Elevated Zeaxanthin Bound to Oligomeric LHCII Enhances the Resistance of Arabidopsis to Photooxidative Stress by a Lipid-protective, Antioxidant Mechanism*

Matthew P. Johnson‡, Michel Havaux‡, Christian Triantaphylidès§, Brigitte Ksas§, Andrew A. Pascal‡, Bruno Robert§, Paul A. Davison‡, Alexander V. Ruban‡, and Peter Horton†‡§¶

From the ‡Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, United Kingdom, the §CEA-Cadarache, Institut de Biologie Environnementale et de Biotechnologie, Laboratoire d’Ecophysiologie Moléculaire des Plantes, UMR 6191, CNRS-CEA-Université Aix Marseille, F-13108 Saint-Paul-lez-Durance, France, the ¶Commissariat à l’Energie Atomique (CEA), Institut de Biologie et Technologies de Saclay (iBiTecS), URA 2096, Gil sur Yvette, F-91191, France, and the §School of Biological and Chemical Sciences, Queen Mary College, University of London, Mile End, Bancroft Road, London E1 4NS, United Kingdom

The xanthophyll cycle has a major role in protecting plants from photooxidative stress, although the mechanism of its action is unclear. Here, we have investigated Arabidopsis plants overexpressing a gene encoding β-carotene hydroxylase, containing nearly three times the amount of xanthophyll cycle carotenoids present in the wild-type. In high light at low temperature wild-type plants exhibited symptoms of severe oxidative stress: lipid peroxidation, chlorophyll bleaching, and photoinhibition. In transformed plants, which accumulate over twice as much zeaxanthin as the wild-type, these symptoms were significantly ameliorated. The capacity of non-photochemical quenching is not significantly different in transformed plants compared with wild-type and therefore an enhancement of this process cannot be the cause of the stress tolerant phenotype. Rather, it is concluded that it results from the antioxidant effect of zeaxanthin. 80–90% of violaxanthin and zeaxanthin in wild-type and transformed plants was localized to an oligomeric LHCII fraction prepared from thylakoid membranes. The binding of these pigments in intact membranes was confirmed by resonance Raman spectroscopy. Based on the structural model of LHCII, we suggest that the protein/lipid interface is the active site for the antioxidant activity of zeaxanthin, which mediates stress tolerance by the protection of bound lipids.

The photosynthetic light reactions produce highly reactive intermediates that combine with the molecular oxygen to become a lethal mixture to the higher plant cell when the rate of light energy use is perturbed (1). There are many environmental changes that can upset the balance between excitation energy absorption by the chlorophyll antenna and its use in photochemistry, such as low temperature, CO₂ limitation, and high irradiance (2). Such disruptions lead to an extended lifetime of the excited chlorophyll singlet state (2Chl*)² within the antenna, increasing the population of triplet states (3Chl*), which are potent photosensitizers leading to the formation of singlet oxygen (¹O₂) that can cause photooxidative damage to the photosynthetic apparatus (reviewed in Ref. 3). A particular potential hazard of the formation of such reactive oxygen species (ROS) arises from their role in initiating lipid peroxidation chain reactions that consume chloroplast membrane lipids forming lipid peroxides as the by-product (4, 5). Lipid peroxides are more polar than their lipid precursors and so compromise membrane integrity if they accumulate. The high concentrations and close packing of polyunsaturated fatty acids in the chloroplast membrane favors the initiation of lipid peroxidation chain reactions in which the radical species is continually regenerated until the substrate is depleted or the reaction is terminated by antioxidants (5, 6).

Higher plants display a number of responses to such photooxidative stress and the xanthophyll cycle, the enzymatic de-epoxidation of the carotenoid violaxanthin to zeaxanthin (7), has a central role. Under light stress conditions, the increase in thylakoid ΔpH activates violaxanthin de-epoxidase and zeaxanthin accumulates (7). Prolonged exposure to stress conditions also results in an increase in the size of the total xanthophyll cycle pool (8). However, the mechanism of action of zeaxanthin in protecting the components of the thylakoid membrane from inhibition and damage is not well understood. Zeaxanthin has a role in the dissipation of excess excitation energy in the photosystem II antenna as heat by a process termed non-photochemical quenching (NPQ) (9). The rapidly reversible, ΔpH-dependent component of NPQ provides photoprotection of the thylakoid membrane under excess light (10). The role of zeaxanthin in NPQ remains controversial; it may act either indirectly, by allosterically promoting the formation of the photo-

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† To whom correspondence should be addressed. Fax: 441142222712; E-mail: p.horton@sheffield.ac.uk.

‡ The abbreviations used are: Chl, chlorophyll; ROS, reactive oxygen species; ChyB, β-carotene hydroxylase; TL, thermoluminescence; MDA, malondialdehyde; ELIP, early light inducible protein; NPQ, non-photochemical quenching; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; A, antheraxanthin; V, violaxanthin; Z, zeaxanthin.
LHCII-bound Zeaxanthin and Oxidative Stress Resistance

Zeaxanthin also appears to have a role in establishing tolerance to photooxidative stress by an NPQ-independent process, which protects the lipids of the thylakoid membrane from oxidative damage (14). Havaux and Niyogi (15) found that the Arabidopsis npq4 mutant, devoid of the PsbS protein, and so lacking the rapidly reversible component of NPQ, was still able to protect itself from lipid peroxidation, whereas the npq1 mutant, lacking a functional violaxanthin de-epoxidase enzyme and so devoid of zeaxanthin, suffered extensive oxidative lipid damage upon exposure to specific conditions of high light and low temperature. The combination of zeaxanthin and the lipophilic compound α-tocopherol that is constitutively present in the thylakoid membrane has been shown to exert a synergistic protection against lipid peroxidation in vitro (16). This effect was explained in terms of prevention of carotenoid consumption by effective scavenging of free radicals by tocopherol, therefore allowing zeaxanthin to effectively quench the primary oxidant \( {\text{O}}_2^\bullet \) in npq1, which lacks zeaxanthin, the synthesis of α-tocopherol is enhanced (17), whereas in the vte1 mutant, which lacks α-tocopherol, the accumulation of zeaxanthin is enhanced (18). In the vte1 mutant lipid peroxidation was more pronounced than in wild-type and in a npq1vte1 double mutant it was further enhanced, suggesting functional overlap between zeaxanthin and α-tocopherol as antioxidants (18).

