Zeaxanthin Has Enhanced Antioxidant Capacity with Respect to All Other Xanthophylls in Arabidopsis Leaves and Functions Independent of Binding to PSII Antennae[1][C][W]

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The *ch1* mutant of Arabidopsis (*Arabidopsis thaliana*) lacks chlorophyll (Chl) *b*. Leaves of this mutant are devoid of photosystem II (PSII) Chl-protein antenna complexes and have a very low capacity of nonphotochemical quenching (NPQ) of Chl fluorescence. Lhcb5 was the only PSII antenna protein that accumulated to a significant level in *ch1* mutant leaves, but the apoprotein did not assemble in vivo with Chls to form a functional antenna. The abundance of Lhca proteins was also reduced to approximately 20% of the wild-type level. *ch1* was crossed with various xanthophyll mutants to analyze the antioxidant activity of carotenoids unbound to PSII antenna. Suppression of zeaxanthin by crossing *ch1* with *npq1* resulted in oxidative stress in high light, while removing other xanthophylls or the PSII protein PsbS had no such effect. The tocopherol-deficient *ch1 vte1* double mutant was as sensitive to high light as *ch1 npq1*, and the triple mutant *ch1 npq1 vte1* exhibited an extreme sensitivity to photooxidative stress, indicating that zeaxanthin and tocopherols have cumulative effects. Conversely, constitutive accumulation of zeaxanthin in the *ch1 npq2* double mutant led to an increased phototolerance relative to *ch1*. Comparison of *ch1 npq2* with another zeaxanthin-accumulating mutant (*ch1 lut2*) that lacks lutein suggests that protection of polyunsaturated lipids by zeaxanthin is enhanced when lutein is also present. During photooxidative stress, α-tocopherol noticeably decreased in *ch1 npq1* and increased in *ch1 npq2* relative to *ch1*, suggesting protection of vitamin E by high zeaxanthin levels. Our results indicate that the antioxidant activity of zeaxanthin, distinct from NPQ, can occur in the absence of PSII light-harvesting complexes. The capacity of zeaxanthin to protect thylakoid membrane lipids is comparable to that of vitamin E but noticeably higher than that of all other xanthophylls of Arabidopsis leaves.

Violaxanthin, a xanthophyll carotenoid present in the photosynthetic apparatus of plants, is rapidly and reversibly de-epoxidized into zeaxanthin via the intermediate antheraxanthin under high-light stress (Demmig-Adams and Adams, 1996; Müller et al., 2001; Horton et al., 2005). This chemical transformation of violaxanthin, called the xanthophyll cycle, is required for the conversion of PSII from a state of efficient light harvesting to a state of high thermal energy dissipation, which is usually measured as a nonphotochemical quenching (NPQ) of chlorophyll (Chl) fluorescence. NPQ protects PSII from photoinhibition, at least under short-term light stress (Niyogi et al., 1998), and increases plant fitness in the field and in fluctuating light (Kulheim et al., 2002). Zeaxanthin synthesis in high light was also found to prevent photooxidative stress and lipid peroxidation (Sarry et al., 1994; Havaux et al., 2000) by a mechanism that seems to be different from NPQ (Havaux and Niyogi, 1999). In a number of cases, accumulation of zeaxanthin was shown to increase tolerance to photooxidative stress while NPQ was not substantially modified (Davison et al., 2002; Havaux et al., 2004; Johnson et al., 2007). Conversely, suppression of zeaxanthin in Arabidopsis (*Arabidopsis thaliana*) mutants affected in other antioxidants, such as lutein, ascorbate, or vitamin E, led to an enhanced susceptibility to oxidative stress that could not be fully explained by a change in NPQ (Niyogi et al., 2001; Baroli et al., 2003; Müller-Moulé et al., 2003; Havaux et al., 2005). Consequently, zeaxanthin was hypothesized to protect plastid lipids and stabilize thylakoid membranes by a more direct mechanism than NPQ. One possibility could be that the light-harvesting complex II (LHCII)/lipid interface is the active site for the antioxidant activity of zeaxanthin, which mediates stress tolerance by protection of LHCII-bound lipids

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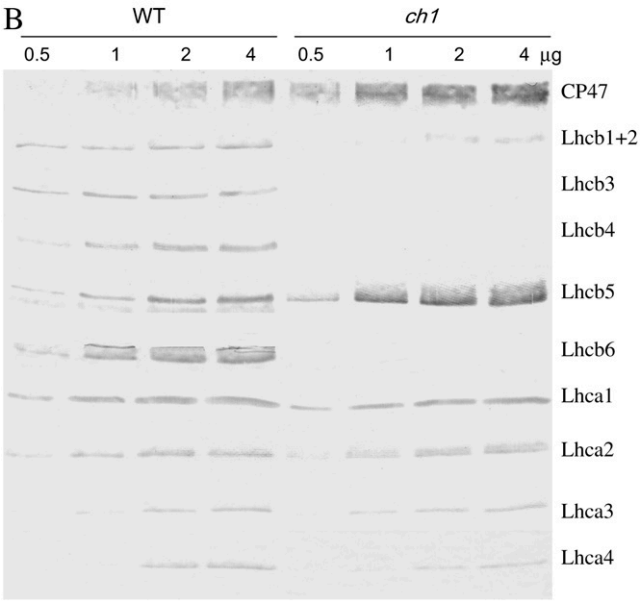

C

	WT	*ch1*
Lhcb1+2	100.0 ± 31.6	< 2.0 ± 0.2
Lhcb3	100.0 ± 14.3	0
Lhcb4	100.0 ± 18.9	0
Lhcb5	100.0 ± 11.4	111.6 ± 12.3
Lhcb6	100.0 ± 16.6	0
Lhca1	100.0 ± 13.6	15.6 ± 1.6
Lhca2	100.0 ± 23.1	24.5 ± 5.8
Lhca3	100.0 ± 6.0	24.6 ± 9.8
Lhca4	100.0 ± 4.2	13.6 ± 2.6

Figure 1. A, Control wild-type and *ch1* Arabidopsis plants showing the pale-green phenotype of the mutant. B, Western blots of Lhcb and Lhca proteins in thylakoids of wild-type and *ch1* Arabidopsis. The internal PSII antenna CP47 was also analyzed for normalization purposes. Amounts of Chl loaded on the gel: 0.5, 1, 2, and 4 μg. The blots were repeated three times with similar results. C, Abundance of Lhca and Lhcb proteins normalized to CP47 in wild-type and *ch1* thylakoids. Data are mean values of three separate measurements ± SD. [See online article for color version of this figure.]

from oxidative damage caused by reactive oxygen species produced in the PSII antenna (Johnson et al., 2007).

In this study, we have examined the antioxidative activity of zeaxanthin and other xanthophylls in a Chl *b*-less Arabidopsis mutant (*ch1*). Because *ch1* is also deficient in LHCII (Espineda et al., 1999), a large fraction of the carotenoids is free in the thylakoid membranes. Therefore, this mutant provides an interesting tool for determining the intrinsic antioxidant property of xanthophylls, which does not depend on their binding to LHCII. In a recent study (Johnson et al., 2007), it was hypothesized that, compared to violaxanthin, the photoprotective function of zeaxanthin is enhanced upon binding to LHCII, like the apoprotein of an enzyme that enhances the reactivity of a bound cofactor. This hypothesis can be tested with the LHCII-deficient *ch1* mutant. Moreover, *ch1* is significantly more sensitive to high light intensities than the wild type (Havaux et al., 2004), so that removal of an antioxidant compound or mechanism is expected to impact more drastically on the chloroplast photostability than in the wild type. The *ch1* mutant was crossed with various xanthophyll mutants to generate double mutants with different xanthophyll compositions. We show here that mutational suppression of zeaxanthin in *ch1* noticeably increased photooxidative stress, while suppression of other xanthophylls or of PsbS-dependent NPQ had no such effect. We conclude that the lipid-protective activity of zeaxanthin is higher than that of the other xanthophylls present in Arabidopsis leaves and this enhanced antioxidant function occurs even in the absence of LHCII.

RESULTS

The Chl Antenna System of the *ch1* Arabidopsis Mutant

Lack of Chl *b* in the Arabidopsis *ch1* mutant leads to a pale-green phenotype, as illustrated in Figure 1A. This was associated with a strong reduction of the light-harvesting Chl *a*/*b*-protein complexes (LHC): western-blot analysis of Lhcb and Lhca polypeptides (Fig. 1B) revealed that all Lhcb proteins, except Lhcb5 (also called CP26), were drastically reduced in the *ch1* mutant relative to the wild type. Lhcb3, 4, and 6 were not detected, while only traces of Lhcb1 and 2 were found in *ch1*. In contrast, Lhcb5 proteins were present in substantial amounts and, when normalized to the CP47 level, their abundance was close to the wild-type level (Fig. 1C). This is surprising when we consider that assembly of Lhcb5 recombinant protein with pigments does not occur in vitro when Chl *b* is absent (Croce et al., 2002). The abundance of Lhca proteins was also reduced in *ch1*, reaching approximately 15% to 25% of the wild-type level. We also examined the abundance of the early light-induced proteins (ELIPs) by western blotting (Supplemental Fig. S1), and we did not find any accumulation of ELIP1 or ELIP2 in *ch1* relative to the wild type, excluding substitution of LHCIIs by these proteins for pigment binding.

Thylakoid proteins were also separated by Tris-sulfate SDS-PAGE and Tris-tricine SDS-PAGE (Supplemental Fig. S2). The former buffer system allows the LHCIs to be well separated from the LHCII antenna proteins, while the latter buffer improves separation of the Lhcb proteins. The results were consistent with the western blots; the electrophoretic patterns of *ch1* thylakoid were dominated by reaction center proteins, while the PSII antenna system was strongly reduced. Particularly, in the Tris-tricine gel, Lhcb4 (=CP29) was found to be

virtually absent, while CP26 was present at wild-type level; Lhcb6 (=CP24) comigrated with Lhca proteins, precluding quantification.

