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Origin of Absorption Changes Associated with Photoprotective Energy Dissipation in the Absence of Zeaxanthin*

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To prevent photo-oxidative damage to the photosynthetic membrane in strong light, plants dissipate excess absorbed light energy as heat in a mechanism known as non-photochemical quenching (NPQ). NPQ is triggered by the trans-membrane proton gradient (ΔpH), which causes the protonation of the photosystem II light-harvesting antenna (LHCII) and the PsbS protein, as well as the de-epoxidation of the xanthophyll violaxanthin to zeaxanthin. The combination of these factors brings about formation of dissipative pigment interactions that quench the excess energy. The formation of NPQ is associated with certain absorption changes that have been suggested to reflect a conformational change in LHCII brought about by its protonation. The light-minus-dark recovery absorption difference spectrum is characterized by a series of positive and negative bands, the best known of which is ΔA_{535} . Light-minus-dark recovery resonance Raman difference spectra performed at the wavelength of the absorption change of interest allows identification of the pigment responsible from its unique vibrational signature. Using this technique, the origin of ΔA_{535} was previously shown to be a subpopulation of red-shifted zeaxanthin molecules. In the absence of zeaxanthin (and antheraxanthin), a proportion of NPQ remains, and the ΔA_{535} change is blue-shifted to 525 nm (ΔA_{525}). Using resonance Raman spectroscopy, it is shown that the ΔA_{525} absorption change in *Arabidopsis* leaves lacking zeaxanthin belongs to a red-shifted subpopulation of violaxanthin molecules formed during NPQ. The presence of the same ΔA_{535} and ΔA_{525} Raman signatures *in vitro* in aggregated LHCII, containing zeaxanthin and violaxanthin, respectively, leads to a new proposal for the origin of the xanthophyll red shifts associated with NPQ.

To ensure the efficiency of photosynthesis, even under low light conditions, the photochemically active Chl³ of the photosystem II (PSII) reaction center are served by additional antenna Chl bound to light-harvesting complexes (LHCII) (1). However, under certain environmental conditions, the amount of light absorbed by the LHCs is in excess of that which can be used in photochemistry. Left unchecked, the excess absorbed light energy can lead to the formation of Chl triplet states in the PSII reaction center that sensitize the production of singlet oxygen (2–4). Singlet oxygen damages the PSII reaction center and other components of the photosynthetic membrane, leading to a sustained decrease in photosynthetic efficiency that is known as photoinhibition (2). To mitigate this, plants have evolved a photoprotective mechanism, known as non-photochemical quenching (NPQ), whereby the excess excitation energy is safely dissipated as heat (4, 5). The major component of NPQ is controlled by the level of the trans-membrane proton gradient (ΔpH) formed as a result of photosynthetic electron transport and is known as qE (6). ΔpH formation triggers the protonation of LHCII (7) and of the PsbS protein (8), as well as activating the violaxanthin de-epoxidase enzyme, which converts the LHCII-bound xanthophyll violaxanthin into zeaxanthin (9). The interaction of these three factors brings about formation of dissipative pigment interactions within the PSII antenna, thus shortening the Chl excited-state lifetime (10, 11).

The precise nature of the quenching pigment interactions responsible for qE remain under debate (12–17). Despite this, many of the photophysical and photochemical features of qE are well characterized experimentally. It has been shown that qE is associated with a series of absorption changes in both the Soret and the Q_y regions (18–24). These absorption changes have been suggested to reflect an altered environment of bound Chl and xanthophyll molecules, brought about by a protein conformational change within LHCII as a result of ΔpH formation (22). The light-minus-dark recovery absorption difference spectrum in the Soret region contains a series of three negative bands below 500 nm and a positive

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³ The abbreviations used are: Chl, chlorophyll(s); PSII, photosystem II; LHCII, light-harvesting antenna complex of photosystem II; NPQ, nonphotochemical Chl fluorescence quenching; qE, rapidly reversible component of NPQ; ΔpH , proton gradient across the thylakoid membrane; Zea, zeaxanthin; F_m , maximum Chl fluorescence in the dark; F_m' , maximum Chl fluorescence after illumination.