A key to understanding the inter-relationship and mechanism of these two protective functions of zeaxanthin is knowledge of where it is located within the thylakoid membrane. Upon solubilization of thylakoid membranes, it was found that virtually all of the xanthophyll cycle carotenoids were bound at peripheral, detergent-labile sites on LHCII proteins, not free in the lipid phase (19), a finding confirmed by the structural model of LHCII, which clearly defines the V1 violaxanthin binding site at the protein surface (20). Application of resonance Raman spectroscopy to intact thylakoids has shown severe distortion of the structure of these carotenoids implying that they are tightly bound to protein (21, 22). However, it has been postulated that under sustained photooxidative stress conditions zeaxanthin is released from its binding site on LHCII to interact with lipids and prevent oxidative damage (23).

Study of the npq mutants has provided valuable insight into the relative importance of the dual roles of zeaxanthin in stress protection. In particular, the constitutive presence of an elevated level of zeaxanthin in npq2lut2 and npq2lor2 mutants was associated with decreased lipid peroxidation (24, 25). An alternative approach that represents a better physiological model is to test the effect of an increased level of zeaxanthin in plants that both retain a functional xanthophyll cycle and have otherwise an unaltered carotenoid composition. Moreover, such an approach allows an assessment of whether the xanthophyll cycle is a limiting factor in the stress response whose increase would enhance stress tolerance. The xanthophyll cycle carotenoids accumulate via the hydroxylation of the two β-rings of β-carotene by one of three β-ring hydroxylase enzymes present in Arabidopsis (26–29). Overexpression of a sense β-carotene hydroxylase 1 gene (sChyB) under the control of a constitutive promoter in Arabidopsis doubled the size of the pool of xanthophyll cycle carotenoids with very little perturbation in the levels of other carotenoids (30). The tolerance of the sChyB plants to high light and high temperature stress was enhanced and there was evidence for less lipid peroxidation relative to wild-type plants. However, no detailed study was made to determine the mechanism of this increased stress tolerance or indeed to find out where the extra zeaxanthin is located under stress conditions in the sChyB plants.

In this paper we have used sChyB plants to investigate how a larger xanthophyll cycle pool size affects tolerance to photooxidative lipid damage and photoinhibition under the most potent conditions of oxidative stress: high light and low temperature. The data presented corroborate the hypothesis that zeaxanthin can protect against lipid damage by an antioxidant mechanism distinct from rapidly reversible NPQ and this theory is developed further by advocating a key role for LHCII in the process as a zeaxanthin-binding protein.

**MATERIALS AND METHODS**

*Arabidopsis thaliana* (ecotype C24) expressing a sense construct of β-carotene hydroxylase 1 (AT4G25700 (ChyB)) have been previously described (30). Plants were grown in a Conviron-controlled environment growth cabinet using fluorescent tube lighting maintained at control conditions, 23/18°C (day/night air temperature), at a photon flux density of 100 μmol of photons \( \text{m}^{-2} \text{s}^{-1} \) (8 h photoperiod). Light stress was imposed by transferring plants aged 5 weeks to a growth chamber at 7.5/12°C (day/night air temperature) and under a photon flux density of 1000 or 1600 μmol of photon \( \text{m}^{-2} \text{s}^{-1} \) (8 h photoperiod). Leaf samples were always taken at the beginning of the photoperiod, after 2 h of illumination.

For analysis of NPQ, fluorescence was measured with a pulse-modulated PAM-101 chlorophyll fluorometer (Heinz Walz). The plants were adapted in the dark for 30 min prior to measurement. Fluorescence quenching was induced by 15 min of actinic illumination with white light obtained from a Schott KL1500 lamp at 1000 μmol of photons \( \text{m}^{-2} \text{s}^{-1} \). The maximal fluorescence in the dark-adapted state (Fm) and during the course of actinic illumination (Fm') and the subsequent dark relaxation period were determined by a 0.8-s saturating (4000 μmol of photons \( \text{m}^{-2} \text{s}^{-1} \)) light pulse applied at 1–2-min intervals. NPQ was defined as ((Fm – Fm')/Fm'). Fv/Fm was measured using a Mini-PAM chlorophyll fluorometer (Heinz Walz) in the growth cabinet under stress conditions after dark adaptation with metal leaf clips (Heinz Walz).

Autoluminescence imaging of spontaneous photon emission in the ultraviolet and visible range was carried out using a highly sensitive charge-coupled device camera, with a liquid N\(_2\) cooled sensor to reduce thermal noise and enable measurement of faint light by signal integration (31). Lipid peroxidation-related luminescence was measured by thermoluminescence (TL) as a strong band peaking at high temperature, 135°C. TL measurements were performed on leaf disks (diameter, 1 cm) with a custom-built apparatus, as previously described (32). The leaf sample was slowly heated from 25 to 150°C at a rate of 6°C min\(^{-1}\).

Lipid peroxidation was assessed indirectly by measuring malondialdehyde (MDA) using HPLC as previously described (18). The sample (3 leaf disks of 12 mm in diameter) was ground
in 1 ml of chilled ethanol:water (80:20, v/v). After centrifugation, 750 ml of the supernatant was mixed with 750 ml of the following reaction mixture: 20% trichloroacetic acid, 0.01% butylated hydroxytoluene, and 0.65% thiobarbituric acid (TBA). After heating at 95 °C for 20 min and centrifugation, the MDA-(TBA)_2 adduct was separated and quantified by HPLC. The analytical column and the HPLC apparatus were similar to those described in Havaux et al. (18). The elution buffer was 85% 50 mM KH2PO4-KOH, pH 7.0, and 35% methanol. The time of chromatography was 12 min, with a flow rate of 0.8 ml min⁻¹, an injected volume of 120 ml, and detection at 532 nm. The average retention time of the MDA-(TBA)_2 adduct was 8 min. The levels of MDA were calculated using tetraethoxyspropane (Sigma) as a standard.