In Figure 2A, the pigmented thylakoid complexes were separated by nondenaturing Deriphat-PAGE. In wild-type thylakoids, the major Chl-binding complexes (B8B, B5, B4, and B2 bands) are LHC complexes or supercomplexes (PSI-LHCI supercomplex, CP29 + CP26 + LHCII, LHCII trimer, and Lhcb monomeric, respectively; Havaux et al., 2004). The B8A band below the B8B band is PSI with reduced LHCI complement (Morosinotto et al., 2005). The B7 and B6 bands represent the PSI and PSII cores, respectively. B3 is the internal PSII antenna CP43. The free pigment band (B1) was small and contained mainly carotenoids, which represented about 4% of the total carotenoid load on the gel (data not shown). The electrophoretic profile of *ch1* thylakoid membranes was very different (Fig. 2A), and the identity of the green bands of *ch1* thylakoids was determined by a detailed analysis of the eluted bands using Tris-sulfate SDS-PAGE (Fig. 2B), spectrophotometry (Fig. 3D; Supplemental Fig. S3), circular dichroism (CD; Fig. 3, A–C), and pigment quantification by HPLC (data not shown). The protein bands in the Tris-sulfate SDS-PAGE of eluted green bands shown in Figure 2B were identified in previous works on wild-type and Chl *b*-less thylakoids (Bassi, 1985; Bassi et al., 1985, 1995). On the basis of their polypeptide composition (Fig. 2B), B3′ and B4′ were identified as the PSII internal antennae CP43 and CP47. B5′ and B6′ corresponded to the D1 + D2 + CP43 supercomplex in the monomeric and dimeric form, respectively. B7′ was the PSII reaction center, and the B8′ and B9′ bands were the PSI core (in the monomeric and the oligomeric state, respectively). These attributions were confirmed by the absorption spectra of the eluted bands (Supplemental Fig. S3). The *ch1* band B2′ corresponding to monomeric LHCIIs in the wild type (band B2) contained proteins that migrated like LHCII or LHCI proteins (Fig. 2B). Because LHCIIs (except Lhcb5) were virtually absent from *ch1* thylakoids and LHCIs were not found in appreciable amounts in the other green bands of the Deriphat gel of *ch1* (and particularly in the PSI bands, B8′ and B9′), it is likely that monomeric Lhca are present in this band, as previously concluded by Espineda et al. (1999). This interpretation is also supported by the presence of a far-red tail, typical of LHCI antennae, in the absorption spectrum of the *ch1* B2′ band (Fig. 3D). Moreover, the CD spectrum of the *ch1* B2′ band (Fig. 3, A–C) was very different from the CD spectrum of the wild-type B2 band and also from the CD spectrum of Lhcb5 reconstituted with the maximum possible Chl *a* (Chl *a*/*b* = 8; see Croce et al., 2002; Fig. 3C). On the contrary, the spectrum resembled the CD spectrum of Lhca1 and Lhca3 containing only Chl *a* (Fig. 3, A and B). This suggests that B2′ contained Lhca antennae only. As a

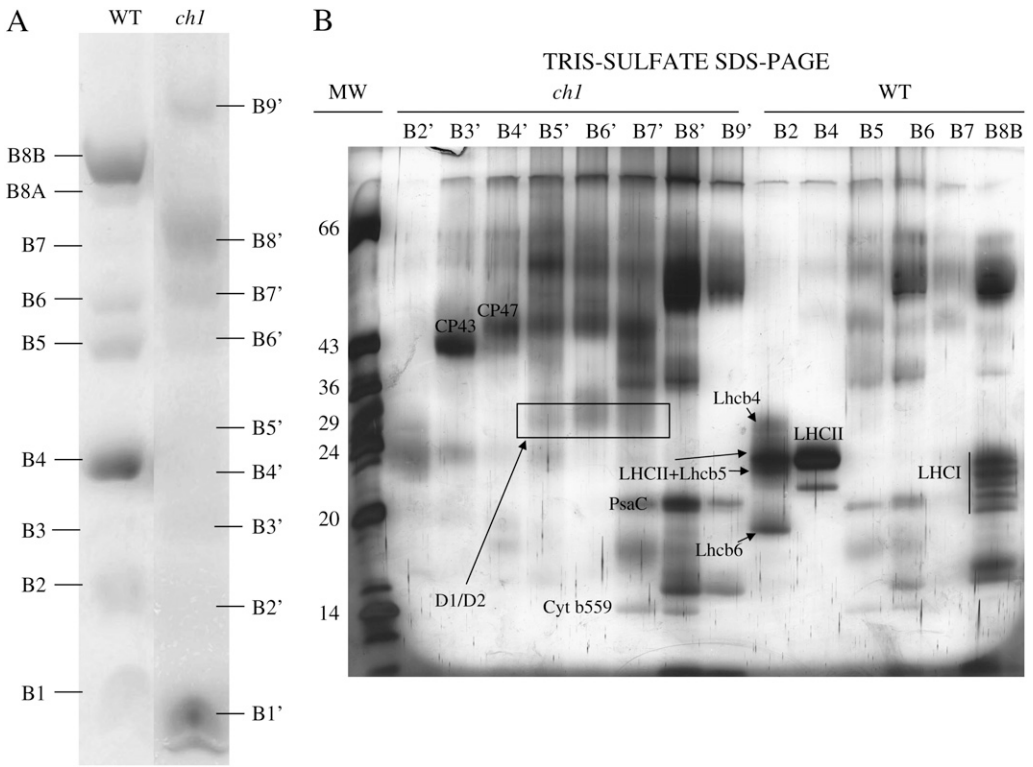

Figure 2. A, Nondenaturing Deriphat-PAGE separation of pigmented thylakoid complexes of wild-type and *ch1* Arabidopsis. Twenty-five micrograms of Chl/lane were loaded on the gel. B, Tris-sulfate SDS-PAGE of the various green bands eluted from the Deriphat gels.

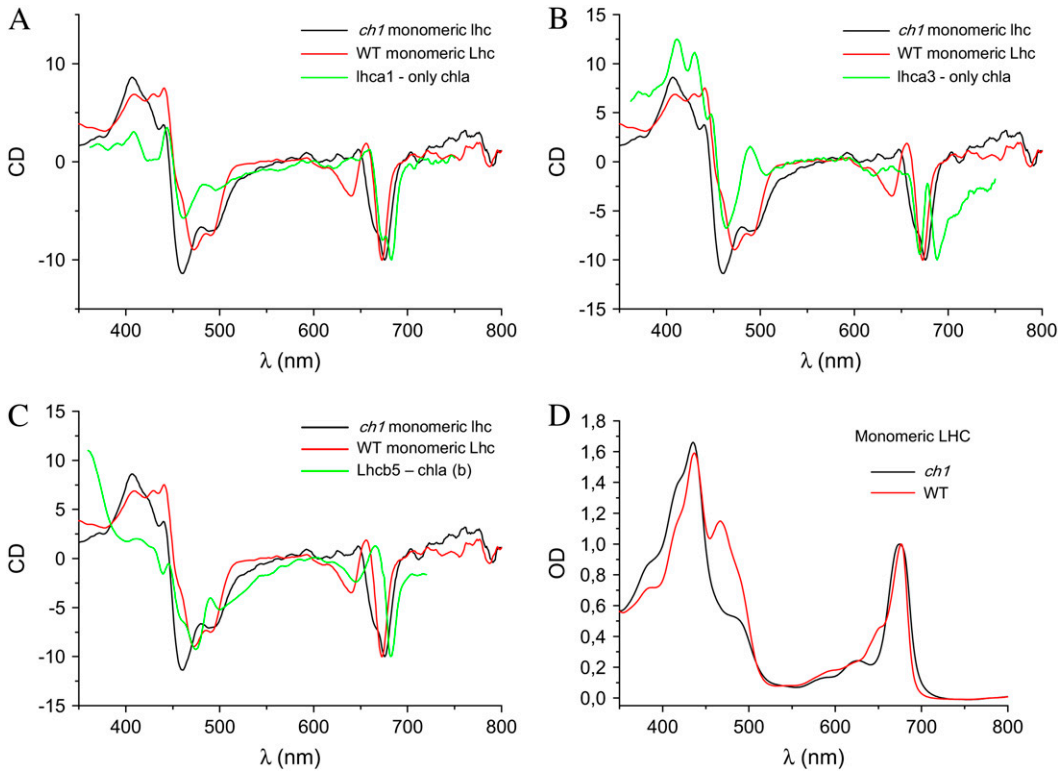

Figure 3. A to C, CD spectra of the B2 or B2′ band of wild-type and *ch1* thylakoids compared with Lhca1 (A), Lhca3 (B), and Lhcb5 (C) recombinant proteins reconstituted with Chl *a*. Because reconstitution of Lhcb5 antenna is impossible in the complete absence of Chl *b*, a small amount of Chl *b* was added to Chl *a* with a Chl *a*:*b* ratio of 8. D, Absorption spectra of the B2 and B2′ bands eluted from Deriphat-PAGE gels of wild-type and *ch1* thylakoids, respectively (see Fig. 2). The spectra were measured on three different preparations with qualitatively similar results.

corollary, the absence of Lhcb5 antennae in this band suggests that this protein was not assembled with pigments in *ch1*. To check this hypothesis, the *ch1* mutant was crossed with a mutant (*lhcb5*) devoid of CP26. The western blots shown in Figure 4A demonstrate the complete absence of Lhcb5 from this mutant and from the *ch1 lhcb5* double mutant. Interestingly, the apparent molecular mass of Lhcb5 in *ch1* (between 27.5 and 28.3 kD) was higher than that of wild-type Lhcb5 (27.7 kD), suggesting that the protein is not fully processed in the *ch1* mutant. We hypothesize that maturation is contemporary with folding and pigment binding; thus, the latter processes are impaired in *ch1*. Importantly, the Deriphat gel of *ch1 lhcb5* was identical to that of *ch1* (Fig. 4B). In particular, the B2 band was not decreased when Lhcb5 was absent, thus showing that this protein did not bind pigments in *ch1*. Moreover, the functional antenna size of PSII was measured by in vivo Chl fluorescence induction measurements in the presence of diuron, and no difference was observed for the fluorescence induction rise between *ch1* and *ch1 lhcb5* (Fig. 4C; Supplemental Fig. S4), confirming that CP26 is not functional in *ch1*. We calculated that the PSII Chl antenna size of *ch1* was reduced to one-third of the antenna size of the wild type (Supplemental Fig. S4).