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band at 535 nm (25). The formation and relaxation kinetics of the latter change known as ΔA_{535} are often monitored as a collective measure of these conformational changes. ΔA_{535} was originally believed to arise from selective light scattering brought about by a change in the membrane thickness linked to Δ pH formation (26, 27). Later ΔA_{535} was shown to depend upon the presence of zeaxanthin and was closely correlated with the formation and relaxation of qE (18–20, 22). In the absence of zeaxanthin, qE is greatly reduced and the ΔA_{535} change has a much smaller amplitude and is blue-shifted to 525 nm (ΔA_{525}) (28). The *npq4* mutant of *Arabidopsis* that lacks the PsbS protein was found to lack rapidly reversible qE and also lacked any ΔA_{535} providing a further link between these two phenomena (29). Resonance Raman spectroscopy has proved of use in identifying the origin of absorption bands both in isolated light-harvesting complexes and in intact chloroplasts and even leaves (30). Resonance Raman spectroscopy confirmed that ΔA_{535} belonged to the 0-0 component of the S_2 electronic transition of zeaxanthin (31). The size of the zeaxanthin resonance enhancement in the Raman quenched-minus-unquenched difference spectrum at 528 nm excitation was also found to depend upon PsbS (31). In addition, the negative absorption changes in the qE difference spectrum below 500 nm were associated with a loss of xanthophyll resonance confirming that they also arise from true electronic transitions rather than light scattering (31). Native isolated PsbS was found able to bind zeaxanthin, producing a red shift in its absorption spectrum mimicking ΔA_{535} (32). The putative binding simultaneously affected protein phenylalanine absorption and circular dichroism (CD), leading to the suggestion that ΔA_{535} may arise from zeaxanthin binding to this hydrophobic protein (32). Indeed, it was suggested that ΔA_{535} may represent the formation of a zeaxanthin-PsbS quenching complex responsible for qE (8). However, several observations have brought this proposal into question. Firstly, reconstituted PsbS was consistently found unable to bind any xanthophylls (33, 34). Secondly, PsbS was still found to perform its function in the absence of zeaxanthin (35), and thirdly, the *npq4* mutant lacking PsbS was found to possess ΔA_{535} and wild-type levels of NPQ, albeit both forming on a much longer timescale than the wild-type (36).

Recently, the origin of one of the main negative bands in the qE difference spectrum peaking at 495 nm (ΔA_{495}) was shown to depend upon the presence of lutein (25). In *Arabidopsis* leaves lacking lutein, the band was shifted to 497 nm (in *lut2*⁻, lutein replaced by violaxanthin) and 501 nm (*lut2npq2*⁻, lutein replaced by zeaxanthin) (25). Very similar negative absorption changes to those observed in leaves below 500 nm have been observed upon aggregation of isolated LHCII and also in quenched but non-aggregated LHCII, suggesting that both *in vivo* and *in vitro* types of quenching may have a common origin (37, 38). However, the characteristic ΔA_{535} change was not observed upon LHCII aggregation, although this may be due to the absence of zeaxanthin in these samples (37). Thus, the question of the exact origin of all elements of the qE-related absorption changes remains. It is known that the energy of an electronic transition within a molecule is strongly dependent on the refractive index of the