Lipid peroxidation (formation of lipid hydroperoxides) was assessed by HPLC analysis of hydroxy fatty acids recovered from plant tissue after NaBH₄ reduction and saponification of total lipids. Arabidopsis leaves from four plants were mixed and sampled (4 × 0.5–1 g), frozen in liquid N₂, and stored at −80 °C before extraction. Extraction was carried out according to the previously described procedure (33). An aliquot of the extract (50 μl) was submitted to straight phase HPLC (Waters, Millipore, St Quentin-Yvelines, France) using a Zorbax rx-SIL column (4.6 × 250 mm, 5-μm particle size, Hewlett Packard, Les Ulis, France), isocratic elution with 70:30:0.25 (v/v/v) hexane: diethyl ether:acetic acid at a flow rate of 1.5 ml min⁻¹, and UV detection at 234 nm. ROS-mediated lipid peroxidation was evaluated from the levels of the different hydroxyoctadecatetraenoic acid isomers as previously described using 15-hydroxy-11,13-(Z,E)-eicosadienoic acid as internal standard (33).

For analysis of pigments and tocopherols 1-cm leaf disks were taken from fully expanded mature leaves and immediately frozen in liquid N₂ and stored at −80 °C until use. Leaf disks were ground in 400 μl of 100% acetone. Pigments from FPLC fractions were first subjected to a phase separation, 0.5-ml samples were combined with 0.5 ml of ethanol and 1 ml of diethyl ether. The colored organic phase containing the pigment was then carefully extracted using a syringe and dried down under a jet of N₂. The dried pigment was resuspended in 200 μl of 100% acetone.

Pigment composition was determined by HPLC using a Dionex reverse phase C18 column and Dionex chromatography system. The solvent system was solvent A: 87% acetonitrile, 10% methanol, 3% 0.1 m Tris, pH 8; solvent B: 80% methanol, 20% hexane. The gradient from solvent A to solvent B was run from 9 to 12.5 min at a flow rate of 1 ml/min. Each peak was integrated at its optimum absorbance and analyzed using Dionex Chromelone software. The system was calibrated using chlorophyll and carotenoid standards of known concentration, obtained from DHI, Hørsholm (Denmark). Tocopherols were assayed using the same HPLC and solvent system and detected using a Dionex fluorimeter, excitation at 295 nm and emission at 340 nm. Peaks were integrated using the Chromelone software and compared with standards of known concentration obtained from Sigma.

Thylakoid proteins were analyzed using immunoblotting essentially as described by Ganeteg et al. (34). Thylakoids prepared as described in Ruban et al. (35) were solubilized in 250 μl of 2× Laemmli buffer, the samples were incubated at 85 °C for 30 min and then proteins were separated by 15% denaturing SDS-PAGE. 0.2 to 2 μg of chlorophyll was loaded per lane. Chlorophyll concentration was determined using the method of Porra et al. (36). Primary antibodies for immunodectection were obtained from S. Jansson (Umeå, Sweden) and were detected using a horseradish peroxidase-linked secondary donkey anti-rabbit antibody, ECL Plus detection reagent, and Hyperfilm ECL photographic film (Amersham Biosciences). The developed film was digitalized and analyzed by the Image Master gel documentation system (Amersham Biosciences) equipped with a Umax Powerlook III high-resolution scanner and one-dimensional software package. For SDS-PAGE analysis of the FPLC fractions 50 μl of each fraction was combined with 2× Laemmli buffer and incubated at 85 °C for 30 min, followed by separation on a 15% denaturing gel. 10 μl of sample were loaded in each lane, alongside broad-range molecular weight markers “Benchmark” (Invitrogen). Protein bands were stained using a silver stain kit (Bio-Rad).

For FPLC analysis, unstacked thylakoid membranes were prepared from either stressed treated or control plants according to Ruban et al. (35), except that MgCl₂ was omitted from all buffers. Samples were diluted to a final Chl concentration of 1.0 mg ml⁻¹ and solubilized by the addition of n-dodecyl β-D-maltoside to a final concentration of 0.5%. The samples were vortexed thoroughly for 1 min, left to stand on ice for 10 min, and then centrifuged for 1 min at 16,000 × g. The super-
natant was then filtered through a 0.45-μm nylon filter and subjected to gel filtration chromatography using a Superdex 200 HR 10/30 column in an Amersham Biosciences ÄKTApuriflr system run at a flow rate of 0.4 ml min⁻¹ using a running buffer containing 20 mM BisTris, 0.03% n-dodecyl β-D-maltoside, pH 6.5, 0.5-ml fractions were collected.

Low temperature resonance Raman spectra were obtained in a helium flow cryostat (Air Liquide, Paris, France) using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid N₂-cooled charge coupled device detector (Spectrum One, Jobin-Yvon, Paris, France) as described by Robert et al. (37). Excitation was provided by Coherent Argon laser (Innova 100) at 488.0 and 514.5 nm, respectively. The choice of wavelength was determined by the absorption maxima of the xanthophyll cycle carotenoids in low temperature absorption spectra (21).

RESULTS

Overexpression of β-Carotene Hydroxylase 1 Increases Resistance to Lipid Peroxidation—Wild-type plants and two independently transformed line sChyB plants grown at low light and moderate temperature (100 μmol of photons m⁻² s⁻¹, 22 °C day/18 °C night, 8 h photoperiod) were tested for their tolerance to a strong photooxidative stress that combined high light and low temperature (1600 μmol of photons m⁻² s⁻¹, 7.5 °C day/12 °C night, 8-h photoperiod). Prior to stress treatment there was no visible difference between the wild-type and sChyB plants (Fig. 1A). However, within 1 and a half days of imposition of this stress the wild-type plants began to exhibit the symptoms of oxidative stress, shriveling and necrosis of leaves (Fig. 1B). In contrast, the plants of both sChyB lines remained healthier. The degree of photooxidative stress in whole plants was investigated by autoluminescence imaging. This technique measures the faint light emitted spontaneously by plants and originating from singlet oxygen and excited triplet state carbonyl groups (31), the by-products of the slow spontaneous decomposition of lipid peroxides and endoperoxides (4). As can be seen in Fig. 1, C and D, the visual symptoms of photooxidative stress seen were reflected in the autoluminescence signal detected in each plant; the wild-type plants were highly luminescent indicative of widespread lipid peroxidation,
whereas the sChyB plants showed much reduced autoluminescence, with signs of oxidative damage only at the margins of the oldest leaves (Fig. 1D, arrows).

