -car, β -carotene.

mutant (relative to those in the wild type) indicated that the β -carotene branch of the carotenoid biosynthetic pathway is affected, and metabolic flux is redirected into the α -carotene branch from which lutein is synthesized.

The NPQ induction curves of the four genotypes plus the heterozygous *szl1/SZL1 npq1/npq1* mutant were compared (Figure 5). When illuminated with $1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, the wild type showed a rapid establishment of NPQ to a value of 2.2 within 5 min, whereas the *npq1* mutant showed a slower rate of NPQ induction and an NPQ value of <1.2. In the *szl1 npq1* plants, the NPQ induction had an even more rapid rise in the first 30 s compared with the wild type, which was probably due to the higher amount of lutein, and the total NPQ reached a value of 1.7, which was substantially higher compared with the *npq1* mutant.

By subtracting the residual NPQ in each genotype after relaxation in the dark, we estimated the qE component after 5 min of light induction to be 1.7, 0.8, and 1.4 in the wild type, *npq1*, and *szl1 npq1*, respectively. The *szl1* single mutant showed the same NPQ induction as that of the suppressor *szl1 npq1*. The heterozygous *szl1/SZL1 npq1/npq1* mutant had NPQ levels identical to the *npq1* mutant, indicating that the *szl1* mutation is recessive for the NPQ phenotype.

The *SZL1* Gene Encodes LCYB

The pigment phenotype of the suppressor *szl1 npq1* suggested that the activity of a carotenoid biosynthetic enzyme is impaired. Since *szl1 npq1* had a very small xanthophyll cycle pool size but twice as much lutein, the most likely candidate for the enzyme affected by *szl1* mutation was LCYB, which functions at the branch point in the carotenoid biosynthetic pathway (Figure 1).

In *Arabidopsis*, LCYB is encoded by a single-copy gene (Cunningham et al., 1996). The *LCYB* gene was amplified directly from the genomic DNAs of the wild type, *npq1*, and *szl1 npq1*. Sequence analysis revealed that *LCYB* from *szl1 npq1* carries a point mutation from G to A at position 1352, which translates into

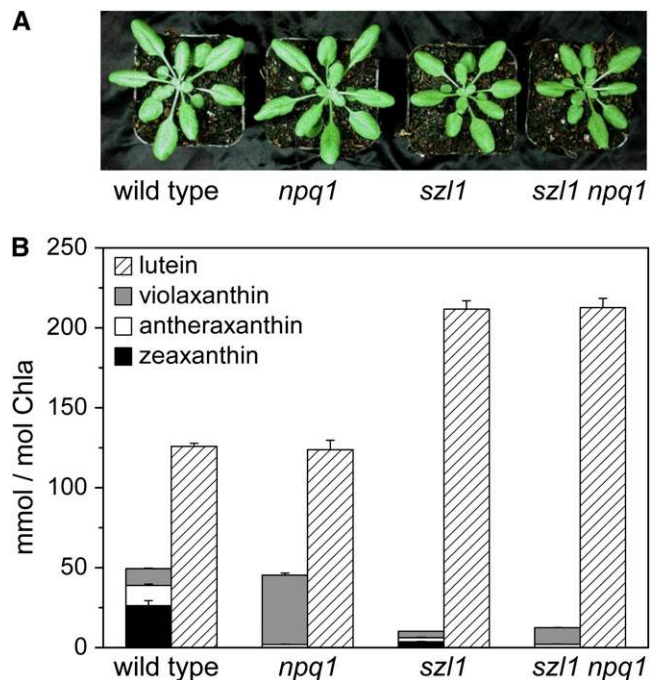


Figure 4. Characteristics of Wild-Type, *npq1*, *szl1*, and *szl1 npq1* Plants.

(A) Growth of the four *Arabidopsis* strains. Plants were grown in LL ($150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) with a short-day photoperiod (10 h light and 14 h dark). Plants were photographed at an age of 4 weeks.

(B) Xanthophyll cycle pigment pool size (V+A+Z) and lutein levels of the four *Arabidopsis* strains. Plants were grown as described in (A). At the end of the dark period, whole plants were exposed to HL ($2000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 30 min, and leaf samples were taken and analyzed by HPLC. Data were normalized to chlorophyll a and shown as the means \pm SD ($n = 9$).

Table 1. Pigment Content of the Wild Type, *npq1*, *szl1*, and *szl1 npq1*

Parameter	Wild Type		<i>npq1</i>		<i>szl1</i>		<i>szl1 npq1</i>	
	LL	HL	LL	HL	LL	HL	LL	HL
Total chlorophyll (nmol cm ⁻²)	21.9 ± 0.5	21.3 ± 1.5	22.5 ± 0.7	22.8 ± 1.2	21.9 ± 2.0	22.3 ± 1.6	20.9 ± 1.3	21.2 ± 0.8
Chl <i>a/b</i>	3.31 ± 0.02	3.29 ± 0.04	3.46 ± 0.07	3.33 ± 0.01	3.37 ± 0.09	3.36 ± 0.07	3.35 ± 0.03	3.40 ± 0.06
Total carotenoids (mmol/mol Chl <i>a</i>)	293.1 ± 11.6	287.9 ± 4.6	273.6 ± 6.8	277.5 ± 6.4	264.8 ± 18.8	277.3 ± 5.3	280.6 ± 2.9	281.6 ± 8.0
VAZ pool size (mmol/mol Chl <i>a</i>)	48.4 ± 3.3	49.6 ± 2.7	41.6 ± 0.8	44.7 ± 2.4	9.4 ± 0.2	10.2 ± 0.2	11.7 ± 0.7	12.5 ± 0.2
Violaxanthin (mmol/mol Chl <i>a</i>)	46.2 ± 3.7	10.6 ± 0.2	39.6 ± 0.7	43.4 ± 1.3	7.2 ± 0.5	3.9 ± 0.0	9.7 ± 0.9	10.4 ± 0.1
Antheraxanthin (mmol/mol Chl <i>a</i>)	2.3 ± 0.4	12.6 ± 0.9	1.8 ± 0.1	1.9 ± 0.2	2.1 ± 0.4	2.5 ± 0.3	2.0 ± 0.2	2.1 ± 0.2
Zeaxanthin (mmol/mol Chl <i>a</i>)	0	26.3 ± 3.1	0	0	0	3.8 ± 0.2	0	0
Lutein (mmol/mol Chl <i>a</i>)	123.2 ± 1.7	125.8 ± 2.0	127.5 ± 4.5	123.8 ± 2.0	200.2 ± 15.0	211.7 ± 5.1	210.7 ± 3.0	212.1 ± 5.7
Neoxanthin (mmol/mol Chl <i>a</i>)	32.3 ± 3.1	32.7 ± 2.7	28.0 ± 0.9	29.5 ± 1.0	6.7 ± 0.7	7.8 ± 0.3	8.5 ± 1.1	9.7 ± 0.7
β-Carotene (mmol/mol Chl <i>a</i>)	76.5 ± 4.3	77.7 ± 3.1	74.7 ± 1.1	77.4 ± 3.6	31.9 ± 2.6	31.9 ± 1.2	32.9 ± 1.7	32.2 ± 1.5
α-Carotene (mmol/mol Chl <i>a</i>)	2.6 ± 0.9	2.2 ± 0.3	1.9 ± 0.1	1.9 ± 0.0	16.8 ± 0.9	15.7 ± 0.6	16.9 ± 0.5	15.1 ± 0.2
β-Branch/α-branch	1.16 ± 0.05	1.16 ± 0.02	1.11 ± 0.02	1.20 ± 0.01	0.22 ± 0.00	0.20 ± 0.00	0.23 ± 0.00	0.24 ± 0.00

Pigment measurements were performed before and after exposure of LL-grown plants to HL (2000 μmol photons m⁻² s⁻¹) for 30 min. Data are presented as the means ± SD (*n* = 9). Chl *a/b*, chlorophyll *a/b*; Chl *a*, chlorophyll *a*.

a change at conserved residue 451 from Gly to Glu (Figure 6A). This point mutation creates an *EcoRI* restriction site, which can be used as a polymorphism marker for the *szl1* allele.