The free pigment zone in the *ch1* green gel was noticeably increased relative to the wild type (Fig. 2), containing 29% ± 1% of the carotenoids (versus 4% in the wild type). The carotenoid composition of the free pigment band was roughly similar to that measured at the leaf level; the predominant xanthophylls were lutein and violaxanthin/zeaxanthin, while relatively little neoxanthin and β-carotene were present in this fraction (data not shown). We also found Chl *a* in the free pigment band of *ch1*, and these unbound Chl molecules likely resulted from disassembly of the PSI-LHCI superstructures that liberated the gap Chls involved in the binding of the antenna system to PSI (Morosinotto et al., 2005).

Crossing of *ch1* with Different Carotenoid Mutants

The thylakoid protein composition of the double mutants *ch1 npq1*, *ch1 npq2*, *ch1 npq4*, and *ch1 lut2* did not differ appreciably from that of the *ch1* thylakoid membranes (Fig. 5A). The total carotenoid concentration (on a leaf area basis) was only slightly reduced (around −20%) in the *ch1* single and double mutants relative to the wild type. Under high-light stress conditions, a substantial fraction of the xanthophyll-cycle pigments (approximately 65%–70%) was in the form of zeaxanthin in *ch1* and also in *ch1 npq4* (Fig. 5B). Compared to the wild type, *ch1* contained less neoxanthin and lutein, as expected from the loss of LHCII. The β-carotene

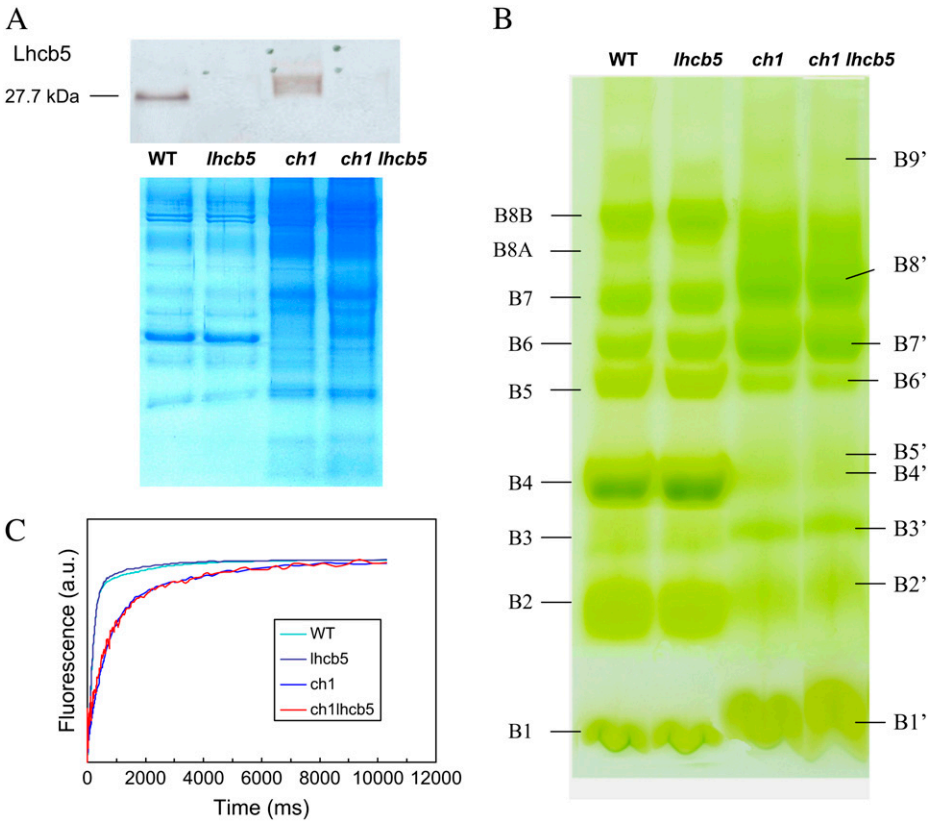

Figure 4. A, Western blot of Lhcb5 in wild-type, *lhcb5*, *ch1*, and *ch1 lhcb5* leaves. One microgram of Chl was loaded on the gel. Coomassie Blue-stained gel was used as loading control. B, Nondenaturing Deriphat-PAGE separation of pigments photosynthetic complexes of wild-type, *lhcb5*, *ch1*, and *ch1 lhcb5* thylakoids. Twenty-five micrograms of Chl/lane were loaded. C, Induction curves of Chl fluorescence in leaves infiltrated with 30 μM diuron. Experiments shown in A to C were repeated two times with similar results.

level was unchanged, while the zeaxanthin content was increased relative to the wild type. As expected, *ch1 npq1* did not synthesize zeaxanthin in high light. *ch1 lut2* is deficient in lutein, which is replaced by the violaxanthin cycle pigments. Thus, in high light, this double mutant contains high amounts of zeaxanthin relative to *ch1*. *ch1 npq2* also contained high amounts of zeaxanthin, but this accumulation was constitutive due to the absence of epoxidized xanthophylls (violaxanthin, neoxanthin, and antheraxanthin) and occurred with normal lutein levels.

NPQ Is Strongly Reduced in *ch1*

NPQ is a protective mechanism that increases thermal energy dissipation in the PSII antennae (Müller et al., 2001; Horton et al., 2005). It requires a transthylakoid pH gradient and is amplified by zeaxanthin. The *ch1* Arabidopsis mutant lacks functional LHCII, and, consequently, NPQ was noticeably inhibited in this mutant (Fig. 5C), as previously reported for Chl *b*-less barley (*Hordeum vulgare*) mutants (Hartel et al., 1996). However, some NPQ activity remained in *ch1*, which was further inhibited in the *ch1 npq1* double mutant that cannot synthesize zeaxanthin in high light and in the *ch1 npq4* double mutant that has no PsbS. Comparison of *ch1 npq4* and *ch1 npq1* is necessary to discriminate antioxidant activities of zeaxanthin due to the molecule itself from those due to zeaxanthin-stimulated NPQ. NPQ in *ch1 npq2* and *ch1 lut2* was intermediate between that of *ch1* and *ch1 npq1*. The effects of the various mutations on NPQ in *ch1* leaves were qualitatively similar to those previously measured in the single mutants *npq1*, *npq2*, *lut2*, and *npq4* (Niyogi et al., 1998; Pogson et al., 1998; Li et al., 2000). Because it is beyond the scope of this work, the small NPQ activity that remains in *ch1* was not studied further, but we tentatively attribute this activity to a reaction center quenching like that previously described by Finazzi et al. (2004).

Absence of Zeaxanthin in the *ch1* Background Results in a Highly Photosensitive Phenotype

The *ch1* single and double mutants were exposed to high-light stress. The *ch1* mutant is known to be more sensitive to high-light treatments than the wild type (Havaux et al., 2004), and we selected in this study stress conditions (1,300 μmol photons $m^{-2} s^{-1}$; 8°C) that caused only moderate photodamage in *ch1*, as judged from the absence of visual symptoms (i.e. leaf bleaching; Fig. 6A) and from autoluminescence imaging (Fig. 7A). The latter technique measures the faint light emitted spontaneously by plants and originating from $^1O_2$ and excited triplet state carbonyl groups (Flor-Henry et al., 2004; Havaux et al., 2006), the byproducts of the slow, spontaneous decomposition of lipid peroxides and endoperoxides (Gutteridge and Halliwell, 1990; Vavilin