solvent environment (39, 40). However, such a strong red shift as seen during the ΔA_{535} change would require a very dramatic change in the solvent environment, possibly due to the arrival of a static dipole or a point charge in the vicinity of the zeaxanthin (41, 42). Alternatively, this shift in absorption may arise from excitonic interactions between pairs of zeaxanthin molecules (43). These zeaxanthin pairs would occur as a result of aggregation of LHCII trimers within the thylakoid membrane. Because zeaxanthin occupies a relatively peripheral position within the LHCII trimer (44), aggregation could result in close associations between xanthophylls belonging to neighboring trimers. Indeed, it has been shown that the formation of excitonically coupled J-aggregates of zeaxanthin (co-linear or “top-to-tail” chains of molecules) in water-ethanol mixtures gives rise to a red shift in the 0-0 transition to 535 nm (34, 45, 46), whereas lutein, antheraxanthin, and violaxanthin J-type aggregates possess red-shifted bands between 500 and 530 nm (45). In the following study, we specifically address the origin of ΔA_{525} . Firstly, using resonance Raman experiments on intact chloroplasts lacking zeaxanthin, we identify the xanthophyll responsible for ΔA_{525} as violaxanthin. In addition, we show that the same Raman signature associated with ΔA_{535} and ΔA_{525} *in vivo* is observed upon aggregation of LHCII binding, respectively, zeaxanthin or violaxanthin in the peripheral V1 site.

EXPERIMENTAL PROCEDURES

Fluorescence and Absorption Measurements—*Arabidopsis thaliana* cv Col0 (wild-type) and two mutants derived from it, *npq1* (lacking zeaxanthin) and *L17* (PsbS overexpressor) (47), were grown for 8–9 weeks in Sanyo plant growth rooms with an 8-h photoperiod at a light intensity of 100 μ mol of photons $m^{-2} s^{-1}$ and a day/night temperature of 22/18 °C. The composition of carotenoids was determined by HPLC for leaf disks rapidly frozen in liquid N_2 as described previously (48). Leaves were vacuum-infiltrated with 20 mM HEPES buffer (pH 7.0) containing 5 mM dithiothreitol (DTT) to inhibit violaxanthin de-epoxidation, whereas control leaves were vacuum-infiltrated with buffer only. Chl fluorescence kinetic analyses of whole leaves was carried out using a Dual-PAM-100 fluorometer (Walz) using varying actinic intensity together with light saturation pulses (4000 μ mol of photons $m^{-2} s^{-1}$) as indicated in the figures. The maximum quantum yield of PSII (F_v/F_m) was defined as $((F_m - F_o)/F_m)$, the quantum yield of PSII (Φ PSII) as $((F_m' - F_s)/F_m')$, qP as $((F_m' - F_s)/(F_m' - F_o'))$, and NPQ as $((F_m - F_m')/F_m')$. qE-related absorption changes were recorded on whole leaves using an Aminco DW2000 spectrophotometer as described previously (25), and difference spectra were calculated by subtracting the “dark recovery” spectrum (5 min of preillumination at 1000 μ mol of photons $m^{-2} s^{-1}$ actinic light followed by 5 min of dark relaxation) from the “light” spectrum recorded in the presence of the actinic light following 5 min of preillumination.

LHCII Isolation—Spinach trimeric LHCII binding either violaxanthin or zeaxanthin was isolated from *n*-dodecyl β -D-maltoside solubilized spinach BBY membranes using sucrose gradient ultracentrifugation as described previously (49). De-epoxidation of violaxanthin to antheraxanthin and zeaxanthin

was achieved by incubating the thylakoids (from which BBYs were derived) for 30 min in 0.33 M sorbitol, 1 mM EDTA, 30 mM HEPES, 20 mM MES, 40 mM ascorbate at pH 5.5 at 20 °C. LHCII was desalted to remove sucrose in a PD10 desalting column (GE Healthcare) in a buffer containing 20 mM HEPES (pH 7.8) and 0.03% (w/v) *n*-dodecyl β -D-maltoside. Quenched LHCII was prepared by removal of detergent by SM-2 bioabsorbent beads (Bio-Rad) allowing for a 10 \times reduction in fluorescence yield as determined by a PAM-101 fluorometer (Heinz Walz). Chl concentration was determined according to the method of Porra *et al.* (50).