To determine whether the *szl1* mutation in the *LCYB* gene is genetically linked with the NPQ suppressor phenotype, the *szl1 npq1* double mutant was backcrossed to the *npq1* mutant, and the NPQ phenotypes were measured in the resulting F2 generation. When scoring F2 plants for the polymorphism marker and NPQ suppressor phenotype, the *szl1* allele cosegregated with the NPQ suppressor phenotype in all of the progeny tested (Figure 6B). Out of 48 F2 plants, 10 were identified as homozygous *szl1/szl1 npq1/npq1* mutants, which is not significantly different (χ^2 , *P* > 0.5) from the expected number (12, or 25%) for a single recessive nuclear mutation.

Reconstitution of the *szl1* Mutant Pigment Phenotype in *Escherichia coli*

To confirm that the *LCYB* point mutation found in the *szl1 npq1* mutant was responsible for the mutant pigment phenotype (low xanthophyll cycle pool size and high lutein content), plasmids pAC-BETA-At and pAC-BETA-At-*szl1* were constructed (see Methods). These plasmids contain bacterial genes for lycopene synthesis plus the wild-type or *szl1* allele of *LCYB*, respectively. The composition of pigments accumulated by *E. coli* strains containing either of these two plasmids with or without an additional plasmid expressing the *Arabidopsis LCYE* gene was compared.

We first examined the products formed when either the wild-type or mutant copy of *Arabidopsis LCYB* was present in the lycopene-accumulating strain of *E. coli*. Figure 7A shows an

HPLC profile of the carotenoid pigments accumulated in *E. coli* cells containing plasmid pAC-BETA-At. As expected from the previous work by Cunningham et al. (1996), these cells accumulated predominantly β-carotene and formed yellow colonies. When the wild-type *LCYB* gene was replaced by the *szl1* mutant

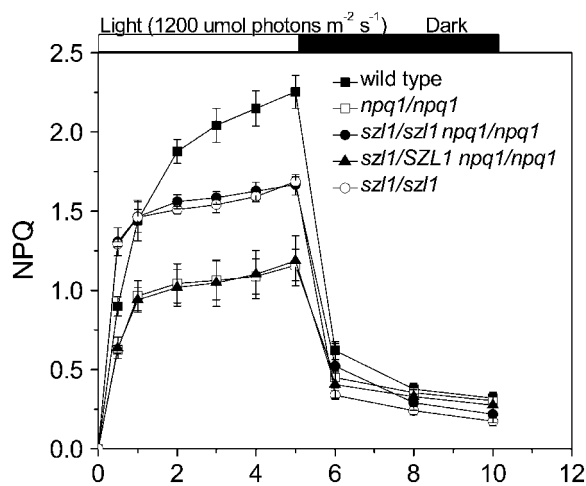


Figure 5. NPQ Induction Curves in the Wild Type, Homozygous *npq1*, *szl1*, and *szl1 npq1*, and Heterozygous *szl1/SZL1 npq1/npq1*.

NPQ was measured during 5 min of illumination with HL (1200 μmol photons m⁻² s⁻¹), followed by relaxation in the dark for 5 min. Data are presented as the means ± SD (*n* = 4).

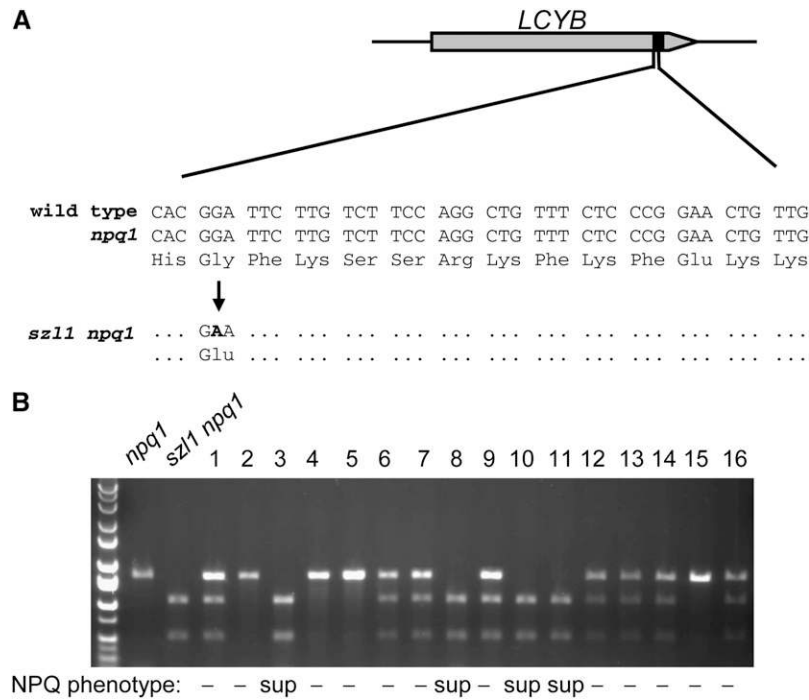


Figure 6. Molecular Genetic Analysis of *szl1*.

(A) Sequence and position of the *szl1* allele of the *LCYB* gene. The *szl1 npq1* suppressor has a single base substitution from G to A, which changes the Gly residue (position 451) in a predicted transmembrane helix (black box) to a Glu. This Gly is invariant in the seven available plant *LCYB* enzymes, including *Arabidopsis*, maize, rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*), tomato, citrus (*Citrus sinensis*), and papaya (*Carica papaya*). Dots in the *szl1 npq1* sequence indicate identity to the wild-type sequence.

(B) Cosegregation analysis of *szl1* and NPQ phenotype. The *szl1 npq1* suppressor was backcrossed to the *npq1* parent to obtain F₂ progeny. Lanes 1 to 16 are the first 16 plants among the total of 48 F₂ progeny that were tested. The PCR fragments amplified from the genomic DNA of F₂ progeny were digested with *EcoRI* and separated on agarose gel.

allele of *LCYB*, *E. coli* cells containing plasmid pAC-BETA-At-*szl1* accumulated approximately one-third as much β -carotene and were paler yellow in color compared with cells containing plasmid pAC-BETA-At (Figure 7B). The lower level of β -carotene accumulation suggests that the *LCYB* gene from *szl1* encodes a still functional but less active β -cyclase relative to the wild-type gene.

The addition of the *Arabidopsis LCYE* gene to cells containing pAC-BETA-At resulted in the production of both α - and β -carotene, and the molar ratio of α -carotene to β -carotene was 2:3 (Figure 7C), which mirrors the accumulation of more β -branch carotenoids than α -branch carotenoids found in both the wild type and *npq1 Arabidopsis* (Table 1). When the same *LCYE* gene was introduced into the *E. coli* strain containing pAC-BETA-At-*szl1*, the composition of the carotenoid pigments was altered, and the molar ratio of α -carotene to β -carotene increased to 4:1 (Figure 7D). This change reflects the significant decrease of β -branch carotenoids and increase of α -branch carotenoids observed in both the *szl1* single mutant and *szl1 npq1* double mutant.