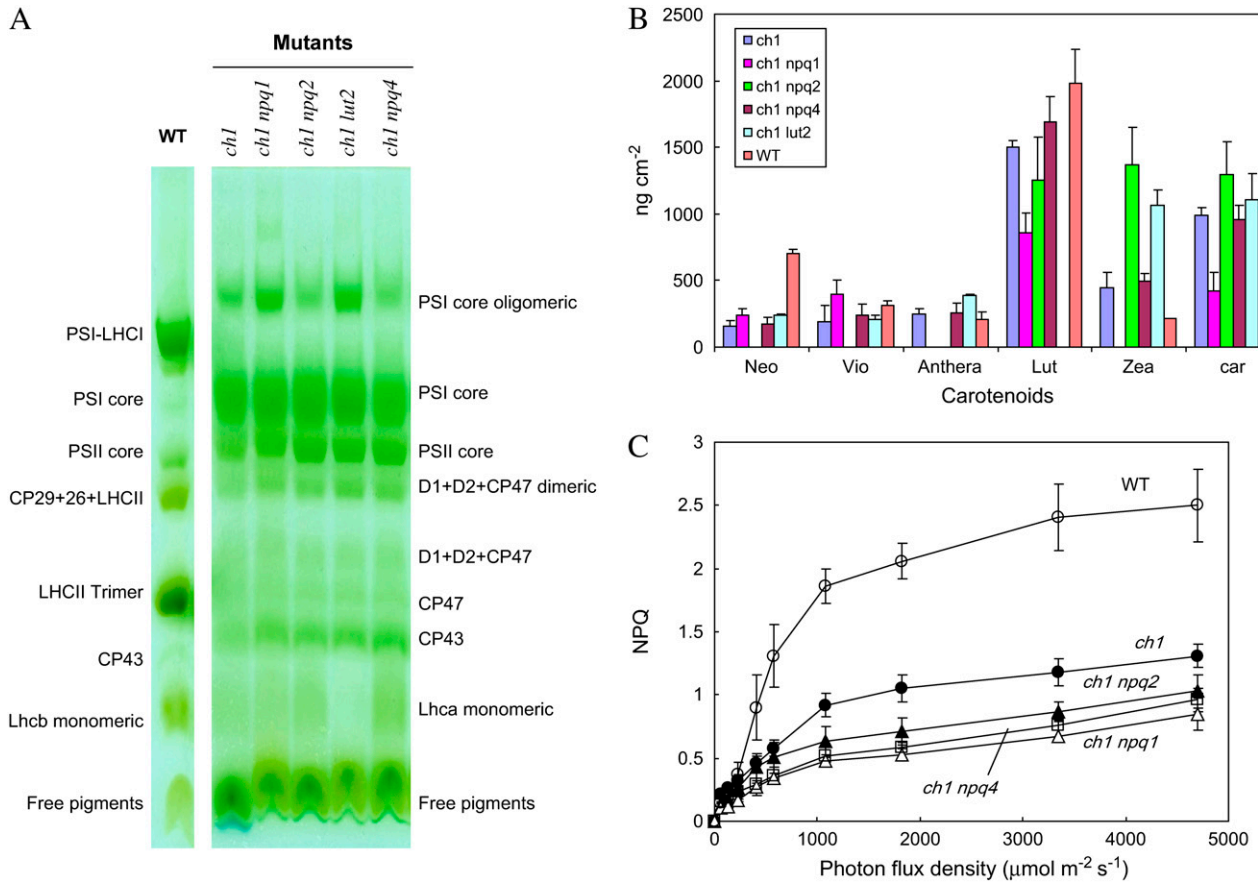

Figure 5. Pigmented photosynthetic complexes, carotenoids, and NPQ in the *ch1* Arabidopsis mutant and in the double mutants *ch1 npq1*, *ch1 npq2*, *ch1 npq4*, and *ch1 lut2*. A, Nondenaturing gel profiles of thylakoids from the wild type, *ch1*, and the double mutants. This experiment was repeated three times with similar results. Twenty-five micrograms of Chl/lane were loaded. The different bands were identified by SDS-PAGE, HPLC, and spectrophotometric analyses of excised bands (see text). Lack of lutein in *ch1 lut2* appeared to increase the stability of LHCI, which remained attached to the PSI core (no monomeric Lhca band). B, Carotenoid concentrations in the leaves of the different mutants. Plants were exposed to high-light stress (1,300 μmol m^{-2} s^{-1}, 8°C, 2 d) before pigment analyses to allow conversion of violaxanthin (Vio) into antheraxanthin (Anthera) and zeaxanthin (Zea). Lut, Lutein; car, β-carotene; Neo, neoxanthin. Data are mean values of four or five measurements + SD. C, NPQ measured in unstressed leaves of the different mutants at different PFDs. For clarity, the NPQ curve of *ch1 lut2* (intermediate between that of *ch1* and *ch1 npq2*) is not shown. Data are mean values of three separate experiments ± SD.

and Ducruet, 1998). Oxidative stress was further quantified by measuring lipid peroxidation using different methods. Thermoluminescence at high temperature (approximately 135°C) was used to measure the photon emission resulting from deactivation of triplet carbonyls generated during thermal breakdown of lipid hydroperoxides (Vavilin and Ducruet, 1998; Havaux, 2003; Fig. 7C). Malondialdehyde (MDA), a 3-carbon aldehyde produced from radical attack on polyunsaturated fatty acids, and Chl *a* were analyzed by HPLC (Figs. 7B and 6B). The photochemical competence of PSII was estimated by Chl fluorescence measurements using the ratio of variable fluorescence to maximal fluorescence (F_v/F_m; Fig. 6C). *ch1 npq4* and *ch1 lut2* behaved like *ch1* in high light; they exhibited few symptoms of oxidative stress or photoinhibition. In striking contrast, *ch1 npq1*, the double mutant that is unable to synthesize zeaxanthin in high light, was found to be highly sensitive to the light treatment compared to *ch1*. All mature leaves bleached (Fig. 6A), with Chl *a* being partially destroyed (Fig. 6B) and extensive lipid peroxidation taking place (Fig. 7). Moreover, PSII photoinhibition was very pronounced in stressed *ch1 npq1* leaves, with F_v/F_m falling from 0.75 to <0.2 (Fig. 6C). On the contrary, the *ch1 npq2* mutant systematically behaved better than *ch1* and the other double mutants. A statistical analysis showed that the difference between *ch1 npq2* and *ch1* was significant for three parameters (luminescence, F_v/F_m, and Chl *a* level). The two zeaxanthin-accumulating double mutants, *ch1 npq2* and *ch1 lut2*, were significantly different for MDA, F_v/F_m, and Chl *a*.

The Protective Function of Zeaxanthin in *ch1* Does Not Rely on CP26

The *ch1 lhcb5* double mutant behaved like *ch1* under high-light stress; both mutants were phototolerant compared to *ch1 npq1* (Fig. 8). Lipid peroxidation in *ch1* and

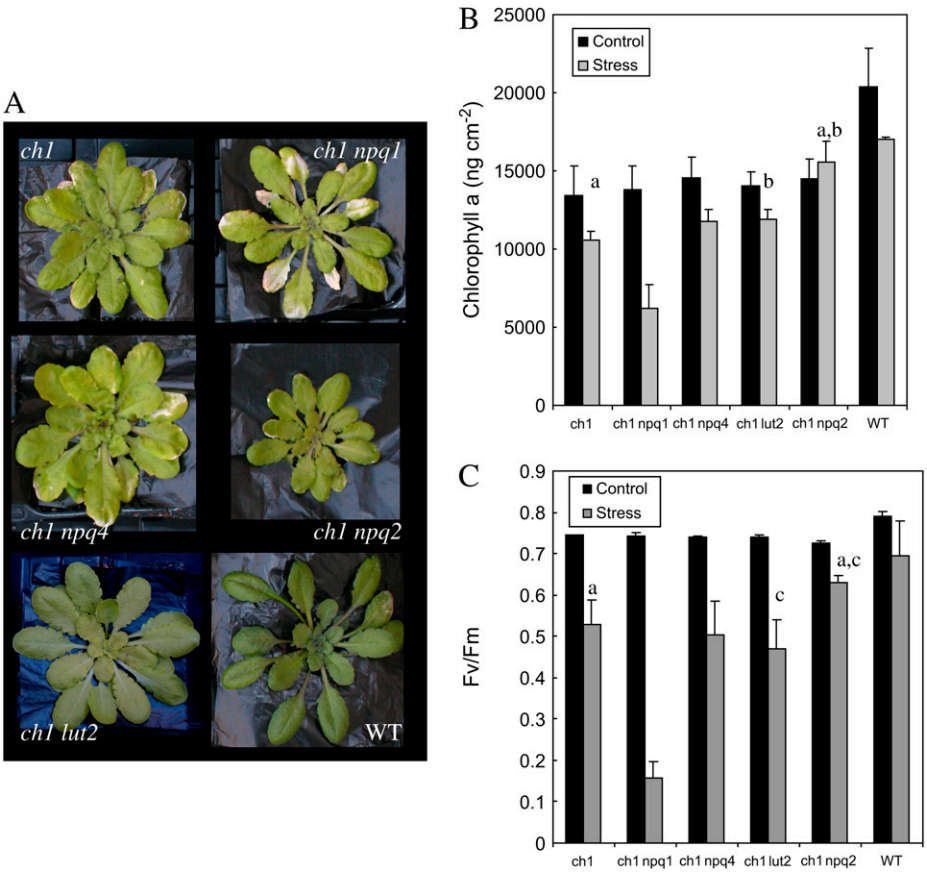

Figure 6. A, Wild-type Arabidopsis and *ch1* single and double mutants after high-light stress (1,300 μmol m^{-2} s^{-1}, 8°C, 2 d). B, Leaf bleaching was estimated from the decrease in Chl *a* concentration after high light. C, PSII photoinhibition was measured by the F_v/F_m Chl fluorescence ratio. Data are mean values of three to six measurements (B) or 8 to 12 measurements (C) + SD. Statistical analysis of the differences between *ch1 npq2* and *ch1* or *ch1 lut2*: a and c, significantly different with $P < 0.05$; b, significantly different with $P < 0.10$ (*t* test). *ch1 npq1* is significantly different from all other mutants for both parameters ($P < 0.05$, *t* test), while *ch1 npq4* was not significantly different from *ch1*.

ch1 lhcb5 leaves, measured autoluminescence imaging (Fig. 8B) and thermoluminescence (Fig. 8C) after high-light stress, was identical and very limited compared to lipid peroxidation in *ch1 npq1* leaves. The *lhcb5* mutant was also crossed with *ch1 npq1*, and the triple mutant *ch1 npq1 lhcb5* exhibited the same sensitivity to high-light stress as the *ch1 npq1* double mutant; the autoluminescence intensity and the amplitude of the 135°C thermoluminescence band were similar in *ch1 npq1* and *ch1 npq1 lhcb5*. We can conclude that the antioxidant function of zeaxanthin in *ch1* does not depend on Lhcb5.

Photoinhibition of PSII Is Not Enhanced in the *ch1 npq1* Double Mutant during Short-Term High-Light Treatments

We checked whether the photoresistance of *ch1* relative to *ch1 npq1* was attributable to a protection by zeaxanthin of PSII reaction centers toward photoinhibition. To this end, leaf discs were exposed to high-light stress at low temperature (Fig. 9), causing a rapid photoinhibition as shown by the decrease in the Chl fluorescence parameter F_v/F_m. However, this decrease was similar in *ch1*, *ch1 npq4*, and *ch1 npq1*. Thus, lack of zeaxanthin does not seem to increase the photoinhibition rate of PSII in *ch1*. This suggests that the enhanced peroxidation of lipids in *ch1 npq1* relative to *ch1* (Fig. 7) was not a secondary effect of PSII photoinhibition. Moreover, when F_v/F_m was measured in whole plants at an early stage of the light stress (before Chl bleaching takes place), we did not observe a significant difference between *ch1* and *ch1 npq1* (data not shown), indicating that the differential lipid peroxidation was not preceded by a differential photoinhibition.