To confirm that the increased autoluminescence in wild-type plants relative to sChyB was indeed related to increased lipid peroxidation, various biophysical and biochemical techniques...
**RESULTS**

To establish that the observed lipid peroxidation was caused by ROS generated as a result of stress treatment the content of lipid hydroperoxides was measured. This allows the degree of peroxidation caused by ROS to be distinguished from that resulting from lipid peroxidation caused by the activity of lipooxygenases, which may be related to stress signaling (41). The amount of peroxidized fatty acid isomers generated from ROS was 56% higher in the wild-type than in either \( sChyB \) line (Fig. 2, A and B). These observations were also confirmed by HPLC assay of the levels of MDA, a 3-carbon low molecular weight aldehyde by-product of free radical attack on polyunsaturated fatty acids. The MDA content in the \( sChyB \) was reduced by 57% relative to wild-type plants (Fig. 2C).

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\( sChyB \) plants accumulated over twice the level of zeaxanthin present in wild-type leaves under photooxidative stress and show a reduction in chlorophyll a loss—To investigate the changes in composition and function of the thylakoid membranes during the development of the stress response it was necessary to use a milder treatment. By reducing the irradiance from 1600 to 1000 \( \mu \)mol of photons m\(^{-2}\) s\(^{-1}\), plants could be studied over a period of several days. This treatment resulted in the onset of the symptoms of oxidative stress after 2 days (data not shown). In both the wild-type and \( sChyB \) plants there was an increase in the size of the xanthophyll cycle pool after 48 h of stress treatment (Fig. 3A). This increase was larger for the wild-type than for \( sChyB \), so that the difference between them was less than 2-fold for stressed plants compared with nearly 3-fold in the controls (Table 1). Within 1 h of stress, violaxanthin was de-epoxidized to zeaxanthin and antheraxanthin via the action of violaxanthin de-epoxidase. In the \( sChyB \) plants there was a greater accumulation of zeaxanthin (Fig. 3B) which, after 3 days, was doubled on a chlorophyll basis relative to wild-type (Table 1). Clearly, a large proportion of the extra violaxanthin in \( sChyB \) was accessible to the violaxanthin de-epoxidase enzyme. It should be noted, however, that despite the higher concentration of zeaxanthin in \( sChyB \), the de-epoxidation state (calculated from the proportions of the xanthophyll cycle pigments \([Z + 0.5A]/[V + A + Z]\)) was essentially the same as in wild-type plants (Fig. 3E, Table 1). There was a decrease in the de-epoxidation state in both plant types during the 3-day stress treatment, which arose not because of a decrease in the total amount of zeaxanthin (Fig. 3B), but rather an increase in violaxanthin and antheraxanthin (Fig. 3, C and D). There was a significant decrease (t test: 95% confidence) in \( \beta \)-carotene content in both wild-type and \( sChyB \) plants after 3 days of stress (Table 1). \( sChyB \) plants had a 15% lower \( \beta \)-carotene content on a chlorophyll basis prior to stress treatment as previously reported (31).

Table 1 also shows that stress treatment caused changes in the content of chlorophylls and tocopherols. On a leaf area basis, the initial chlorophyll content of \( sChyB \) was less than the wild-type. There was a decrease in the content of both chlorophyll \( a \) and chlorophyll \( b \) in both plant types upon stress treatment, the loss of chlorophyll \( a \) being proportionately larger (t test: 90% confidence) in the wild-type than in \( sChyB \). The content of \( \alpha \)-tocopherol increased after stress in both wild-type and \( sChyB \) plants, and there was also some increase in \( \gamma \)-tocopherol. Significantly the \( \alpha \)-tocopherol content of the wild-type plants was 25% higher (t test: 95% confidence) than in the \( sChyB \) plants after stress treatment (Fig. 3F).

**TABLE 1**

Pigment and tocopherol composition of wild-type and \( sChyB \) plants under control and stress conditions

| Pigment           | Control | Stress | Control | Stress | Control | Stress |
|-------------------|---------|--------|---------|--------|---------|--------|
| Chlorophyll \( a \) | ND      | 0.31   | ND      | 0.43   | ND      | 0.17   |
| Chlorophyll \( b \) | 0.23    | 0.24   | 0.25    | 0.19   | 0.26    | 0.21   |
| Chlorophyll \( a/b \) | 1.45    | 1.48   | 1.46    | 1.51   | 1.53    | 1.52   |

\( * \)Significantly different by Student’s \( t \) test from respective wild-type sample, 95% confidence limits.
\( \dagger \)ND, not detected.
\( \ddagger \)Significantly different by Student’s \( t \) test from respective control sample, 95% confidence limits.
\( \ddagger\ddagger \)Significantly different by Student’s \( t \) test from respective wild-type sample, 90% confidence limits.

were used. First, using TL, the degree of lipid peroxidation in leaf disks harvested from stressed plants was accurately quantified from the emission band at 135 °C (27). The amplitude of this TL band is related to the amount of lipid cycloperoxides that are broken down during heating, leading to the formation of carbonyl species in the excited triplet state (38). Previous studies have correlated this amplitude with other indices of lipid peroxidation (15, 38–40). After stress treatment, this band was found to be ~70% higher in the wild-type than in either \( sChyB \) line (Fig. 2, A and B). These observations were also confirmed by HPLC assay of the levels of MDA, a 3-carbon low molecular weight aldehyde by-product of free radical attack on polyunsaturated fatty acids. The MDA content in the \( sChyB \) was reduced by 57% relative to wild-type plants (Fig. 2C).