Effect of Pigment Alteration on the Composition of PSII and Photosystem I

To investigate how accumulation of lutein in the suppressor *szl1 npq1* suppresses the *npq1* mutation, we first tested whether the

pigment alteration in *szl1 npq1* might indirectly suppress the low NPQ phenotype of *npq1* by affecting the composition of the PSII antenna. Immunoblot analysis showed that the Lhcb4 (CP29) and Lhcb5 (CP26) protein levels were reduced in both *szl1 npq1* and *szl1* compared with wild type and *npq1*, whereas the amounts of other LHC proteins were unchanged (Figure 8A). There were no differences in the levels of the PsbS protein and the PSII reaction center protein, D1 (Figure 8A). Further quantitative analysis showed that Lhcb4 and Lhcb5 protein levels were decreased to 50% of the wild-type level, while Lhcb6, Lhca1, and PsaF protein levels were slightly reduced in both *szl1* and *szl1 npq1* (Figures 8B and 8C). Because the pigment alteration in the suppressor affected only the levels of the Lhcb4 and Lhcb5 proteins, and it was reported that a relatively minor effect on qE was observed in antisense or knockout plants that lack detectable Lhcb4 or Lhcb5 (Andersson et al., 2001; Betterle et al., 2009), we then hypothesized that the accumulation of lutein in *szl1* directly suppresses the *npq1* mutation by replacing the role of zeaxanthin in qE at the molecular level.

TA Spectroscopy of *szl1 npq1* Thylakoids

To determine if the spectroscopic signature of CT quenching, the formation of a carotenoid radical cation, was restored in *szl1*

npq1, we measured ultrafast time-resolved TA spectra and kinetics in isolated thylakoids in the presence or absence of light-induced qE. Figure 9A shows the near infrared (NIR) TA spectra of thylakoid membranes of the *szl1 npq1* double mutant at 15 ps delay between pump and probe laser pulses. The TA signal in the qE state was measured every 20 nm from 880 to 1040 nm under an actinic light ($\sim 600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) (red line). The spectrum without qE (black line) was measured after the sample was darkened for 10 min to relax the light-induced ΔpH . Because chlorophylls and carotenoids have no ground-state absorbance or emission in this NIR region, we selectively measured excited-state absorbance of transient species. The spectrum without qE exhibits a gradual increase with wavelength mainly due to chlorophyll excited-state absorbance (Polivka et al., 2002; Holt et al., 2005). The qE spectrum revealed an enhanced absorbance in the wavelength region from

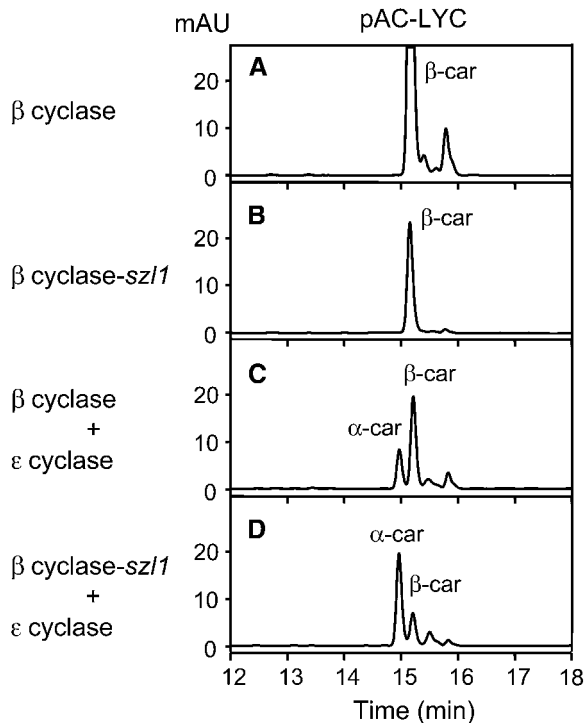


Figure 7. HPLC Analysis of Products Formed from Lycopene in *E. coli* Expressing the *Arabidopsis* Wild-Type ϵ -cyclase and Wild-Type or Mutant β -Cyclase.

Carotenoid pigment composition was examined in cultures of *E. coli* containing the plasmids and genes indicated above and to the left. The *Arabidopsis* wild-type and mutant copy of β -cyclase were cloned directly in the pAC-LYC plasmid to give the plasmid pAC-BETA-At and pAC-BETA-At-*szl1*, respectively (see Methods). Carotenoids were extracted with acetone from equal numbers of cells (based on A_{600}), and pigments were separated by HPLC and detected by absorbance at 445 nm. α -car, α -carotene; β -car, β -carotene.

- (A) pAC-LYC plus the *Arabidopsis* wild-type β -cyclase.
 (B) pAC-LYC plus the *Arabidopsis* mutant β -cyclase.
 (C) pAC-LYC plus the *Arabidopsis* wild-type β -cyclase and ϵ -cyclase.
 (D) pAC-LYC plus the *Arabidopsis* mutant β -cyclase and wild-type ϵ -cyclase.

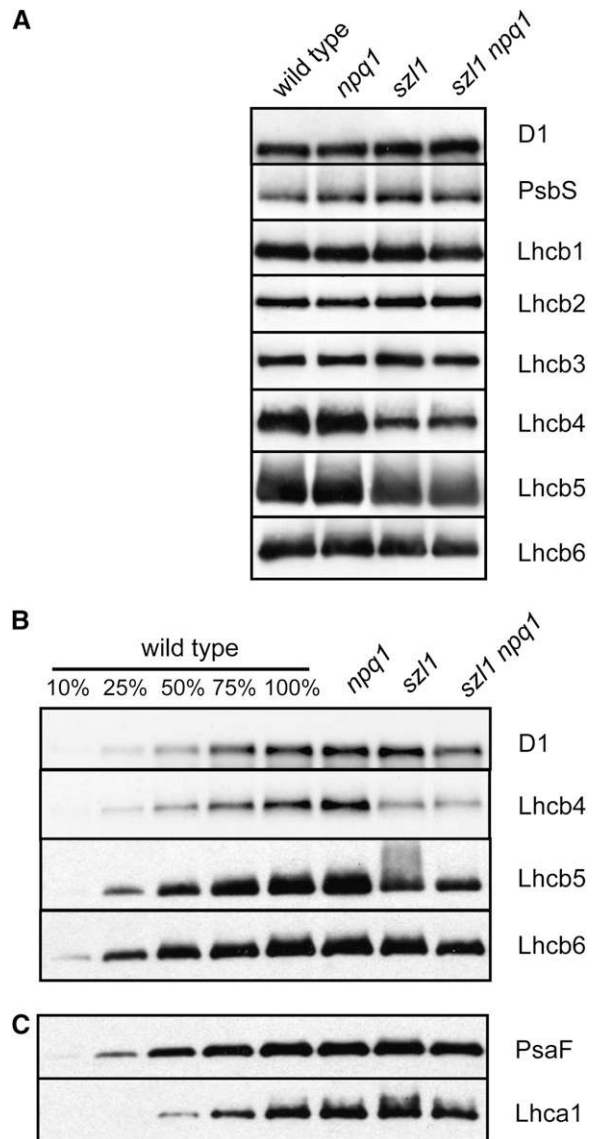


Figure 8. PSII and PSI Protein Levels in LL-Grown Wild Type, *npq1*, *szl1*, and *szl1 npq1*.