The Photosensitive Phenotypes of *ch1 npq1* and *ch1 vte1* Are Similar and Additive

The *vte1* Arabidopsis mutant is deficient in tocopherol and accumulates the tocopherol precursor 2,3-dimethyl-phytyl-1,4-benzoquinol (DMPBQ; Maeda et al., 2006). We designed a double mutant, *ch1 vte1*, deficient in vitamin E, and a triple mutant, *ch1 vte1 npq1*, deficient in both vitamin E and zeaxanthin. The responses of those mutants to high-light stress were compared with those of *ch1* and *ch1 npq1*. *ch1 vte1* behaved like *ch1 npq1*, showing similar bleaching of the mature leaves (Fig. 10A), similar lipid peroxidation (Fig. 10, B and C), and similar photoinhibition (Fig. 10D). The triple mutant *ch1 vte1 npq1* was extremely photosensitive, exhibiting an almost complete bleaching of the leaves and suffering extensive lipid peroxidation within approximately 1 d. The Chl *a* content of the *ch1 vte1 npq1* leaves fell from approximately 14,000 ng cm^{-2} to 3,620 ng cm^{-2} (versus 6,220 and 5,915 ng cm^{-2} in *ch1 npq1* and *ch1 vte1*, respectively). PSII photoinhibition was very marked in *ch1 vte1 npq1* ($F_v/F_m < 0.05$) compared to *ch1 npq1* and *ch1 vte1* (F_v/F_m approximately

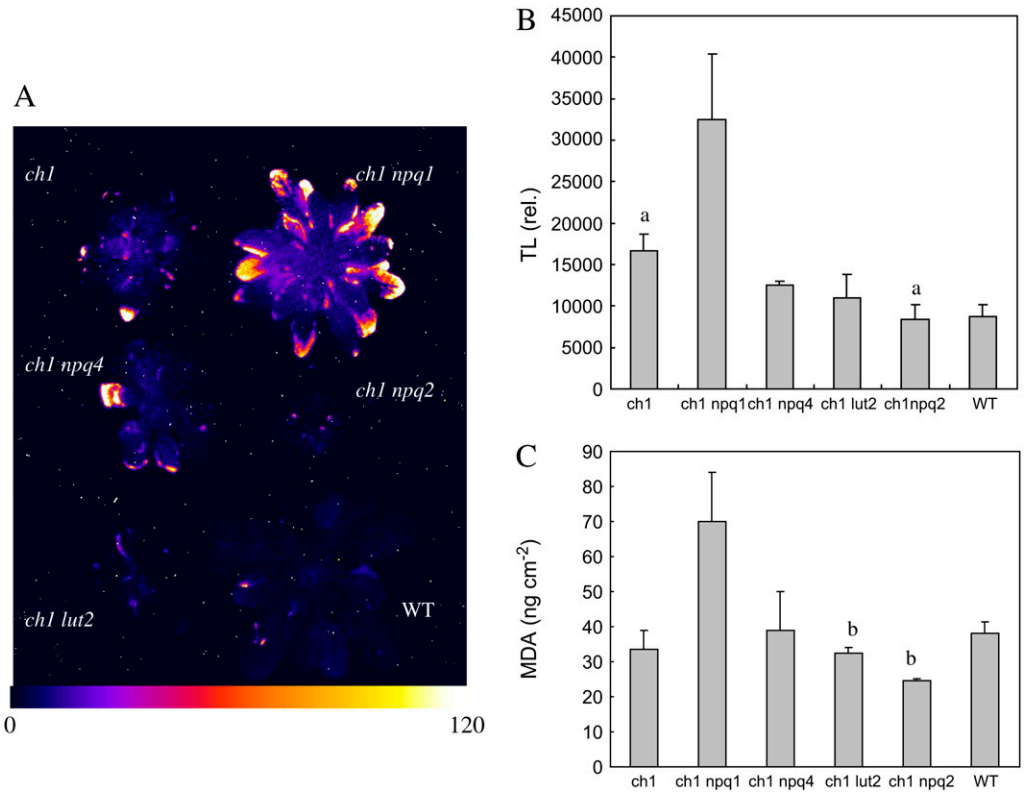

Figure 7. Lipid peroxidation and photooxidative stress in the *ch1* single and double mutants exposed to high-light stress (1,300 μmol m^{-2} s^{-1}, 8°C, 2 d). A, Autoluminescence imaging of oxidative stress in the different mutants after high-light stress. The experiments were repeated four times with similar results. Lipid peroxidation was also estimated by thermoluminescence (B) and MDA concentration (C). Data are mean values of three to six measurements + SD. Statistical analysis of the differences between *ch1 npq2* and *ch1* or *ch1 lut2*: a and b, significantly different with $P < 0.05$ and 0.10, respectively (*t* test). *ch1 npq1* is significantly different from all other mutants for both parameters ($P < 0.05$, *t* test), while *ch1 npq4* was not significantly different from *ch1*.

0.12). Thus, removing zeaxanthin or removing vitamin E and accumulating DMPBQ appeared to result in very similar phenotypes, and removing both led to additive effects.

We also examined the α-tocopherol level in the *ch1* single and double mutants before and after high-light stress (Fig. 11). Leaves of all mutants had similar tocopherol content under control conditions. High-light stress brought about an increase in the tocopherol content in all mutants. However, the increase was very marked in *ch1 npq2* and was relatively modest in *ch1 npq1* compared to *ch1*, *ch1 lut2*, and *ch1 npq4*. Thus, it appears that a high concentration of zeaxanthin and lutein preserved vitamin E under high-light stress, while vitamin E consumption was enhanced when zeaxanthin was absent. However, we cannot exclude that vitamin E synthesis was inhibited in the absence of zeaxanthin.

DISCUSSION

The *ch1* Mutant Does Not Contain Any Functional LHCII Antenna

Lhcb5 was the only PSII antenna protein present in significant amount in the Chl *b*-less *ch1* Arabidopsis mutant. However, this study has shown that this protein is not assembled with pigments and is not functional. It is not clear why Lhcb5 apoproteins were stable in *ch1* thylakoids while all other Lhcb proteins were degraded. However, Lhcb5 is known to behave very differently from the other Lhcb proteins in response to perturbations of pigment composition. For instance, in the *lut2 npq2* Arabidopsis mutant that contains zeaxanthin as the only xanthophyll, Lhcb5 was the only minor LHCII that was down-regulated (Havaux et al., 2004). A recent study of the *aba4* mutant showed a selective degradation of Lhcb5 in the absence of neoxanthin (Dall'Osto et al., 2007), and Lhcb5 was completely absent from a lutein-only mutant that contains no β-carotene-derived xanthophylls (L. Dall'Osto and R. Bassi, unpublished data). Clearly, Lhcb5 requires β–β-xanthophylls for its stability. Although Lhcb5 antennae cannot assemble in the absence of Chl *b*, we speculate that the presence of rather high amounts of β–β-xanthophylls in the lipid phase of *ch1* thylakoids somehow stabilized and protected the unfolded apoprotein from degradation. In the Chl *b*-less barley mutant *Chlorina f-2*, the LHC composition depends on the stage of the mutant's chloroplast development and also on the growth conditions (Krol et al., 1995; Preiss and Thornber, 1995).

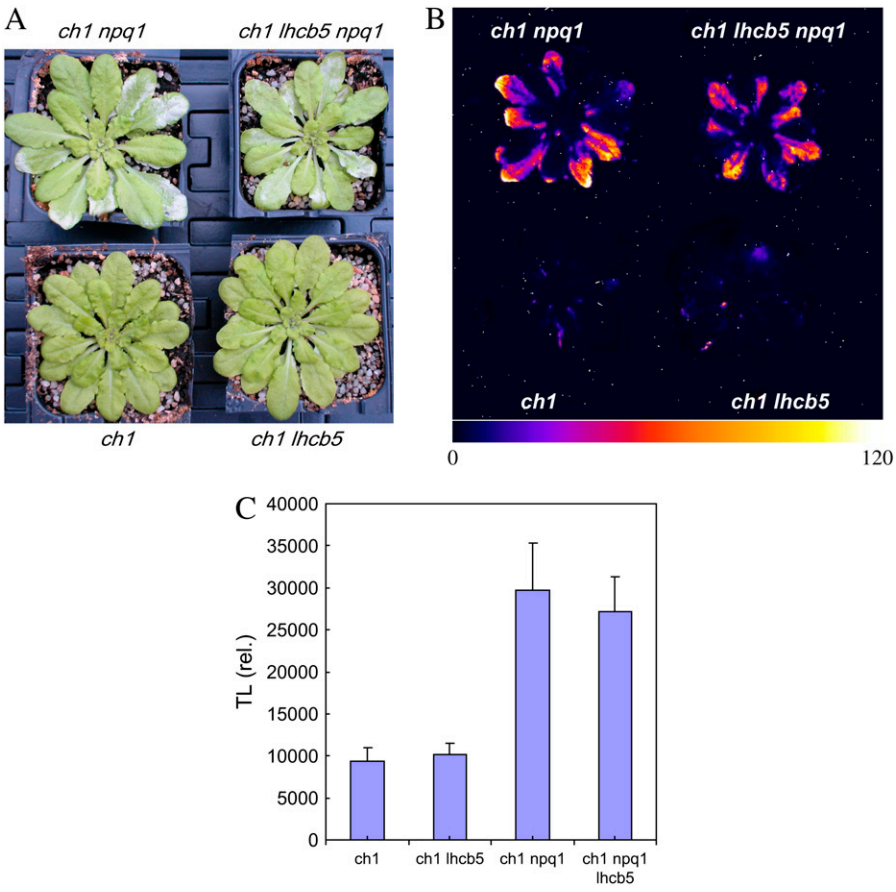

Figure 8. A, Oxidative stress and lipid peroxidation in *ch1 lhcb5* and *ch1 npq1 lhcb5* compared to *ch1* and *ch1 npq1* after high-light stress (1,300 μmol m^{-2} s^{-1}, 8°C, 2 d). B, Autoluminescence imaging of lipid peroxidation. C, High-temperature thermoluminescence (amplitude of the 135°C TL band). The experiment was done three times with similar results.