Resonance Raman Spectroscopy—Low temperature (77 K) resonance Raman spectra were measured on leaves immediately frozen following 5 min of preillumination at 1000 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (light) or alternatively preilluminated and then frozen following an additional period of 5 min of dark relaxation (dark recovery) and on isolated LHCII prepared in the trimeric and aggregated states as described above. Purified xanthophylls prepared as described previously (51, 52) were

dissolved in pyridine for 77 K measurements. Samples were measured in a helium flow cryostat (Air Liquide, France) using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid nitrogen-cooled CCD detector (Spectrum One, Jobin-Yvon, France) as described (51). Excitation was provided by a Coherent Argon (Innova 100) laser (488.0 and 528.7 nm).

RESULTS

The well known light-minus-dark recovery absorption difference spectrum in *Arabidopsis* wild-type leaves is presented in Fig. 1, exhibiting the usual positive maximum at 535 nm consistent with the presence of zeaxanthin (Table 1). In the *npq1* difference spectrum, the positions of the negative bands below 500 nm are virtually identical, whereas the 535 nm maximum is blue-shifted to 525 nm, consistent with the absence of zeaxanthin (and antheraxanthin) (Fig. 1, Table 1). The amplitude of all bands in the difference spectrum is also smaller in *npq1* than in the wild type, consistent with the lower NPQ in the former sample (Fig. 1). Using resonance Raman difference spectroscopy, the origin of the 525 nm band in the *npq1* absorption difference spectrum was probed. Two Raman spectra were recorded using 528.7 nm excitation, one for leaves frozen under illumination (5 min of preillumination at 1000 μmol of photons $\text{m}^{-2} \text{s}^{-1}$) and the second one frozen after a further 5 min of dark recovery. A light-minus-dark recovery Raman difference spectrum should then reveal a selective gain of resonance for the xanthophyll species responsible for the absorption difference (30). However, although a resonance gain was detected in the *npq1* leaves, the signal-to-noise ratio was such that it was impossible to identify accurately the xanthophyll species responsible (data not shown). Thus, to identify the origin of the ΔA_{525} band, we used the L17 mutant that overexpresses PsbS and thus has a much higher level of NPQ when compared with the wild type (47). It has been shown previously that PsbS overexpression in this mutant also enhances qE in the absence of zeaxanthin (35). Using the same principle, leaves were vacuum-infiltrated with DTT, an inhibitor of xanthophyll cycle activity, such that no zeaxanthin (or antheraxanthin) was formed during illumination (Table 1). In these leaves, the level of ΔA_{525} was enhanced by around a factor of 2 when compared with *npq1*, reflecting the larger NPQ, although there was no difference in the de-epoxidation state between the two samples (Fig. 1). Consistent with this enhanced ΔA_{525} , the light-minus-dark recovery resonance Raman difference spectrum for L17 leaves treated with DTT had a significantly improved signal-to-noise