Thylakoid protein samples were loaded on the basis of total protein ($5 \mu\text{g lane}^{-1}$), and immunoblot analysis was performed with polyclonal antibodies directed against each of the indicated proteins. D1 is a PSII reaction center protein; PsbS is a PSII protein that is essential for qE; Lhcb1, Lhcb2, and Lhcb3 are components of LHCII trimers; Lhcb4, Lhcb5, and Lhcb6 (also called CP29, CP26, and CP24, respectively) are monomeric, minor antenna proteins of PSII; PsaF is a PSI reaction center protein; Lhca1 is a PSI antenna protein. For comparison to mutant samples, dilutions were made from wild-type samples.

(A) Immunoblot analysis of D1, PsbS, and Lhcb protein levels in the four genotypes.

(B) Quantification of Lhcb4, Lhcb5, and Lhcb6 protein levels in *szl1* and *szl1 npq1*.

(C) Quantification of PsaF and Lhca1 protein levels in *szl1* and *szl1 npq1*.

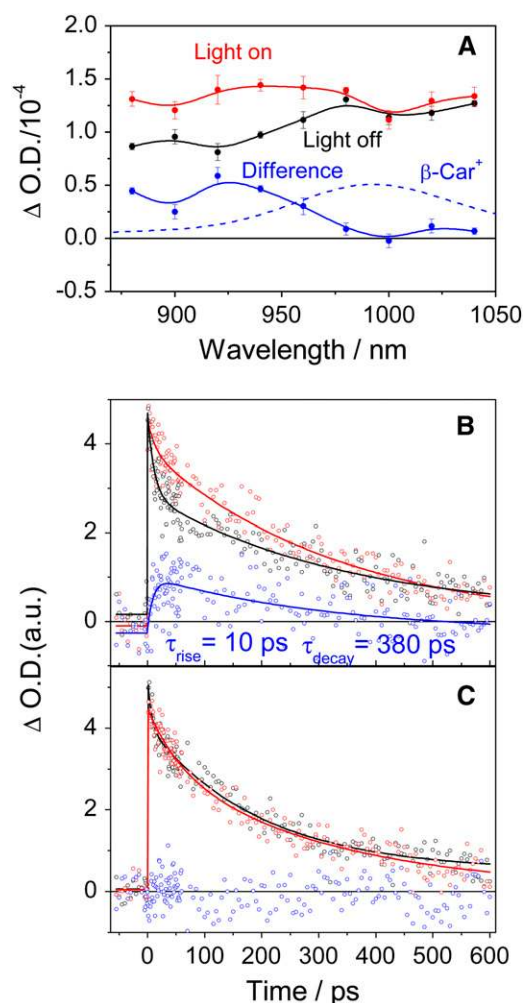


Figure 9. TA Spectroscopy of *szl1 npq1* Thylakoids.

(A) TA spectrum from 880 to 1040 nm of *szl1 npq1* mutant thylakoids with qE (red line) or without qE (black line) at 15 ps delay after pump pulse. The blue line shows the difference kinetics between the red and the black lines, and the blue dotted line is the spectrum of a β -carotene radical cation (β -Car $^+$) for comparison (Tracewell and Brudvig, 2003). Data are presented as the means \pm SE ($n = 5$).

(B) TA kinetics probed at 950 nm.

(C) TA kinetics probed at 1000 nm.

880 to 960 nm. To remove the chlorophyll excited-state absorption and to emphasize the difference, we subtracted the black trace under darkness from the red one under actinic light, resulting in the blue reconstructed spectrum (Figure 9A). The broad reconstructed spectrum provided evidence of CT quenching in *szl1 npq1*, but the spectrum was maximized at \sim 920 nm, which is substantially blue-shifted relative to the spectrum of a β -carotene radical (blue dotted line) or a zeaxanthin radical cation with a broad spectrum centered \sim 980 to 1000 nm (Holt et al., 2005; Amarie et al., 2007). Instead, the spectrum observed in *szl1 npq1* was consistent with the reported absorption spectrum of a lutein radical cation, which was centered at

\sim 920 to 950 nm depending on the solvent used (Mortensen and Skibsted, 1997; Edge et al., 1998; Galinato et al., 2007).

Individual TA kinetic traces of the *szl1 npq1* thylakoids at 950 and 1000 nm are shown in Figures 9B and 9C, respectively. The qE trace under actinic light at 950 nm (red line in Figure 9B) revealed distinctly slower kinetics than the trace without qE (black line in Figure 9B), whereas both the traces at 1000 nm were similar (Figure 9C). Thus, unlike the wild type, which has a difference in kinetics at 1000 nm but not 950 nm (Holt et al., 2005), the different kinetics in the *szl1 npq1* thylakoids showed the characteristic pattern of a carotenoid radical cation at 950 nm (blue line in Figure 9B) but not at 1000 nm (blue line in Figure 9C).

TA Spectroscopy of Isolated LHC Complexes

To determine if the NIR absorption changes detected in *szl1 npq1* thylakoids can be associated with LHC complexes, as in the case of zeaxanthin radical cation formation (Ahn et al., 2008; Avenson et al., 2008), we investigated the effect of substituting β -xanthophylls by lutein, as observed in the *szl1 npq1* mutant, in reconstituted LHC complexes. CP24, CP26, and CP29 complexes were refolded in vitro from apoproteins expressed in *E. coli* and chlorophylls *a* and *b* plus lutein only or a total carotenoid mix. The resulting complexes were characterized (Table 2) by having lutein in both carotenoid binding sites L1 and L2 (LL complexes), whereas in the control complexes, the L2 binding site could be occupied by lutein, violaxanthin, or neoxanthin (LNV complexes) (Pagano et al., 1998; Ruban et al., 1999; Ballottari et al., 2009), except for CP24, which cannot bind neoxanthin either in vivo or in vitro (Pagano et al., 1998). LHCII trimers were purified from the wild type or the *chy1 chy2 lut5* mutant, which has lutein as the only xanthophyll (Fiore et al., 2006). Both LHCII samples have lutein in L1 and L2 xanthophyll binding sites; however, LHCII trimers from *chy1 chy2 lut5* have lutein instead of violaxanthin bound in the external V1 binding sites, while site N1 remained empty (Liu et al., 2004; Mozzo et al., 2008). All these complexes were analyzed by NIR TA spectroscopy. LHCII-LL trimers did not show any TA difference compared with LHCII-LNV complexes; the TA kinetics in the lutein radical cation absorption region were characterized only by excited states absorption decay (see Supplemental Figure 1 online). This is similar to the case of CP26-LL, which has been previously analyzed (Avenson et al., 2009). As shown in Figure 10, CP24 and CP29 kinetics at 920 nm were instead characterized by the presence of an additional rise component only in the CP24-LL and CP29-LL complexes. The difference kinetics reported in Figures 10C and 10D clearly show the formation of a lutein radical cation in CP24 and CP29 binding lutein as the only xanthophyll, with a rise time of 5 ps and main decay of \sim 50 to 60 ps. Lutein radical cation formation in CP24 and CP29 complexes was confirmed by the reconstructed NIR TA spectra reported in Figures 10A and 10B, showing a peak at 920 nm, consistent with result obtained for *szl1 npq1* thylakoids upon qE induction (Figure 9).