When grown in intermittent light, Lhcb5 was also the only Lhcb protein that was detected by immunoblotting in *Chlorina f-2* (Krol et al., 1995).

Zeaxanthin Protects *ch1* Thylakoid Membranes by a Mechanism That Takes Place outside the LHCIIs

Zeaxanthin plays a central role in the response of plants to high-light stress through its participation in NPQ (Demmig-Adams and Adams, 1996; Müller-Moulé et al., 2003; Horton et al., 2005). Besides NPQ, zeaxanthin has been suggested to fulfill another protective function by stabilizing thylakoid membranes and protecting membrane lipids from peroxidative damage (Havaux et al., 2000, 2004; Müller et al., 2001; Baroli et al., 2003). The existence of a protective role of zeaxanthin independent of NPQ is confirmed in this study. Removing zeaxanthin from the LHCII- and NPQ-deficient *ch1* mutant resulted in a phenotype very sensitive to photooxidative stress and lipid photodestruction. In striking contrast, removing epoxidized xanthophylls (violaxanthin, antheraxanthin, and neoxanthin) or lutein had no such effect, indicating that the protection was specific to zeaxanthin. Moreover, suppression of NPQ by crossing *ch1* with *npq4* did not enhance photooxidative stress, confirming that the photosensitivity of *ch1 npq1* was due to the absence of the zeaxanthin molecule itself and not to the inhibition of zeaxanthin-stimulated NPQ. Those findings confirm, in the *ch1* background, previous studies of Arabidopsis mutants that have shown that removing lutein or epoxidized xanthophylls had little impact on the photoresistance of the photosynthetic apparatus (Pogson et al., 1996; Hurry et al., 1997; Tardy and Havaux, 1996), while suppression of zeaxanthin can increase oxidative stress and photoinhibition under specific light stress conditions (Havaux and Niyogi, 1999).

The mechanism by which zeaxanthin could exert its NPQ-independent photoprotection is unknown. Because this antioxidative activity takes place in a mutant devoid of LHCIIs, it is not restricted to triplet Chl and $^1O_2$ quenching in the PSII antennae, as suggested by some authors (Kalituho et al., 2006). It is also unlikely that the main effect of zeaxanthin was in the PSII core antennae of *ch1* thylakoids. Indeed, if the function of zeaxanthin was to quench $^1O_2$ in the PSII core, we would have expected an increased photoinhibition of PSII photochemistry in the absence of zeaxanthin (Nishiyama et al., 2006), and this was not observed in light-stressed leaf discs using Chl fluorescence measurements.

Chilling temperatures have been shown to induce a preferential photoinhibition of PSI in some plant species (Sonoike, 1996). However, a previous study did not

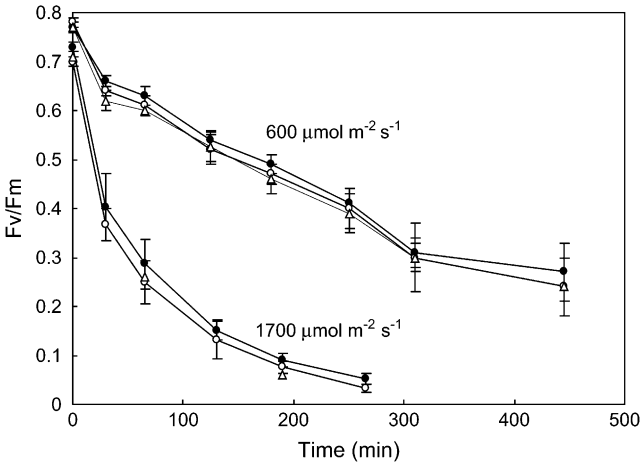

Figure 9. PSII photoinhibition in *ch1*, *ch1 npq1*, and *ch1 npq4* leaf discs exposed to high-light stress at low temperature (600 or 1,700 μmol photons m^{-2} s^{-1} and 8°C). Photoinhibition was measured by the decrease in the Chl fluorescence parameter F_v/F_m. Black circles, *ch1*; white circles, *ch1 npq1*; white triangles, *ch1 npq4*. Data are mean values of three separate experiments ± SD.

reveal any significant damage to PSI in wild-type Arabidopsis exposed to stress conditions very similar to those used in this study (8°C and 1,000 μmol photons m^{-2} s^{-1}), while PSII was substantially photoinhibited (Havaux and Kloppstech, 2001). Moreover, the absence of the xanthophyll cycle in *npq1* was not accompanied by an increase in the sensitivity of PSI to chilling-induced photoinhibition (Havaux and Kloppstech, 2001). Thus, the photoprotective function of the xanthophyll cycle seems to be rather specific to PSII in Arabidopsis despite the fact that zeaxanthin synthesis occurs in both PSI and PSII (Thayer and Björkman, 1992; Verhoeven et al., 1999; Wehner et al., 2004). Nevertheless, because LHCI proteins accumulated in *ch1* to approximately 20% of the wild-type level, we cannot completely exclude that part of the photosensitivity of the *ch1 npq1* double mutant was due to the lack of zeaxanthin in the PSI antenna system.

The total carotenoid content of *ch1* leaves was close to that of wild-type leaves. Therefore, the complete absence of LHCII antennae and the marked reduction of the PSI antenna system in *ch1* suggest that a relatively high concentration of free carotenoids can be maintained in the lipid phase of the thylakoid membranes in this mutant, and this is supported by the high amount of carotenoids found in the free pigment band in nondenaturing green gels. When dissolved into artificial lipid membranes, zeaxanthin has been shown to be an efficient terminator of lipid peroxidation (Lim et al., 1992; Woodall et al., 1997). Thus, we can speculate that free zeaxanthin molecules present in the lipid matrix or zeaxanthin molecules located at the protein-lipid interfaces directly protected *ch1* thylakoid lipids by free radical scavenging and $^1O_2$ quenching. This effect is unique to zeaxanthin because removal of the other xanthophylls from *ch1* did not result in a photosensitive phenotype. Possible factors that could account for the unique lipid-protective activity of zeaxanthin in native thylakoid membranes include its orientation in the membrane lipid bilayer and physico-chemical interactions with lipids (Oshima et al., 1993; Woodall et al., 1997; McNulty et al., 2007), its polarity (Wisniewska et al., 2006), and the elevated number of double bonds (Mathews-Roth et al., 1974). Moreover, because of their chemistry, carotenoid epoxide isomers, like violaxanthin, do not bind singlet oxygen, and therefore they have a low capacity of chemical scavenging of singlet oxygen compared to zeaxanthin (Kopsell and Kopsell, 2006). This study shows that unbound lutein and epoxidized xanthophyll molecules are less protective than unbound zeaxanthin. However, when bound to LHCIIs, those xanthophylls can have specific antioxidant functions, as recently shown for neoxanthin and lutein (Dall'Osto et al., 2006, 2007).

Zeaxanthin and Vitamin E Protect Lipids in an Additive Manner

Protection of thylakoid membrane lipids against peroxidative damage is a function that is traditionally attributed to vitamin E (Fryer, 1992), although alternative functions have been suggested for vitamin E (Maeda et al., 2006; Munné-Bosch et al., 2007). Thus, unbound zeaxanthin in *ch1* thylakoid membranes could fulfill a protective function similar to that of vitamin E. A previous study has shown that zeaxanthin and vitamin E are functionally redundant (Havaux et al., 2005), and this is supported here by the similarity of the phenotypes obtained by suppression of zeaxanthin or tocopherols in *ch1*. The relative photosensitivity of *ch1* allowed us to readily reveal the antioxidant role of zeaxanthin and vitamin E. Contrary to *ch1 npq1* and *ch1 vte1*, the single mutants *vte1* and *npq1* are quite tolerant to high-light stress (Havaux et al., 2005). In the wild-type background, it was necessary to simultaneously remove several protective compounds, e.g. zeaxanthin and vitamin E, to obtain a photosensitive phenotype. Also, removing only one carotenoid impacted very little on photoresistance (Pogson et al., 1996; Tardy and Havaux, 1996; Hurry et al., 1997), but xanthophyll mutants became photosensitive when two mutations were combined, e.g. *lut2 npq1* (Niyogi et al., 2001) or *aba4 npq1* (Dall'Osto et al., 2007). Here, with the *ch1* mutant, we succeeded in evaluating the lipid-protective antioxidant capacity of individual classes of xanthophylls (namely lutein, zeaxanthin, and epoxidized xanthophylls) without combining various mutations.