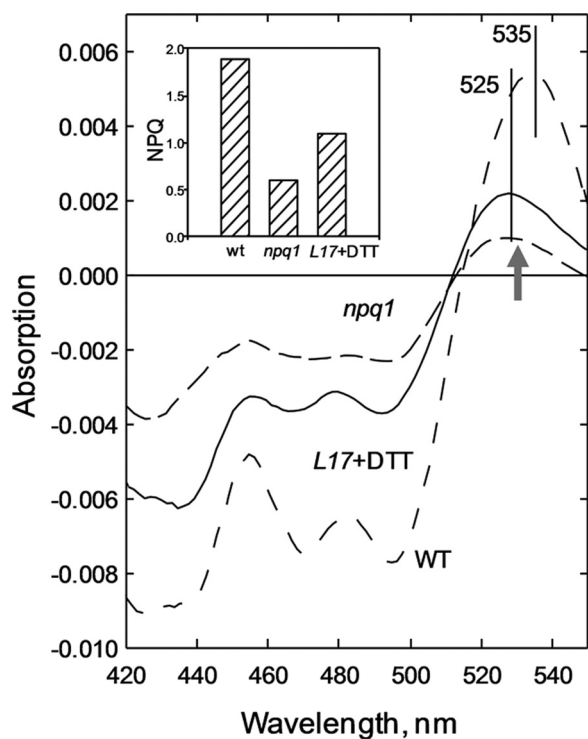


FIGURE 1. Light-treated-minus-dark recovery absorption difference spectra in *Arabidopsis* leaves. Wild-type *npq1* and L17+DTT leaves were measured immediately after 5 min of illumination at 1000 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ (light-treated) or following a further 5 min of dark relaxation (dark recovery). *Inset*, amplitude of NPQ for each sample, \pm S.E.

TABLE 1

Pigment composition of *npq1* and L17 + DTT leaves

Samples were taken from leaves, either dark-adapted for 120 min or after 5 min of illumination at 1000 μmol of photons $\text{m}^{-2} \text{s}^{-1}$. Data are normalized to 100 Chl *a* + *b* molecules and are means \pm S.E. from four replicates. Neo, Lut, Vio, Ant, Zea, DEPs and Chl *a/b*: neoxanthin, lutein, violaxanthin, antheraxanthin, zeaxanthin, de-epoxidation state ($Z + 0.5A$)/($V + A + Z$), and Chl *a/b* ratio, respectively; ND, none detected.

Plant	Neo	Lut	Vio	Ant	Zea	DEPs	Chl <i>a/b</i>
						%	
WT dark	5.2 \pm 0.5	17 \pm 1	4.4 \pm 0.2	0.2 \pm 0.1	0	4 \pm 0.9	3.1
WT light	5.1 \pm 0.9	16 \pm 1	2.0 \pm 0.4	0.8 \pm 0.3	1.7 \pm 0.4	46 \pm 1.2	3.1
<i>npq1</i>	5.3 \pm 0.9	19 \pm 2	4.4 \pm 0.8	ND	ND	0	3.1
L17 + DTT dark	5.2 \pm 0.6	16 \pm 1	4.4 \pm 0.2	ND	ND	0	3.1
L17 + DTT light	5.2 \pm 0.5	17 \pm 2	4.6 \pm 0.4	ND	ND	0	3.1

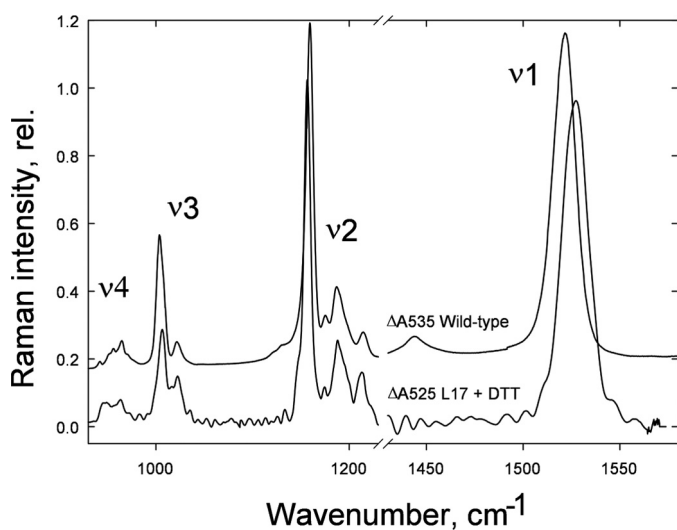


FIGURE 2. Comparison of the qE-associated Raman spectrum (difference spectrum of light-treated-minus-dark recovery) induced by 528.7 nm excitation in *Arabidopsis* leaves from wild type and L17 treated with DTT. *rel.*, relative.

ratio. Thus, a meaningful comparison with individual pigment spectra was possible, allowing us to attribute the band(s) (see below).