Light-Induced Absorbance Changes

A slower light-induced absorbance change, ΔA_{535} , depends on both zeaxanthin and the Δ pH and is closely associated with the

Table 2. Pigment Composition of the CP24 and CP29 Recombinant Proteins

	Chl <i>a</i>	Chl <i>b</i>	Chl <i>a/b</i>	Chl/Car	Car, No.	N	V	L	Z
CP24-LL	6.07 ± 0.05	3.93 ± 0.05	1.54 ± 0.03	4.76 ± 0.09	2.08 ± 0.04	ND	ND	2.08 ± 0.03	ND
CP24-LV	5.99 ± 0.10	1.99 ± 0.01	1.50 ± 0.06	4.74 ± 0.10	2.11 ± 0.04	ND	0.90 ± 0.08	1.21 ± 0.06	ND
CP29-LL	6.01 ± 0.01	4.01 ± 0.10	3.02 ± 0.02	4.20 ± 0.10	1.90 ± 0.04	ND	ND	1.90 ± 0.02	ND
CP29-LNV	5.58 ± 0.06	2.42 ± 0.06	2.31 ± 0.08	3.69 ± 0.17	2.17 ± 0.11	0.37 ± 0.05	0.61 ± 0.04	1.19 ± 0.01	ND

Pigments of the different complexes were normalized to 10 chlorophylls (*a+b*) per CP24 and 8 chlorophylls per CP29 (Bassi and Dainese, 1992). Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; Chl/Car, ratio of total chlorophylls to total carotenoids; Car, number of carotenoids per polypeptide; N, neoxanthin; V, violaxanthin; L, lutein; Z, zeaxanthin; ND, not detectable. Data are presented as the means ± SD (*n* = 3).

induction and relaxation of qE (Ruban et al., 1993; Bilger and Björkman, 1994; Li et al., 2004). Figure 11 shows a typical qE spectrum for wild-type *Arabidopsis*, taken as the difference in absorbance changes between 10 and 60 s after illumination to eliminate contributions from the electrochromic shift (see Bilger and Björkman, 1994). The peak absorbance change occurred at ~530 to 535 nm. By contrast, peaks observed in the *szl1*, *szl1 npq1*, and *npq1* mutants were blue-shifted by ~5 to 10 nm to ~525 to 530 nm (Figure 11). In each strain, the qE absorbance signals decayed in the dark with half times of 5 to 10 s, suggesting that all signals reflected similar processes.

DISCUSSION

Isolation of the suppressor mutant *szl1 npq1*, which contains a point mutation in the *LCYB* gene and thus results in a dramatic change in pigment profile and qE capacity, has provided insights into carotenoid biosynthesis and the role of lutein in qE.

Regulation of the Carotenoid Biosynthetic Pathway at the Branch Point

The plant carotenoid biosynthetic pathway branches at the cyclization reactions to produce carotenoids with either two β-rings or one β- and one ε-ring. In brief, lycopene is either cyclized twice by *LCYB* to produce β-carotene and derivatives thereof or once each by *LCYB* and *LCYE* to produce α-carotene that is the precursor to lutein (Figure 1). It has been hypothesized that partition of flux into the β- and α-branches of the pathway is controlled by the relative activities of *LCYB* and *LCYE* (Cunningham et al., 1996; Pecker et al., 1996; Pogson et al., 1996). Indeed, transgenic manipulations to increase or decrease *LCYE* expression in *Arabidopsis* resulted in lutein levels ranging from 5 to 180% of the wild type (Pogson and Rissler, 2000). Similar results were reported recently with *LCYE* transgenic potato (*Solanum tuberosum*) and *Brassica* plants (Diretto et al., 2006; Yu et al., 2007). It has also been shown that natural genetic variation at the *LCYE* locus in maize (*Zea mays*) changes the ratio of α- versus β-branches of the carotenoid pathway (Harjes et al., 2008).

Here, by affecting *LCYB* instead of *LCYE*, we provide complementary experimental evidence to support the above hypothesis. The suppressor *szl1 npq1* has very low levels of violaxanthin, antheraxanthin, and neoxanthin, but it accumulates nearly twice as much lutein and approximately eight times more α-carotene compared with the wild type (Table 1). Molecular

genetic analysis demonstrated that the *szl1* mutation affects the structural gene encoding *LCYB* by changing a Gly residue in a highly conserved predicted transmembrane helix to a Glu (Figure 6A). It is clear that *szl1* is not a complete loss-of-function mutation affecting *LCYB* activity, as evidenced by the decreased but detectable levels of β-carotene when the *szl1* allele of *LCYB* was expressed in a lycopene-accumulating strain of *E. coli* (Figure 7B). In *E. coli* cells that express both the *szl1* allele of *LCYB* and the wild-type *LCYE*, the molar ratio of α-carotene to β-carotene increased to 4:1 (Figure 7D), which is in agreement with the high levels of α-branch carotenoids and low levels of β-branch carotenoids accumulated in the suppressor *szl1 npq1*.

LCYB Is the Principal Lycopene β-Cyclase in *Arabidopsis*

The enzyme encoded by the *Arabidopsis LCYB* gene belongs to the CrtL protein family, which includes the β- and ε-cyclases in some cyanobacteria and plants (Cunningham et al., 1994, 1996; Stickforth et al., 2003). Many bacteria, including the green sulfur bacterium *Chlorobium tepidum* and cyanobacteria, have an *LCYB* that is different from that of plants. The first member of this new family, CruA, was recently identified from *C. tepidum* in a complementation assay with a lycopene-accumulating strain of *E. coli* (Maresca et al., 2007). Two homologs of CruA, denoted CruA and CruP, were found and characterized in the cyanobacterium *Synechococcus* sp PCC 7002. A genome search revealed that *Arabidopsis* contains a CruP homolog, which is encoded by At2g32640 (Maresca et al., 2007). Whether the CruP homolog also has lycopene β-cyclase activity in *Arabidopsis* is not yet known, but the dramatic change of the pigment profile observed in the suppressor *szl1 npq1* suggests that *LCYB* is the major lycopene β-cyclase in *Arabidopsis*.

Does *LCYB* Have Neoxanthin Synthase Activity in *Arabidopsis*?

Conversion of violaxanthin to neoxanthin is catalyzed by the enzyme neoxanthin synthase (NSY) (Figure 1). Genes encoding NSY activity have not yet been identified conclusively in *Arabidopsis*. Two homologous NSY genes have been cloned from either tomato (*Solanum lycopersicum*) or potato based on their ability to convert all-*trans*-violaxanthin to all-*trans*-neoxanthin in vitro or in transient expression systems (Al-Babili et al., 2000; Bouvier et al., 2000). Polypeptides encoded by NSY genes are homologous to *LCYB*, and the tomato NSY gene product has β-cyclase activity and accounts for the fruit-specific high