The amount of peroxidized fatty acid isomers generated from ROS was 56% higher in the wild-type than in \( sChyB \) (Fig. 2D). Lipooxygenase-dependent lipid peroxidation was not induced by stress treatment (data not shown). Together, these data conclusively show that, whereas under control conditions the plants show no significant differences in background lipid peroxidation, when exposed to photooxidative stress wild-type plants suffer far more lipid peroxidation than \( sChyB \) plants.
was not significantly different when measured after a 15-min exposure to an actinic light intensity of 1000 μmol of photons m⁻²s⁻¹, with values of 2.03 ± 0.10 in wild-type and 1.90 ± 0.17 in sChyB recorded at room temperature. The values of NPQ formed by illumination at low stress temperature were 2.42 ± 0.13 and 2.11 ± 0.22, respectively. During stress treatments, NPQ was further investigated by measurement of the reversibility of the decline in Fv/Fm: the proportion of Fv/Fm recovering within 30 min upon restoration of the plants to low light at room temperature is the result of rapidly relaxing NPQ, whereas the proportion that did not recover represents oxidative damage to PSII. During the first few hours of stress treatment the Fv/Fm declined rapidly by about 50% in both wild-type and sChyB plants (Fig. 4A). This was followed by a slower rate of decline, which was more exaggerated in the wild-type than in sChyB. After 2 h of stress treatment, most of the decline in Fv/Fm was reversible, in both plant types (Fig. 4B). The reversibility at 24 h was much less, but again not significantly different in sChyB compared with wild-type. However, after 64 h of stress the wild-type Fv/Fm was not only significantly lower (t test: 90% confidence), but it recovered less, from 0.26 to 0.34, whereas in sChyB, Fv/Fm recovered from 0.38 to 0.51.

Immunoblotting was used to further investigate possible differences in function of wild-type and sChyB plants after stress (Fig. 4C). A 25% net loss of D1 protein was detected, but there was no difference between the responses of wild-type and sChyB plants. There was a small (approximately 15%) increase in the level of PsbS protein in both wild-type and sChyB, but the level in each was not significantly different. The early light inducible proteins (ELIPs) accumulated under stress: ELIP1 and ELIP2, possibly implicated in sustained energy dissipation (42), both increased, but again the content was not significantly different in sChyB when compared with the wild-type.

**The Extra Zeaxanthin in sChyB Plants Is All Bound to Protein within an Oligomeric LHCII-enriched Domain**—The location of the xanthophyll cycle carotenoids in the thylakoid membranes of wild-type and sChyB plants was investigated, before and after exposure to 2 days of stress treatment. Because the xanthophyll cycle carotenoids are susceptible to removal from their protein-binding sites upon detergent treatment (19), extremely careful procedures are needed to determine the extent of binding to the various pigment protein complexes of the thylakoid membrane. A rapid and gentle procedure, using a low concentration of n-dodecyl-β-D-maltoside to solubilize thylakoid membranes has been used to localize xanthophyll cycle carotenoids to an oligomeric LHCII fraction in spinach (19). This approach was used here, thylakoids being prepared from stressed and control plants from each line, but using FPLC rather than sucrose gradient centrifugation to resolve the resulting fractions, thereby greatly reducing the length of exposure of the sample to detergent.

Fig. 5A and B, present FPLC elution profiles recorded at 445 nm to detect both chlorophyll and carotenoid containing complexes in solubilized control thylakoids. In both WT and sChyB plants four distinct bands were obtained, which were present both in stressed and unstressed thylakoids (data not shown). Samples collected during elution were analyzed by SDS-PAGE (Fig. 5C and D) and by observation of their absorption spectra.
(data not shown). Whereas the first three bands contained the PSI core and LHCII proteins (I and II) and PSII core proteins (III), all of the LHCII polypeptides were found in band IV, a broad peak with a retention time around 30 min. The molecular weight of this band was higher than that of solubilized LHCII trimers (dotted lines, band V), and therefore more likely to be the oligomeric LHCII particles as described previously (19, 22). Negligible absorbance was detected in the regions where monomeric Lhcb proteins (VI) or free pigment (VII) would elute. The gel profile of the oligomeric LHCII band revealed a number of polypeptides in the 25-kDa region, suggesting that the Lhcb proteins of trimeric LHCIIb and monomeric CP24, CP26, and CP29 were present (Fig. 5, C and D), confirmed using immunoblotting against Lhcb1 and Lhcb3–6 antibodies (Fig. 5E). In addition, ELIPs 1 and 2 were detected in the LHCII oligomeric band, but only in the samples isolated from the stressed plants. To further investigate the composition of the oligomeric LHCII band a second separation was carried out by FPLC, using the detergent present in the running buffer. Fig. 5, A and B, shows the results of this separation, which yielded as expected a very small PSII core contamination (arrow), along with a large peak containing trimeric LHCII (V) and a smaller peak comprising monomeric Lhcb proteins (VI). The pigment composition was consistent with the protein composition of the oligomeric band: the chlorophyll a/b ratio was found to be 1.61, lower than that of an PSII-LHCII supercomplex, but higher than that of an LHCII trimmer; virtually all of the neoxanthin and chlorophyll b was associated with the oligomeric LHCII fractions (data not shown); and the fraction contained β-carotene and large amounts of lutein (Table 2).

Fig. 5F presents a distribution profile of the total amount of violaxanthin in the solubilized control fractions from both wild-type and sChyB plants, whereas Fig. 5G presents zeaxanthin distribution in the stressed plants. In the wild-type, 83% of the violaxanthin was bound in the LHCII oligomeric band in the control, rising to 90% of zeaxanthin in the stressed sample. In sChyB, 90% of violaxanthin and 90% of zeaxanthin were bound to this band in the control and stressed samples, respectively. Specifically, in thylakoids from both the non-stressed control and stressed plants all of the xanthophyll cycle carotenoids were bound by protein and no pigments were detected in fractions 12 and 13 where free pigment would elute. The remaining 10–15% of xanthophyll cycle carotenoids present eluted with the PSI-LHCI fractions in both wild-type and sChyB.

Table 2 presents the pigment composition of the fractions making up the oligomeric LHCII band. The pool of xanthophyll cycle carotenoids was ~3 times larger in sChyB compared with the wild-type in both stressed and unstressed plants. After stress treatment, the pool size increased by 25% in wild-type and by 27% in sChyB. 52% of the xanthophyll cycle pool was present in a de-epoxidized form under stress in wild-type and sChyB. This de-epoxidation state was higher than that in the PSI-LHCI fractions where values of 25% in wild-type and 28% in sChyB were measured (not shown). An increase in the lutein content of ~15% was seen in the LHCII oligomers in both wild-type and sChyB plants under stress, whereas the neoxanthin content decreased by ~10%. After fractionation of the oligomeric band into its constituent trimers and monomers (Fig. 5, A and B), over 95% of the zeaxanthin was still bound: zeaxanthin distribution between the trimer, monomer, and free pigment bands was, respectively, 57, 36, and 3% in WT, and 66, 31, and 1% in sChyB.