Fig. 2 presents the total light-minus-dark recovery resonance Raman difference spectrum using 528.7 nm excitation of L17 leaves treated with DTT. The four characteristic frequency regions of carotenoids have been assigned as follows: ν_1 , C=C stretching mode; ν_2 , C–C stretches coupled with C–H in-plane bending or C–CH₃ stretching; ν_3 , CH₃ in-plane rocking vibrations; ν_4 , C–H out-of-plane bending modes (30). The light-minus-dark recovery resonance Raman difference spectrum at 528.7 nm excitation of wild-type leaves containing zeaxanthin had a ν_1 position at 1522 cm⁻¹ (Fig. 2), consistent with the position of isolated zeaxanthin in pyridine as observed previously (31). However, the ν_1 position in spectra of L17+DTT leaves was significantly different, peaking at 1528 cm⁻¹. Because the ν_1 position is highly dependent upon the conjugation length of the C=C chain (conjugated double bonds) of a carotenoid, lutein, violaxanthin, and neoxanthin have respectively higher ν_1 positions (51). In the case of neoxanthin, the presence of a 9-*cis* configuration results in a further up-shift in ν_1 when compared with violaxanthin, despite them both possessing nine conjugated double bonds. The ν_1 position is thus a unique “molecular fingerprint” of the xanthophyll species involved in a particular resonance enhancement in the Raman difference spectrum resulting from an absorption change (30). Therefore, the up-shifted ν_1 position in L17+DTT leaves when compared with the wild-type confirms that a xanthophyll other than zeaxanthin is involved in the ΔA_{525} change (Fig. 2).

Several features of the Raman difference spectrum using 528.7 nm excitation in L17+DTT leaves give clues as to the identity of the xanthophyll species responsible for the ΔA_{525} change. Firstly, the ν_1 position at 1528 cm⁻¹ is identical to that of isolated violaxanthin dissolved in pyridine (Fig. 3), whereas ν_1 for isolated lutein is at 1524 cm⁻¹ and ν_1 for neoxanthin is at 1533 cm⁻¹ (Fig. 3). Examination of the ν_3 region

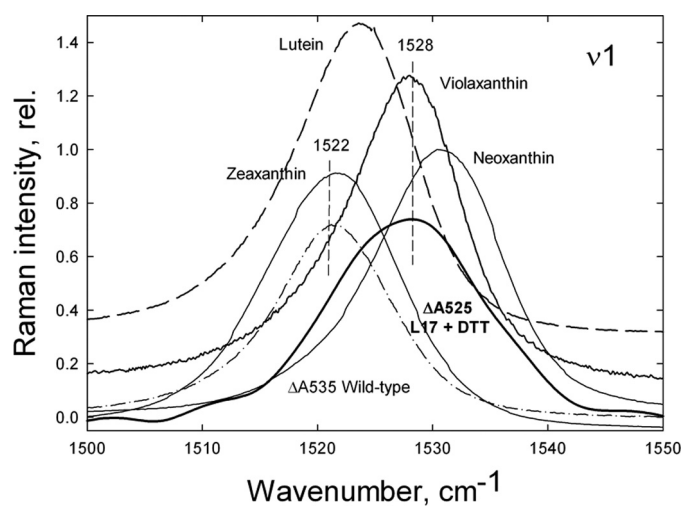


FIGURE 3. Comparison of the ν_1 region of the qE-associated Raman spectrum (difference spectrum of light-treated-minus-dark recovery) induced by 528.7 nm excitation in *Arabidopsis* leaves from wild type and L17 treated with DTT. The ν_1 region of the Raman spectra induced by 528.7 nm excitation of isolated zeaxanthin, violaxanthin, lutein, and neoxanthin dissolved in pyridine are also shown for comparison. *rel.*, relative.