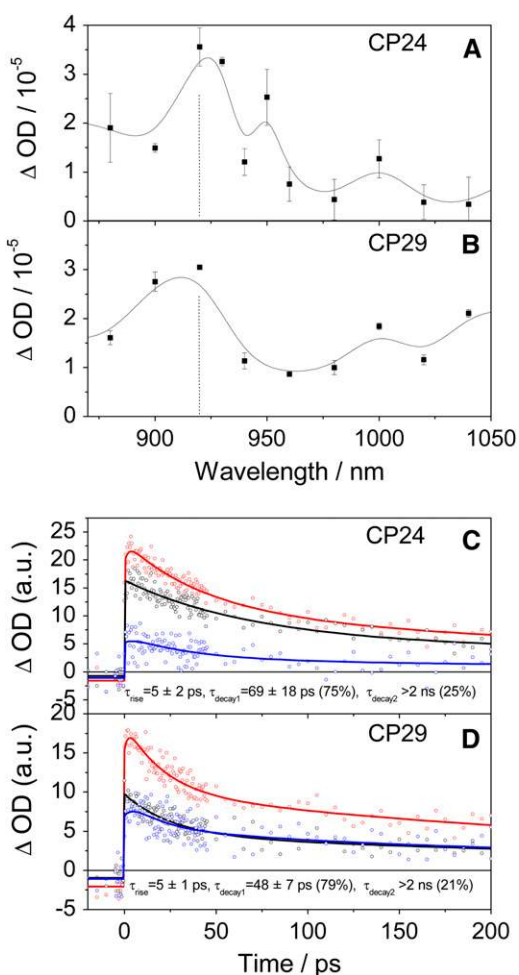


Figure 10. TA Spectroscopy of LHC Complexes.

(A) Difference NIR-TA spectrum (from 880 to 1040 nm) between CP24 complexes binding lutein in both L1 and L2 sites (CP24-LL) and CP24 with violaxanthin in site L2 (CP24-LV). Each point represents the difference between the ΔA value obtained at 20 ps delay after pump pulse in CP24-LL and the corresponding value in CP24-LV. Data are presented as the means \pm SE ($n = 5$).

(B) Difference NIR-TA spectrum (from 880 to 1040 nm) between CP29 complexes binding lutein in both L1 and L2 sites (CP29-LL) and CP29 with violaxanthin or neoxanthin in site L2 (CP29-LNV). Each point represents the difference between the ΔA value obtained at 20 ps delay after pump pulse in CP29-LL and the corresponding value in CP29-LNV. Data are presented as the means \pm SE ($n = 5$).

(C) TA kinetics probed at 920 nm of CP24-LL (red trace) and CP24-LV (black trace). Difference kinetic trace is reported in blue with rise and decay times indicated.

(D) TA kinetics probed at 920 nm of CP29-LL (red trace) and CP29-LNV (black trace). Difference kinetic trace is reported in blue with rise and decay times indicated.

β -carotene content of the tomato *B* mutant (Ronen et al., 2000). Based on these findings, it has been proposed that NSY has a dual function in converting both violaxanthin to neoxanthin and lycopene to β -carotene (Hirschberg, 2001). Because *Arabidopsis* LCYB is encoded by a single-copy gene and shows signif-

icant identity to tomato and potato NSYs, it is possible that *Arabidopsis* LCYB is also a bifunctional enzyme that has both LCYB and NSY activity. Isolation of the *szl1 npq1* and *szl1* mutants provides an opportunity to test this hypothesis. If LCYB does function as NSY, the *szl1* mutation that impairs LCYB activity might also decrease NSY activity; thus, we would expect a preferential reduction of neoxanthin content compared with that of violaxanthin in the *szl1 npq1* or *szl1* mutant. On the other hand, if LCYB does not have NSY activity, then we would expect to observe a proportional reduction of neoxanthin and violaxanthin in the *szl1 npq1* or *szl1* mutant. Pigment analysis of LL-grown plants showed that the molar ratio of neoxanthin to violaxanthin in *szl1 npq1* and *szl1* was the same as that of *npq1* (Table 1), indicating that LCYB does not have NSY activity. It is more likely that in *Arabidopsis* a novel type of NSY is responsible for the conversion of violaxanthin to neoxanthin, and the recently identified ABA4 protein would be a very good candidate (North et al., 2007).

A Direct Role of Lutein in qE

How does the increased lutein content caused by the *szl1* mutation restore qE in the *npq1* mutant that lacks zeaxanthin? Based on the very similar chemical structures of lutein and zeaxanthin, an analogy has previously been drawn with zeaxanthin when speculating about the functions of lutein in qE, and a possible role of lutein in qE has been proposed. Indeed, an effect of lutein on qE has been demonstrated in several mutants and

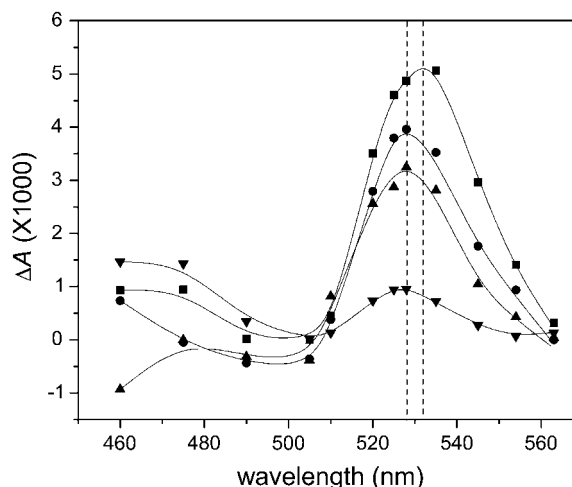


Figure 11. Light-Induced Spectral Absorbance Changes in Leaves.

Intact leaves of wild type (squares), *szl1* (circles), *szl1 npq1* (triangles), and *npq1* (inverted triangles) were illuminated with $\sim 1150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ red light for 10 min to induce qE, and absorbance changes (from 460 to 563 nm) were measured during a 1-min dark interval. The qE spectrum was calculated as the difference in absorbance between 10 and 60 s after illumination to eliminate contributions from the electrochromic shift. The dashed lines indicate the peak positions of ~ 530 to 535 nm in the wild type and ~ 525 to 530 nm in the *szl1*, *szl1 npq1*, and *npq1* mutants.

transgenic plants with altered lutein levels (Niyogi et al., 1997, 2001; Pogson et al., 1998; Pogson and Rissler, 2000; Lokstein et al., 2002), but whether and how lutein plays a direct or an indirect role in qE has been unclear.

Previously characterized mutants that accumulate extra lutein have only been obtained together with either the total depletion of β -xanthophylls (Dall'Osto et al., 2007), which strongly affects antenna protein composition, or the normal accumulation of zeaxanthin (Pogson and Rissler, 2000), thus obscuring the specific lutein-associated qE phenotype. The *szl1 npq1* mutant accumulates nearly double the wild-type amount of lutein, has lower total carotenes (increased α -carotene but lower β -carotene), retains low levels of violaxanthin, antheraxanthin, and neoxanthin, and lacks zeaxanthin (Figure 4), but the total carotenoid (and chlorophyll) content does not change (Table 1). Thus, the increase in α -carotenoids occurs at the expense of β -carotenoids, and the extra lutein and α -carotene are likely bound at sites that are normally occupied by β -carotenoids.

In principle, the enhancement of qE in the absence of zeaxanthin could result from an indirect effect of the substitution of lutein for β -xanthophylls on the composition and/or structure of the PSII antenna (Lokstein et al., 2002) or by a direct role of lutein in qE (either by CT quenching or an S1 energy transfer mechanism). We did detect a 50% decrease in the levels of Lhcb4 and Lhcb5 in *szl1 npq1* (Figure 8), but it seems unlikely that this could explain the increased level of qE because only a limited decrease of the qE amplitude was observed when a complete absence of Lhcb4 or Lhcb5 was induced in a wild-type background by antisense or knockout (Andersson et al., 2001; Betterle et al., 2009). Therefore, we considered the possibility of a direct role of lutein in enhancing a qE mechanism. At present, it is not possible to test directly for the occurrence of the S1 quenching mechanism in isolated thylakoids (Ruban et al., 2007), but we were able to investigate formation of a carotenoid radical cation, a key molecular species in the CT quenching mechanism (Holt et al., 2005) by TA spectroscopy.