**Binding of Zeaxanthin to LHCII in Whole Thylakoids Prepared from Stressed Plants Is Confirmed by Resonance Raman Spectroscopy**—To confirm the conclusions made from the biochemical experiments outlined above, the binding of the xanthophyll cycle carotenoids was probed in vivo in intact thylakoids using resonance Raman spectroscopy. This method allows selective excitation of specific pigments using a series of narrow laser lines to assess their configuration and state of binding within intact membranes (37). Resonance Raman spectra were collected after excitation at each wavelength and control-minus-stressed or stressed-minus-control difference spectra were calculated for both wild-type and sChyB thylakoids. The H region of the resulting spectra, which arises from C-H bond wagging modes normally resonance forbidden in planar carotenoids, was used as a fingerprint of protein binding (19, 22, 43). Difference spectra at 488 nm, where violaxanthin is in resonance, confirms that the extra violaxanthin is bound in sChyB and that this binding gives an identical fingerprint to that of the wild-type (Fig. 6A). Comparison of the difference spectra at 514 nm, where zeaxanthin is in resonance, shows that the extra zeaxanthin in sChyB is also all bound (Fig. 6B).
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**FIGURE 6.** Analysis of thylakoid membranes of wild-type and sChyB by resonance Raman spectroscopy. Control-minus-stressed difference spectra in the v4 region at 488.0 nm (violaxanthin in resonance) and stressed-minus-control spectra at 514.5 nm (zeaxanthin in resonance) for wild-type and sChyB thylakoids. The dashed lines are the spectra of violaxanthin and zeaxanthin as free pigments in lipid/detergent micelles. Stress treatment was given for 3 days, as described in the legend to Fig. 3, before thylakoid isolation. Spectra were normalized to the amplitude of the v3 band (37).

![Image](22614.png)

6B). The thylakoid spectra are very different from those of zeaxanthin and violaxanthin as free pigments, which displayed rather flat featureless spectra (Fig. 6, dashed lines). There is an enhanced structure in the thylakoid spectra of zeaxanthin compared with that of violaxanthin, and this is accompanied by increases in amplitude, which was estimated relative to the intensity of the v1 band in the Raman spectrum, a region not affected by binding (37). The v4/v1 ratio for 488 nm excitation (violaxanthin) was ~0.04 for both wild-type and sChyB, whereas for 514 nm excitation (zeaxanthin) it was ~0.07, almost 2 times higher. This suggests not only that the pigments are equally bound in the wild-type and sChyB plants, but that there could be a stronger association of zeaxanthin to proteins than for violaxanthin.

**DISCUSSION**

The Xanthophyll Cycle Content Limits the Tolerance of Arabidopsis to Oxidative Stress—Previously it has been shown that reduction in the level of zeaxanthin increases the sensitivity of plants to photooxidative stress (for review, see Refs. 44 and 45), whereas constitutive presence of an enhanced zeaxanthin content was associated with increased stress tolerance (24, 25). The sChyB plants used here not only had an elevated zeaxanthin content, but possessed a functional xanthophyll cycle and had little other change in thylakoid composition compared with wild-type plants. These plants therefore presented an ideal model to study the effects of a larger xanthophyll cycle pool on the response of Arabidopsis plants when challenged with photooxidative stress induced by high light and low temperature. The larger pool size resulted in a dramatically enhanced tolerance to the photooxidative stress. An enhanced tolerance of a high temperature/high light stress was previously found for the sChyB plants (30). Here, the tolerance to photooxidative stress conferred by increased zeaxanthin content in the sChyB plants was manifested in several ways: first, by reduced chlorophyll a bleaching; second, by an increased resistance to lipid peroxidation; and third, by a reduced level of photoinhibition determined from the irreversible decline in Fv/Fm.

Plants grown under excess light usually have a larger xanthophyll cycle pool size than plants grown under light limiting conditions (8, 46, 47). Interestingly, both the wild-type and sChyB plants showed a larger pool size under stressed conditions, yet the difference between them was maintained. Some of this extra xanthophyll could be explained by the utilization of existing β-carotene, possibly becoming available during stress-induced D1 turnover (48). Clearly, not only is the regulation of this pool an important part of the response to oxidative stress, but the response is not saturated in wild-type plants: an increased pool size generated by overexpression of ChyB leads to an improved response to stress. Thus, the ability to form sufficient quantities of zeaxanthin is a limiting factor in the ability of plants to withstand photooxidative stress.

Zeaxanthin Promotes Increased Protection of Thylakoid Membrane Lipids by an NPQ-independent Process—Although the capacity for the rapidly reversible component of NPQ increases in high light grown plants, it appears that the concentration of the PsbS protein is the limiting factor in this response rather than the size of the xanthophyll cycle pool; acclimation of plants to high light has been associated with an increase in PsbS concentration (49, 50). The fact that the amplitude of NPQ can be increased by overexpression of PsbS without any increase in zeaxanthin content indicates that the xanthophyll cycle content is in excess of that needed for fulfillment of its requirement in this process (10). Under the stressed conditions the wild-type and sChyB plants exhibited only a small increase of PsbS level, and there was no difference between wild-type and sChyB. Thus, the observation that the sChyB plants do not exhibit a larger NPQ is consistent with this conclusion: there is no difference in NPQ capacity between wild-type and sChyB plants under either control or stress conditions. This leaves the following questions concerning the role of the xanthophyll cycle in stress tolerance: what is the function of the extra xanthophyll cycle pigment; why does the pool size increase under excess light conditions; and how does a larger pool size confer increased stress resistance? We propose that the answers reside in a second function of zeaxanthin, the protection of thylakoid membrane lipids from oxidative damage, as first proposed by Sarry et al. (14). This protective function of zeaxanthin was shown to operate without the presence of PsbS and independently from NPQ (15). In this study we have obtained conclusive evidence to corroborate this view. We have demonstrated decreased ROS-induced lipid peroxidation in the presence of an enhanced content of zeaxanthin. The levels of α-tocopherol, known to accumulate under photooxidative stress conditions (17) were found to be 25% higher in wild-type compared with sChyB plants after stress treatment. This result is further evidence of a link between these two compounds consistent with compensatory increases in zeaxanthin in the vte1 mutant and tocopherol in npq1, and points to the perturbation of the anti-
oxidant defense system in the thylakoid membranes of sChyB plants.