The antioxidant mechanism of α-tocopherol in lipid bilayers involves the active hydroxyl group on the chromanol ring of the molecule terminating lipid peroxidation by interacting with various lipid radical species to form the stable chromanoxyl radical. As mentioned above, zeaxanthin also functions as a chemical trap for hydroxyl radicals and $^1O_2$ apart from its physical quenching capability. Moreover, both zeaxanthin and vitamin E have membrane-stabilizing properties (Fryer, 1992;

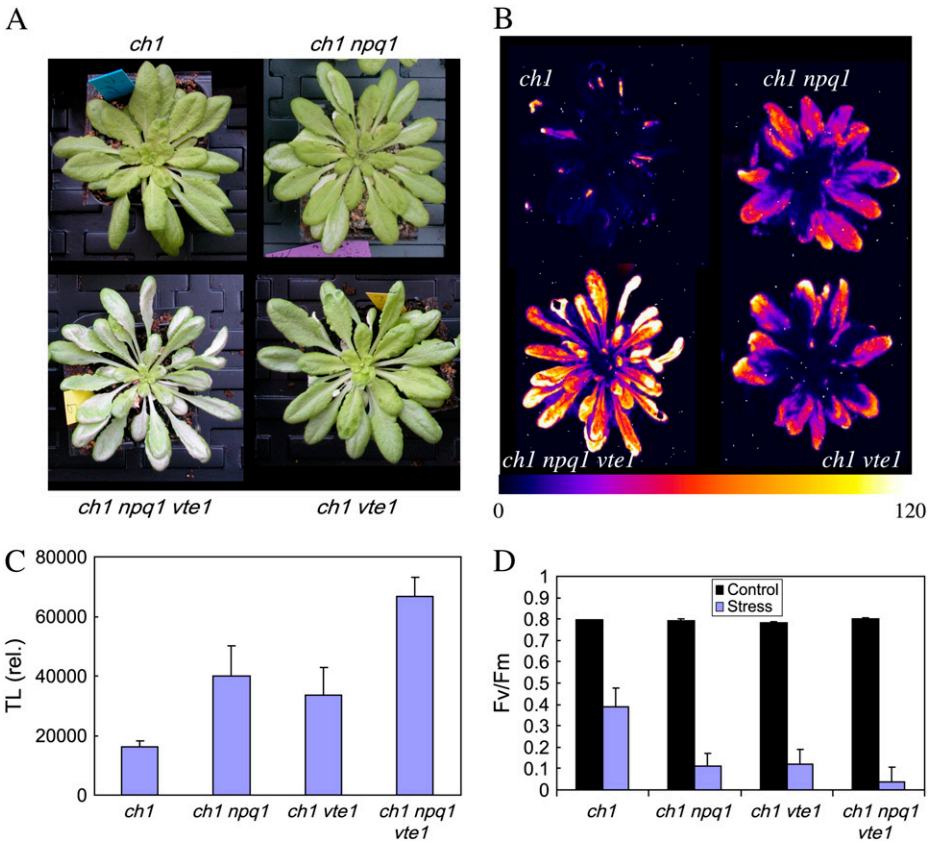

Figure 10. Responses of *ch1 vte1* and *ch1 vte1 npq1* in comparison with *ch1* and *ch1 npq1* to high-light stress (1,300 μmol m^{-2} s^{-1}, 8°C, 2 d). A, Picture of the different mutants after high-light stress. B, Autoluminescence imaging. C, Lipid peroxidation measured by the amplitude of the thermoluminescence band at 135°C. D, PSII photoinhibition measured by the decrease in the F_v/F_m Chl fluorescence ratio. Mature, well-developed leaves were selected for the fluorescence measurements. A and B, The experiment was repeated three times with similar results; C and D, data are mean values of three (C) or five to 10 (D) measurements + SD.

Woodall et al., 1997; Gruszecki and Strzalka, 2005; Wisniewska et al., 2006). While membrane stabilization is considered to be a minor function of tocopherols (Fryer, 1992; Suzuki et al., 1993), the antioxidant activity of carotenoids in membranes is strongly influenced by their physical-chemical interactions with lipids (Oshima et al., 1993; Woodall et al., 1997; McNulty et al., 2007). In this way, the two types of antioxidants might complement each other in lipid bilayers. Moreover, combinations of carotenoid, especially zeaxanthin and α-tocopherol, have been shown to exert a synergistic protection against lipid peroxidation in vitro (Palozza and Krinsky, 1992; Böhm et al., 1997; Wrona et al., 2004). This effect was explained in terms of preservation of free radical-mediated degradation of one antioxidant by the other. More precisely, the chromanoxyl radical may be reconverted to vitamin E by reaction with carotenoids (Böhm et al., 1997). This interpretation is supported by our data because α-tocopherol was substantially decreased in *ch1 npq1* relative to *ch1*, suggesting increased consumption of vitamin E when zeaxanthin was absent.

Protection against photooxidative stress as the main function of vitamin E has recently been questioned because the *vte1* mutant is not sensitive to high light (Maeda et al., 2006). This study shows that vitamin E does protect plants against photooxidation because removing vitamin E from *ch1* thylakoids resulted in a highly photosensitive phenotype, comparable to that of *ch1 npq1*. When zeaxanthin and vitamin E were removed simultaneously, plants were extremely sensitive to high-light stress and photooxidative stress, confirming that the *npq1* and *vte1* mutations are additive (Havaux et al., 2005). Therefore, the site (or one of the sites) of action of zeaxanthin could be similar to that of vitamin E, strengthening the idea that one of the functions of zeaxanthin is protection of thylakoid lipids. However, it should be stressed that the tocopherol-deficient *vte1*

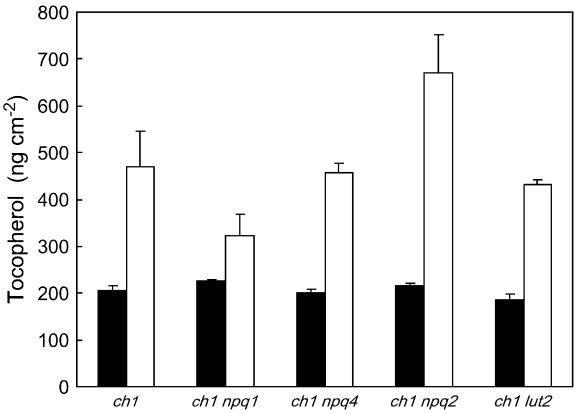

Figure 11. α-Tocopherol level in leaves of *ch1* single and double mutants before (in black) and after (in white) high-light stress at low temperature (1,300 μmol photon m^{-2} s^{-1}, 8°C, 2 d). Data are mean values of four measurements + SD.

mutant accumulates the quinol biosynthetic intermediate DMPBQ. The presence of this compound can interfere with the responses of the mutant to stress conditions (Maeda et al., 2006). Therefore, it must be kept in mind that the photosensitivity of the *ch1 vte1* and *ch1 vte1 npq1* mutants could be due to the lack of tocopherols and also to the accumulation of DMPBQ. In other words, we cannot exclude the possibility that the sensitivity of *ch1 vte1* to lipid peroxidation was partly caused by the presence of high amounts of DMPBQ in the thylakoids.

Tocopherols have also been proposed to play a role in the PSII reaction centers as scavengers of $^1O_2$ produced in PSII that control D1 degradation (Krieger-Liszkay and Trebst, 2006). Additionally, $^1O_2$ scavenging may be important for the repair of photodamage to PSII. The strong photoinhibition of PSII measured in *ch1 vte1* leaves after high-light stress is compatible with this function. However, *ch1 npq1* was as photoinhibited as *ch1 vte1*, but the rate of PSII photoinhibition was not increased by the absence of zeaxanthin in *ch1 npq1* leaf discs exposed to high-light stress. Therefore, we believe that photoinhibition was a secondary effect of photooxidative destruction of thylakoid membranes in the absence of tocopherol or zeaxanthin.

A recent study has shown that, after transfer to low temperature, Arabidopsis tocopherol-deficient mutants, including *vte1*, exhibit defects in photoassimilate export from source leaves, resulting in massive accumulation of carbohydrates (Maeda et al., 2006). However, this phenomenon is relatively slow, with sugar accumulation occurring after more than 5 d at low temperature (7.5°C). Therefore, it is very unlikely that sugar accumulation was involved in the photooxidative damage of *ch1 vte1* leaves reported here because our stress experiments were much shorter (2 d). Moreover, the increased photosensitivity of *ch1 vte1* and *ch1 vte1 npq1* compared to *ch1* was also observed when plants were exposed to very high light (1,800 μmol photons m^{-2} s^{-1}) at 22°C (Supplemental Fig. S5). After 1 d under those conditions, *ch1 vte1 npq1* leaves bleached and exhibited extensive lipid peroxidation (as measured by the amplitude of the thermoluminescence peak at 135°C), while the level of lipid peroxidation was low in *ch1* leaves and intermediate in the double mutant *ch1 vte1*. Consequently, we can exclude that the phenomena reported in this study are indirectly due to cold-induced blockage of photoassimilate export.

The *ch1 npq2* double mutant that constitutively contains high amounts of zeaxanthin with normal levels of lutein and vitamin E appeared to be more tolerant to high-light stress than *ch1*. Association of lutein with high amounts of zeaxanthin was more protective than zeaxanthin alone because *ch1 npq2* was more phototolerant than *ch1 lut2*. Thus, the combination zeaxanthin-lutein-vitamin E could represent the most efficient combination of lipid-soluble antioxidants for preventing oxidation of polyunsaturated lipid membranes. This combination of antioxidants could also be the most stable one because *ch1 npq2* leaves were found to contain the highest level of α-tocopherol under high-light stress. However, although NPQ was not increased in *ch1 npq2* relative to *ch1* or *ch1 lut2*, we cannot exclude that zeaxanthin present constitutively acted differently from zeaxanthin synthesized transiently from violaxanthin in the xanthophyll cycle and induced an additional (yet unidentified) protective mechanism in *ch1 npq2* relative to *ch1* or *ch1 lut2*.

Interestingly, lutein and zeaxanthin are the only carotenoids found in the human eye, especially the macula and the lens epithelium and cortex (Krinsky et al., 2003; Whitehead et al., 2006), where they are associated with vitamin E. Human skin also contains carotenoids that are thought to play a significant part in the skin's antioxidant defense system and may help prevent cutaneous malignancy (Hata et al., 2000; Ermakov et al., 2005). Interestingly, a recent clinical trial has shown that the combined administration of zeaxanthin and lutein provides the highest degree of antioxidant protection against skin lipid peroxidation under UV stress (Palombo et al., 2007). Thus, functional synergism between zeaxanthin and lutein seems to be a general feature of biological tissues challenged with radiation-induced reactive oxygen species toxicity.