We hypothesized that lutein might be able to take the place of zeaxanthin in directly quenching $^1\text{Chl}^*$ during qE through the CT mechanism via the formation of a lutein radical cation instead of a zeaxanthin radical cation, thus restoring qE to a higher level in the *szl1 npq1* suppressor. Indeed, NIR TA traces revealed a carotenoid radical cation in *szl1 npq1* by probing at 950 nm (Figure 9B) but not at 1000 nm (Figure 9C), in contrast with wild-type thylakoids (Holt et al., 2005). The TA spectrum of the suppressor thylakoids showed a maximum at \sim 920 nm (Figure 9A), which is blue-shifted relative to the spectrum of a β -carotene or zeaxanthin radical cation and is in agreement with the reported spectrum of a lutein radical cation (Mortensen and Skibsted, 1997; Edge et al., 1998; Galinato et al., 2007). Similarly, the ΔA_{535} leaf absorbance change associated with zeaxanthin-dependent qE was missing in *npq1* (Niyogi et al., 1998) and replaced with a blue-shifted absorbance change in *szl1 npq1* (Figure 11A). Thus, the results of the TA spectroscopy (Figure 9) and leaf absorbance (Figure 11) experiments strongly suggest that lutein can substitute for zeaxanthin in qE.

We suspect that the high amount of lutein accumulated in the suppressor probably magnifies a TA signal that is normally present at a relatively low level and is obscured by the stronger

zeaxanthin radical cation signal in the wild type. Similarly, we propose that the residual qE in the *npq1* mutant is lutein dependent, but the lutein radical cation signal is likely below the detection limit of the TA experiment. We are currently trying to improve the sensitivity and signal-to-noise ratio of our TA setup to test this idea. A basal level of CT quenching by lutein (that could be enhanced by PsbS overexpression) could explain previous observations of zeaxanthin-independent qE, which indicated that zeaxanthin (and antheraxanthin) is not required for qE (Crouchman et al., 2006). Indeed, a blue-shifted absorbance change very similar to that observed here (Figure 11A) was detected in *npq1* leaves (Johnson et al., 2009) and in wild-type leaves treated with DTT to phenocopy the *npq1* mutation by inhibition of VDE activity (Crouchman et al., 2006).

The spectroscopic signature of CT quenching has been detected recently in recombinant CP29 (Lhcb4), CP26 (Lhcb5), and CP24 (Lhcb6) (Ahn et al., 2008; Avenson et al., 2008, 2009). Each of these pigment-protein complexes can exhibit zeaxanthin radical cation formation through CT from zeaxanthin to a strongly coupled chlorophyll dimer. A lutein radical cation was so far detected only in CP26, but only when zeaxanthin is also present (Avenson et al., 2009). Because zeaxanthin is lacking in *szl1 npq1*, it is unlikely that lutein radical cation formation of the type observed in CP26 in vitro (Avenson et al., 2009) could explain the TA signal detected in *szl1 npq1* thylakoids (Figure 9). Instead, we detected the formation of lutein radical cations in CP24 and CP29 complexes reconstituted with only lutein (Figure 10). Our results in vitro and in vivo thus highlight the possibility of lutein to substitute for zeaxanthin as a quencher of excess excitation energy through formation of radical cations. The fraction of lutein-only CP24 and CP29 complexes undergoing CT quenching in vitro, however, is rather low (\sim 1%), as in the case of zeaxanthin binding complexes (Avenson et al., 2008), indicating the fundamental role of other factors, such as PsbS and ΔpH , for the induction of qE in vivo. In wild-type plants, CP24, CP26, and CP29 bind lutein and violaxanthin/zeaxanthin in carotenoid binding sites named L1 and L2, respectively. An increase in the lutein content of these complexes, substituting for violaxanthin/zeaxanthin in the L2 site as in the case of the *szl1 npq1* mutant, was previously reported to induce a decrease of fluorescence quantum yield (Formaggio et al., 2001). Although we favor the hypothesis that the extra lutein bound in the L2 site of LHC complexes in the *szl1 npq1* mutant is directly involved in CT quenching, we cannot at this point exclude the possibility that it acts as an allosteric factor, similar to zeaxanthin in CP26 (Avenson et al., 2009), inducing lutein radical cation formation in site L1. It is worth noting that carotenoid radical cation reactions have distinct patterns and dependence on xanthophyll composition in each Lhcb protein, thus contributing to explain the existence of multiple, conserved gene products in the plant antenna systems.

METHODS

Plant Material and Growth Conditions

All *Arabidopsis thaliana* plants were of the ecotype Columbia-0. The *npq1* mutant is affected in VDE and lacks zeaxanthin under high light (Niyogi

et al., 1998). The *szl1 npq1* double mutant was crossed with the wild type, and the *szl1* single mutant was isolated from F2 progeny. Plants were grown on Sunshine Mix 4 potting mix (Sun Gro Horticulture Distribution) in controlled conditions of 10 h light, 22°C/14 h dark, 23°C, with a light intensity of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For physiological studies, plants at the age of 5 to 6 weeks (prior to bolting) were used. For mutant screening and cosegregation analysis, plants were grown for 2 weeks on minimal plant nutrient agar medium (Haughn and Somerville, 1986) at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (continuous light) at 23°C and then transferred to soil.

Isolation of Suppressors of the *npq1* Mutant

M2 seedlings were derived from mutagenesis of *npq1* seeds with 0.3% (v/v) ethyl methanesulfonate. Suppressors of *npq1* were identified by chlorophyll fluorescence video imaging (Niyogi et al., 1998) for the NPQ phenotype and by HPLC for pigment composition after exposure to high light.

Chlorophyll Fluorescence Measurement

Chlorophyll fluorescence was measured at room temperature from attached, fully expanded rosette leaves using an FMS2 fluorometer (Hansatech). After an overnight dark period, leaves were exposed to actinic light (1250 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 5 min followed by 5 min of darkness. The maximum fluorescence levels after dark adaptation (F_m) and in the light-adapted condition (F_m') were recorded after applying a saturating pulse of light. NPQ was calculated as $(F_m - F_m')/F_m'$.

Pigment Analysis

HPLC analysis of carotenoids and chlorophylls was done as previously described (Müller-Moulé et al., 2002). A total of nine samples (three independently grown sets of plants with three samples each) were measured. Carotenoids were quantified using standard curves of purified pigments (VKI) and normalized to chlorophyll *a*.

Genetic Mapping and Cosegregation Analysis

The *szl1* mutation was mapped by candidate gene approach. The *LCYB* gene was amplified by PCR from genomic DNA of the wild type, *npq1*, and *szl1 npq1* mutants using primers LBC1 (5'-CCATTTCTCAATCCCTCTGGT-3') and LBC2 (5'-AGTATATCCGCATTGCAAGTC-3'), and 1.7-kb PCR products were used for DNA sequencing.