Accumulation of Zeaxanthin Resulted in Reduced Photo-inhibition—In the sChyB plants the extra zeaxanthin content also conferred an increased resistance to photoinhibition measured by the higher ratio and faster recovery of Fv/Fm. Photoinhibition of PSII occurs at low temperature because photosynthesis and protein turnover are inhibited, leading to the accumulation of damaged PSII centers (51). One mechanism of protection of the PSII reaction center by zeaxanthin at low temperatures is thought to be its involvement in sustained ΔpH-independent energy dissipation, which may involve interactions between ELIP proteins and an aggregated LHClI antenna (42, 52). However, no increase in the levels of ELIPs in sChyB or a larger decrease in Fv/Fm indicative of more sustained quenching was detected. Zeaxanthin may have a role in protecting the reaction center during the D1 repair cycle in a more direct way (53), although the loss of D1 is similar in wild-type and sChyB plants. The apparent increased protection of PSII in sChyB could therefore result from an effect on Fv/Fm not related to less inactivation of PSII reaction centers, but to reduced damage to the antenna. Such an effect would be consistent with the increase in chlorophyll bleaching in the wild-type plants.

90% of the Zeaxanthin Is Bound within the Oligomeric LHClI Antenna under Photooxidative Stress—Critical to an understanding of the mechanism by which zeaxanthin is able to protect the thylakoid lipids from oxidative damage is knowledge of whether these molecules are bound by proteins under stress conditions or if they are free to diffuse in the lipid phase of the membrane. In the previous study on sChyB most of the extra violaxanthin present in unstressed sChyB plants was found to be bound to LHClI (30). However, no data concerning the location of zeaxanthin under stress conditions was reported. In this previous study, a significant increase was also seen in thefree pigment fraction, which may reflect the relatively high detergent concentration used to solubilize the thylakoids prior to and/or during the lengthy analysis by sucrose gradient centrifugation. Here, by application of a rapid but extremely gentle solubilization and separation procedure, we were able to obtain an oligomeric LHClI fraction that retained the in vivo bound complement of xanthophyll cycle carotenoids. Moreover, this analysis was carried out on both control plants containing only violaxanthin, and stressed plants, with a high de-epoxidation state. Remarkably, in both cases, the extra xanthophyll cycle carotenoids present in sChyB plants were virtually all present in the oligomeric LHClI band, with no free pigment detected. This band is very similar in pigment and polypeptide composition to that described by Ruban et al. (19, 22), which was highly enriched in Lhcb proteins and depleted in PSII core proteins. The presence of monomeric minor LHClI components in this band suggests that it is formed by the selective solubilization of PSII cores from the C252M2 LHClI/PSII supercomplex, leaving the LHClI antenna in an oligomeric intact form. Under these conditions, it was previously shown by electron microscopy that the supercomplexes are unstable (54). Thus, we predict that the LHClI oligomer contains 4 LHClI trimers, and two each of CP24, CP26, and CP29.

In the structural model of trimeric LHClIb, 1 neoxanthin and 1 xanthophyll cycle carotenoid is bound by each protein monomer (20). In control wild-type plants under the growth conditions used here the xanthophyll cycle carotenoid to neoxanthin ratio was less than 1 (see Table 2). Therefore, because most of the neoxanthin in the thylakoid membrane is bound to LHClIb, the V1 sites (where xanthophyll cycle carotenoid is bound) must be only partially occupied by violaxanthin in these plants. It is therefore likely that in the sChyB plants the extra xanthophyll cycle carotenoids preferentially fill up these sites. The ratio of xanthophyll cycle carotenoids to neoxanthin in the oligomeric LHClI band gives a predicted V1 occupancy for control plants of 0.58 in wild-type and 1.48 in sChyB. However, the presence of monomeric LHClI complexes, which bind more than one violaxanthin per monomer and substoichiometric amounts of neoxanthin (10, 55, 56) would raise the maximum potential value of this ratio to greater than 1. Given the above suggested composition of the oligomer, and the upper limit of 4.5 violaxanthin per neoxanthin (19) in the three minor complexes, a maximum ratio of 1.5 is predicted. A more conservative estimate of the number of violaxanthin bound by the minor complexes (55), would give a ratio of around 1.3. However, the ratio increases to 0.85 in the wild-type and 2.11 in sChyB under stressed conditions. Here it is possible that there is an increase in the amount of zeaxanthin bound to the lutein sites on minor Lhcb proteins as suggested by Morosinotto et al. (57). Alternatively some zeaxanthin may be bound by other proteins associated with the LHClI oligomer: ELIPS, which have capacity for carotenoid binding (58), were found in the oligomeric band, consistent with previous observations (59). The low molecular mass (10–15 kDa) proteins, SEPS (60) or LHClIe (61) may also bind zeaxanthin, an increase in amount of proteins in this molecular mass range was observed in the SDS gel of the oligomer prepared from stressed plants (see Fig. 5D). It should be emphasized, however, that most of the zeaxanthin was associated with the oligomeric LHClI fraction. Moreover, in both wild-type and sChyB around 90% of it was retained by Lhcb proteins following further fractionation. This suggests that the vast majority of zeaxanthin was bound predominantly at the V1 site of Lhcb proteins, a finding supported by the application of resonance Raman spectroscopy to probe intact thylakoid membranes. Both violaxanthin and zeaxanthin were coordinated in a well defined hydrophobic environment in both wild-type and sChyB plants and the near identity of the wild-type and sChyB spectra for both pigments provided no evidence that the extra pigment in the sChyB plants was bound differently from that in the wild-type.