The Antioxidant Function of Zeaxanthin in Wild-Type Arabidopsis

The Arabidopsis *ch1* mutant has a lower tolerance to high-light intensities than the wild type (Havaux et al., 2004). The same phenomenon was also reported for the Chl *b*-less barley mutant *chlorina-f2* (Leverenz et al., 1992; Havaux and Tardy, 1997). This can be attributed to the photoprotective functions of the LHCII system, which include thermal energy dissipation via NPQ, quenching of triplet Chl and $^1O_2$ by the LHCII-bound xanthophylls, and, possibly, the screening and stabilizing effect of the LHCIIs on the PSII reaction centers. The increased photosensitivity of *ch1* may thus be due to a higher $^1O_2$ production within the PSII reaction center in the absence of the LHCII-related protective and/or stabilizing mechanisms. It should also be mentioned that the PSII antenna size of *ch1* is much smaller than that of the wild type (Supplemental Fig. S4). Therefore, the number of photons entering the wild type is higher than the photons entering *ch1*, and the photosensitivity of *ch1* is actually underestimated when a given light intensity is used to compare the wild type and *ch1*.

Wild-type thylakoid membranes contain much less free carotenoids in the lipidic phase than *ch1* thylakoids, although the operation of the xanthophyll cycle could involve the transient release of xanthophylls into the lipid phase (Morosinotto et al., 2002; Hieber et al., 2004; Goss et al., 2005). However, it is well known that violaxanthin is only loosely bound to LHCII and exposed on the periphery of the complex, presumably in contact with membrane lipids and in close vicinity of LHCII-bound lipids (Caffarri et al., 2001). Therefore, we can speculate that the protective and stabilizing effect

of zeaxanthin on membrane lipids, as observed here for free zeaxanthin in *ch1*, actually occurs at the LHCII-lipid interface in wild-type plants. In particular, protection of the lipid boundary of the photosystems could preserve the stability of the antennae and prevent membrane lipid peroxidation by cutting out the initiation phase. There are, however, environmental conditions that tend to increase the amount of unbound carotenoids in plants. For instance, long-term acclimation of barley or *Vinca* to high-light intensity and low or high temperature was reported to induce carotenoid decoupling from the Chl antennae (Havaux et al., 1998) and increased level of free xanthophylls (Havaux et al., 1998; Verhoeven et al., 1999). Similarly, in the green alga *Dunaliella salina*, long-term acclimation to high-light irradiance caused the appearance of a zeaxanthin fraction separated from the Chl proteins and presumably imbedded in the thylakoid lipid bilayer (Yokthongwattana et al., 2005). Thus, increased level of unbound carotenoids seems to be associated with long-term acclimation of plants to excess light energy, and the lipid protective function of free zeaxanthin, observed here in *ch1*, is physiologically relevant to those situations.

Based on resonance Raman spectroscopic data, it has been recently suggested that the relative strengths and configurations of binding of violaxanthin and zeaxanthin to LHC proteins participate in their differential antioxidant activity, e.g. by favoring interaction of zeaxanthin with LHC-bound lipids (Johnson et al., 2007). Although our results do not exclude this possibility in wild-type Arabidopsis, it is nevertheless clear that the differential antioxidant activity of zeaxanthin and the other Arabidopsis xanthophylls, including violaxanthin, is conserved even in the absence of LHCII and therefore is not restricted to an enhanced reactivity of the xanthophyll upon binding to LHCII. As shown in this study, the intrinsic antioxidant activity of zeaxanthin is higher than that violaxanthin, and we believe that this characteristic of zeaxanthin is of critical importance to its function within the xanthophyll cycle. In other words, the xanthophyll cycle may be seen as a system that substitutes, within the LHCII antennae, a xanthophyll (violaxanthin) by a more powerful antioxidant (zeaxanthin) that can efficiently protect LHCII-associated lipids. Whether the antioxidant capacity of zeaxanthin is further enhanced when it binds to LHCII should be investigated in the future.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Stress Treatments

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants were grown on compost in a phytotron under controlled conditions, as previously described (Havaux et al., 2005). The *ch1-1* mutant is affected in chlorophyllide *a* oxygenase and lacks Chl *b*. Absence of chlorophyllide *a* oxygenase and Chl *b* impacts the import and stabilization of Lhcb proteins, resulting in a loss of major and minor LHCIIs (Espineda et al., 1999; Reinbothe et al., 2006). This mutant was crossed with the following mutants: the *npq1* mutant deficient in zeaxanthin (Niyogi et al., 1998), the *npq4* mutant lacking the PSII protein PsbS (Li et al., 2000), the *lut2* mutant deficient in lutein (Pogson et al., 1996), the *npq2* mutant deficient in neoxanthin and violaxanthin that are replaced by zeaxanthin (Niyogi et al., 1998), the *vte1* mutant deficient in vitamin E (Havaux et al., 2005), and the *lhcb5* mutant lacking the PSII antenna CP26 (Dall'Osto et al., 2005). All double and triple mutants were constructed for this work, except *ch1 npq4*, which was received from Dr. Kris Niyogi. Light stress was imposed by transferring plants aged 5 weeks to high light (1,300 μmol photons m^{-2} s^{-1}, photoperiod of 8 h) and low temperature (8°C/8°C, day/night). Plants were transferred to high light at 10 AM (local time) after 1 h of illumination. Leaf samples were harvested from stressed plants between 10 AM and 11 AM.

Chl Fluorescence, Thermoluminescence, and Luminescence Imaging

Chl fluorescence from leaf discs was measured with a PAM-2000 fluorometer (Walz; Havaux et al., 2005). The F_v/F_m parameter was used as a measure of the maximal efficiency of PSII photochemistry (Kitajima and Butler, 1975). F_m is the maximal fluorescence level and F_v is the difference between F_m and the initial fluorescence level F_o. F_v/F_m was measured in light-stressed leaf discs after 15-min adaptation to darkness. NPQ was calculated as $(F_m/F_m') - 1$, where F_m' is the maximal fluorescence level in the light. For the PSII photoinhibition experiments, light stress was imposed by exposing leaf discs (1 cm in diameter) at constant temperature (8°C) to white light (photon flux density [PFD], 600 or 1,700 μmol photons m^{-2} s^{-1}), as previously described (Havaux et al., 2005). PFD was measured with a LI-COR quantum meter. The functional antenna size of PSII was estimated by measuring the induction of Chl fluorescence emitted by leaves infiltrated with diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. Leaves were infiltrated with 30 μM diuron in 150 mM sorbitol. Chl fluorescence was induced with green light produced by a light-emitting diode (PFD, 7 μmol m^{-2} s^{-1}).

Lipid peroxidation was measured in leaf discs by thermoluminescence using a custom-made apparatus that has been described previously (Havaux, 2003). The amplitude of the thermoluminescence band peaking at approximately 135°C was used as an index of lipid peroxidation (Ducruet, 2003; Havaux, 2003). The samples (three leaf discs of 6 mm in diameter) were slowly heated from 25°C to 150°C at a rate of 6°C min^{-1}. Photon emission associated with lipid peroxidation was also imaged at room temperature using a CCD camera cooled with liquid nitrogen (VersArray LN/CCD 1340-1300B; Roper Scientific), as described elsewhere (Havaux et al., 2006). Plants were preadapted to complete darkness for 2 h before recording spontaneous luminescence. Acquisition time was 20 min.

HPLC Analyses

Lipid peroxidation was assessed by measuring MDA extracted in ethanol:water (80%:20%), using HPLC as previously described (Havaux et al., 2005). Chl and carotenoids were extracted in pure methanol and separated by HPLC as described by Havaux et al. (2005).

Denaturing and Nondenaturing Electrophoreses of Thylakoid Proteins

Thylakoid membranes were solubilized in 0.8% dodecyl-D-maltoside and fractionated into a PAGE system using Deriphat as charged detergent (Havaux et al., 2004). After separation, green bands were excised from gel and eluted by incubating overnight at 4°C with 10 mM HEPES, pH 7.0, 0.06% dodecyl-D-maltoside. After filtration through a 10-μm nylon net, the eluate was characterized by spectrophotometry, HPLC analysis of pigments (Gilmore and Yamamoto, 1991), CD (Croce et al., 1996), and protein analyses. For the latter analyses, eluate was concentrated into a Vivaspin 100 device (Sartorius) and fractionated by Tris-sulfate SDS-PAGE (Shagger and von Jagow, 1987; Caffarri et al., 2001).

For immunotitration, thylakoid samples corresponding to 0.5, 1, 2, and 4 μg of Chl were loaded for each sample and electroblotted on nitrocellulose membranes. Filters were incubated with antibodies raised against Lhcb1 + 2, Lhcb3, CP29 (Lhcb4), CP26 (Lhcb5), CP24 (Lhcb6), Lhca1-4, or CP47 (PsbB) and were revealed with alkaline phosphatase conjugated antibody. Signal amplitude was quantified using the GelPro 3.2 software (Bio-Rad).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_202241 (*CH1*), NM_100728 (*NPQ1*),

NM_202242 (*NPQ4*), NM_125085 (*LUT2*), NM_180954 (*NPQ2*), NM_119430 (*VTE1*), and NM_117102 (*LHCB5*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Western blot of ELIP1 and ELIP2 in wild-type and *ch1* thylakoids.

Supplemental Figure S2. Tris-sulfate SDS-PAGE and Tris-tricine SDS-PAGE separation of thylakoid proteins from the wild type and *ch1*.

Supplemental Figure S3. Absorption spectra of the different bands eluted from the Deriphat-PAGE of thylakoid pigmented complexes of *ch1* and the wild type.

Supplemental Figure S4. Functional PSII antenna size in wild-type, *lhcb5*, *ch1*, and *ch1 lhcb5* leaves.

Supplemental Figure S5. Exposure of *ch1*, *ch1 npq1*, *ch1 vte1*, and *ch1 vte1 npq1* to high-light stress at 22°C.

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