Cosegregation of the *szl1* mutation with the mutant (high NPQ) phenotype was determined by backcrossing the homozygous *szl1 npq1* double mutant to the *npq1* mutant. For cosegregation analysis, a 1.5-kb PCR product was amplified from the genomic DNA of the segregating F2 plants using the forward primer ZL49 (5'-GCAGGTTTCTGAAGCTGGAC-3') and the reverse primer ZL50 (5'-TGAACCTTGATGTTGGCTTGC-3'). The PCR products were digested overnight with *EcoRI* whose site is present only in the sequence from the *szl1* mutant, giving two smaller fragments.

Plasmid Construction

Plasmids pAC-LYC, y2, and y8 were kindly provided by Francis X. Cunningham (University of Maryland). Cells of *Escherichia coli* containing plasmid pAC-LYC accumulate lycopene and form pink colonies (Cunningham et al., 1994). Plasmids y2 and y8 are *Arabidopsis* cDNA library plasmids that contain functional genes for *Arabidopsis* *LCYE* and *LCYB*, respectively (Cunningham et al., 1996).

Plasmid *y8-szl1* was constructed by introducing a point mutation G1352A into *LCYB* of y8 plasmid according to the QuickChange site-directed mutagenesis kit guidelines (Stratagene). The sequence of the forward primer was (mutagenic positions underlined) ZL61, 5'-ATCTG-

CAACCTCATTACTGGCACGAATTCTGTCTCCAGGCTGTTTCTC-3'; the reverse primer ZL62 was complementary to the forward primer.

Plasmid pAC-BETA-At and plasmid pAC-BETA-At-*szl1* were constructed by cloning a 1.8-kb *XbaI* fragment (partially filled recessed termini with T and C) from plasmids y8 and y8-*szl1*, containing the *Arabidopsis* wild-type and mutant (*szl1*) copy of *LCYB*, respectively, in the *HindIII* site (partially filled recessed termini with A and G) of pAC-LYC.

SDS-PAGE and Immunoblot Analysis

To prepare thylakoid membranes, rosette leaves were harvested, frozen immediately in liquid nitrogen, and stored at -80°C . Thylakoid membranes were prepared and analyzed by SDS-PAGE and immunoblotting as previously described (Li et al., 2002). Thylakoid protein samples containing equal amounts of total protein (5 μg) were loaded in each lane. The D1 antibody was kindly provided by Anastasios Melis (University of California, Berkeley, CA), the Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5, Lhcb6, and Lhca1 antibodies were kindly provided by Stefan Jansson (Umeå University), and the PsaF antibody was kindly provided by Anna Haldrup (The Royal Veterinary and Agricultural University, Copenhagen, Denmark). Proteins were blotted onto Protran nitrocellulose membranes (BA83; Whatman) and probed with antibodies. After incubation with peroxidase-conjugated secondary antibodies, the chemiluminescence signal (ECL; Amersham Pharmacia) was detected with Kodak BioMax Light film with exposure times in the linear range.

NIR TA Spectroscopy

Thylakoids for the NIR TA measurements were prepared as previously described (Gilmore et al., 1998). The NIR TA laser system has been previously described (Holt et al., 2005; Avenson et al., 2008). Briefly, the repetition rate was 250 kHz, and the pump pulses were tuned to $\sim 650 \text{ nm}$ (i.e., the chlorophyll *b* Qy transition). The maximum pump energy and full width half maximum of the pulse autocorrelation trace were 48 nJ/pulse and $\sim 40 \text{ fs}$, respectively. We chose 650 nm as our excitation wavelength because the output power of our optical parametric amplifier was higher than that at 680 nm, yielding higher signal-to-noise ratios. White light continuum probe pulses were generated in a 1-mm quartz plate. The cross-correlation function of the pump and probe overlap was $\sim 85 \text{ fs}$. The diameters of the pump and probe beams at the sample holder were estimated to be 146 and 83 μm , respectively. The mutual polarizations of the pump and probe beams were set to the magic angle (54.7°). The time resolution of the TA measurements was 5 ps/point (-60 to -10 ps), 0.5 ps/point (-10 to 60 ps), and 5 ps/point (65 to 600 ps). A monochromator (Spectra Pro 300i; Acton Research) with a spectral resolution of 2.7 nm, a InGaAs photodiode (DET410; Thorlabs), and a chopper-associated lock-in amplifier (SR830; Stanford Research Systems) were used to monitor transmission. The path length of the cuvette used for isolated thylakoids was 2 mm and was continuously translated during experiments to avoid sample degradation. NIR TA kinetic profiles were obtained during steady state actinic illumination and 10 min following a light-to-dark transition by photoexcitation of the thylakoids at 650 nm and probing at 880 to 1040 nm. We obtained transient traces (-10 to 20 ps) at every 20 nm (880 to 1040 nm), averaged five times, and plotted the TA spectrum at 15 ps with standard error.

Leaf Absorbance Measurements

Absorbance spectra were measured using the diffused optics flash spectrophotometer (Kramer and Sacksteder, 1998) and the appropriate narrow band-pass interference filters (Omega Optics). Detached leaves were gently clamped into the spectrophotometer and illuminated with $\sim 1150 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ red light, supplied by a high-flux, red light emitting diode (Luxeon LXHL-PD09; Phillips Lumileds) for 10 min, and leaf

absorbance changes (from 460 to 563 nm) were measured during a 1-min dark interval. Within this interval, spectral contributions from the xanthophyll cycle are negligible, since the conversion of zeaxanthin to violaxanthin is relatively slow. Typically, spectral changes attributable to the electrochromic shift (which peak at 518 to 520 nm) are most prominent during the first second of the dark interval, whereas beyond this window qE spectral contributions (which peak at ~535 nm) dominate. The qE absorbance spectra were partially corrected for the contributions of the residual fast electrochromic shift by subtraction of the 10-ms spectrum, which contains only electrochromic shift changes.

In parallel experiments, chlorophyll fluorescence was measured in a modified laboratory-built nonfocusing optics spectrophotometer (NoFOSpec) (Sacksteder et al., 2001), which used laboratory-built, small aperture compound parabolic concentrators (CPCs; both solid, clear-cast acrylic and hollow aluminum) designed specifically for work with intact *Arabidopsis* plants. Leaves from intact plants were gently clamped between the CPCs with the upper surface of the leaf against the solid acrylic CPC and the lower surface against the hollow CPC, through which water saturated air was pumped. The modified design uses two separate detectors to collect fluorescence and absorbance data. Surface fluorescence from the leaf returns through the acrylic CPC and is reflected, using a hot mirror (NT43-453; Edmund Optics), through a far-red cut-off filter (RG-710; Schott) onto the fluorescence detector. High-flux, red LEDs were used with collimating optics (Polymer Optics) for actinic and saturating light sources, and measuring pulses were supplied by a green LED (NSPG500S; Nichia). Maximum fluorescence values obtained from saturating flashes (1 s, >16,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ red light) were measured at steady state (F_m') (with actinic illumination of ~1150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ red light for 10 min) and during a 10-min dark recovery (F_m''). qE was calculated as $(F_m'' - F_m')/F_m''$.

Isolation and Reconstitution of LHC Complexes

Native LHCII trimers were isolated from the wild type and *chy1 chy2 lut5* mutant (Fiore et al., 2006) as previously described (Caffari et al., 2001). Recombinant apoproteins CP24 and CP29 were refolded in vitro in the presence of chlorophylls *a* and *b* and total carotenoids, or lutein as the only xanthophyll, as described previously (Pagano et al., 1998; Gastaldelli et al., 2003).

Accession Number

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number U50739 (LCYB).

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Transient Absorption Spectroscopy of LHCII Trimers.

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