Prevention of Lipid Peroxidation by Zeaxanthin Involves Interaction with LHClI—We propose that the function of zeaxanthin as an antioxidant preventing thylakoid lipid damage is enhanced by its binding to LHClI. Because all of the extra zeaxanthin in sChyB plants is bound within the LHClI oligomers, the increased resistance to lipid peroxidation is mediated by an increased protection afforded by this antioxidant mechanism, an idea that is supported by data obtained in the chl1 mutant of Arabidopsis (25). This mutant has only trace amounts of LHClI and was found to be highly sensitive to photooxidative stress relative to wild-type plants despite accumulating zeaxanthin.
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The \textit{chaos} mutant defective in thylakoid targeting of LHC-type proteins, including ELIPs is highly sensitive to lipid peroxidation despite unchanged zeaxanthin levels, this sensitivity being rescued by overexpression of ELIPs (62). However, in apparent contradiction, an \textit{elip1elip2} double mutant did not have a lipid peroxidation phenotype (63). These two results can now be reconciled by the zeaxanthin-binding model: perhaps it is the lack of sufficient zeaxanthin-binding proteins (in the LHC superfamily) rather than of any particular binding protein that increases sensitivity to lipid peroxidation in both \textit{ch1} and \textit{chaos} mutants. Because proteins for light harvesting are thought to have evolved from photoprotective ELIPs (64) it seems logical to propose that the apparent ability of these proteins to prevent lipid peroxidation has been retained in the LHC proteins of both photosystems. Thus, the V1 binding site would constitute an ancestral site preserved through the evolution of LHCII from the ELIPs. Interestingly, the thylakoid membrane shares many features with the membranes of the human retina: both contain large quantities of endogenous photosensitizers; both are exposed to high fluxes of incident light and oxygen tension; and both systems contain the same three key antioxidants, zeaxanthin, lutein, and \(\alpha\)-tocopherol. Recently it has been found that zeaxanthin in the retina is bound in a specific manner by xanthophyll-binding proteins (65, 66) and that this binding enhances the antioxidant activity of zeaxanthin in protecting against lipid peroxidation \textit{in vitro} (67).

Binding of zeaxanthin to LHCII may have a role in protection of the specific lipids associated with the complexes. Examination of the structural model of LHCIIb reveals that the xanthophyll cycle carotenoid present in the V1 binding site tightly embraces the bound phospholipid (Fig. 7), which is essential for maintaining the trimeric structure (68, 69). The V1 site is also in close proximity to the bound molecule of digalactosyl diacylglycerol. Thus the principal antioxidant activity of zeaxanthin could be protection of the bound lipids from oxidative damage caused by reactive oxygen species produced in the PSII antenna. The protection of the bound lipid would both preserve antenna stability during photooxidative stress and prevent the initiation of lipid peroxidation chain reactions by cutting out the potential initiator.

Binding to LHCII could also provide an explanation of the differential antioxidant activities of zeaxanthin and violaxanthin. When zeaxanthin and violaxanthin are dissolved in benzene, their rate constants of \(^{1}\text{O}_2\) quenching are not very different (70), suggesting that the differential antioxidant activity stems from other factors. Conversely, it has been proposed that, because of their chemistry, carotenoid epoxide isomers, like violaxanthin, do not bind \(^1\text{O}_2\) and therefore has a low capacity for chemical scavenging of \(^1\text{O}_2\) (71). However, based on our data, we propose that the activity of zeaxanthin is enhanced by the specific nature of its binding to the V1 site on the protein. Thus, the LHC protein, rather like the apoprotein of an enzyme, enhances the reactivity of the bound co-factor, in this case zeaxanthin. Possibly, this enhancement arises from both the distortion of zeaxanthin in this site, shown in our Raman data as well as its close proximity to the lipids. An alternative explanation may be found in the observation that the capacity of zeaxanthin to quench \(^1\text{O}_2\) in lipid micelles decreases at high concentration, a phenomena ascribed to the formation of zeaxanthin aggregates lacking antioxidant activity (72). Thus, binding to LHC proteins may prevent the formation of zeaxanthin aggregates in the lipid phase, maintaining a high efficiency of \(^1\text{O}_2\) quenching. The differential antioxidant effect of violaxanthin and zeaxanthin may then result mainly from their relative strengths or configurations of binding to LHC proteins. The contrasting Raman signatures of violaxanthin and zeaxanthin reported here and observed previously (21) together with the greater resistance of zeaxanthin to extraction from LHCII by detergent (19) provide direct support for differences in binding. The dissimilar hydrophobicities and head group orientations of violaxanthin and zeaxanthin (73) can readily explain such differences.
The stabilization of the thylakoid membrane by zeaxanthin has also been suggested to include a control over membrane fluidity (74). Whereas such an effect could explain the increased tolerance of sChyB plants to heat stress (30), it would provide no simple explanation for the enhanced resistance to cold stress reported here. Although an effect on fluidity was suggested to arise from “free” zeaxanthin in the lipid phase (75), we suggest that it could again arise from its binding to LHCII: as well as an interaction with boundary lipids discussed above, zeaxanthin is known to increase the strength of interaction between LHCII trimers in vitro (76); both could enhance membrane rigidity.

The Dual Function of the Xanthophyll Cycle—We propose that both functions of the xanthophyll cycle, in NPQ and as an antioxidant, involve its binding to proteins of the LHC family. These same differences in properties of violaxanthin and zeaxanthin and the resultant effects on protein binding have also been proposed to explain the role of the xanthophyll cycle in NPQ (77). Thus, the ancestral V1 antioxidant catalytic site could have evolved to also become the NPQ allosteric site. This binding site for zeaxanthin is important because the LHCII/lipid interface is the key site of its antioxidant activity during photooxidative stress. At the same time, de-epoxidation of violaxanthin to zeaxanthin at this site potentiates the pH- and PsbS-dependent transition of the LHCII antenna into the non-photochemically quenched state (12) that enables dissipation of excess excitation energy and hence inhibition of a major source of photooxidative stress. We have shown that the macroorganization of the LHCII antenna is essential for the Raman binding fingerprint of violaxanthin and zeaxanthin (22). It is suggested that this binding, stabilized within the macrostructure that is retained in the isolated LHCII oligomer, is of critical importance to its function. The importance of the thylakoid macrostructure for the formation of NPQ has also been reported (78). Therefore, the dual function of zeaxanthin in photoprotection involves the same conserved binding sites, and may depend on the same features of macroorganization of the thylakoid membrane.

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