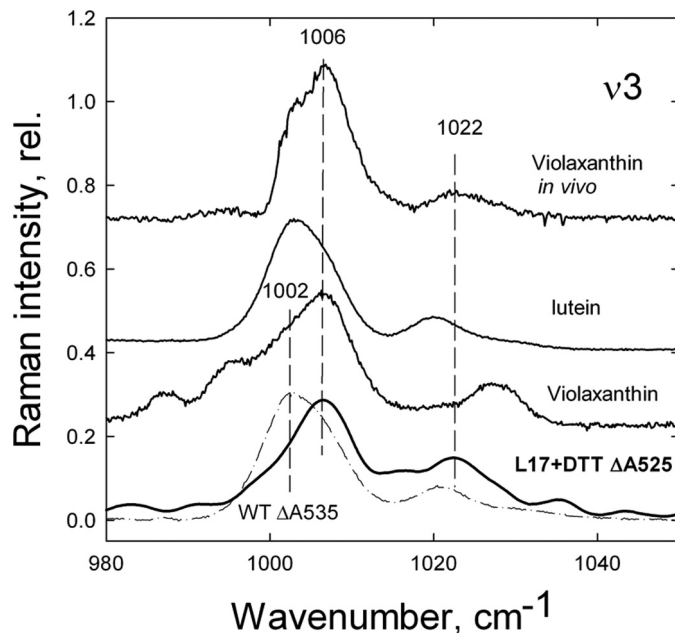


FIGURE 4. Comparison of the ν_3 region of the qE-associated Raman spectrum (difference spectrum of light-treated-minus-dark recovery) induced by 528.7 nm excitation in *Arabidopsis* leaves from wild type and L17 treated with DTT. The ν_3 region of the Raman spectra induced by 528.7 nm excitation of isolated violaxanthin and lutein dissolved in pyridine and the *in vivo* violaxanthin spectrum induced by 488.0 nm excitation (difference spectrum of wild-type dark adapted-minus-dark recovery leaves) are shown for comparison. *rel.*, relative.

of the spectrum provides further evidence of the involvement of violaxanthin in the ΔA_{525} change (Fig. 4). The ν_3 peak position is at 1006 cm⁻¹ in the L17+DTT Raman difference spectrum (Fig. 4), again consistent with the position of isolated violaxanthin (Fig. 4, trace 2), whereas for both isolated lutein and isolated zeaxanthin, ν_3 is at 1003 cm⁻¹ (Fig. 4). It should be noted, however, that the satellite of the main ν_3 band in the L17+DTT difference spectrum is at ~1022 cm⁻¹, deviating slightly from the corresponding band for isolated violaxanthin

(1030 cm^{-1}), whereas it is still higher than that of isolated lutein at 1020 cm^{-1} (Fig. 4). To account for this deviation, the L17+DTT spectrum was compared with the previously reported *in vivo* violaxanthin Raman spectrum (dark adapted-minus-dark recovery spectrum of wild-type leaves, where a preillumination period has caused violaxanthin de-epoxidation). The resulting Raman difference spectrum at 488.0 nm excitation reveals a selective loss of resonance from violaxanthin, thus allowing the *in vivo* features of this pigment to be observed. Comparison of the *in vivo* violaxanthin spectrum with that of the L17+DTT leaves presented here reveals that all features of the ν_3 region are identical, including the position of this satellite band (Fig. 4).

The ν_4 region of the Raman spectrum has been shown to provide information on the molecular distortion of xanthophylls upon binding to proteins because the C–H wagging modes are formally resonance-forbidden in planar carotenoids (30). In L17+DTT leaves, the ν_4 region of the spectrum shows the two sharp modes at 949 and 962 cm^{-1} , characteristic for the *in vivo* violaxanthin Raman spectrum previously reported (51) and in contrast to the structureless ν_4 region of violaxanthin dissolved in lipid micelles (Fig. 5). In addition, the ν_4 region in L17+DTT leaves is slightly broadened with

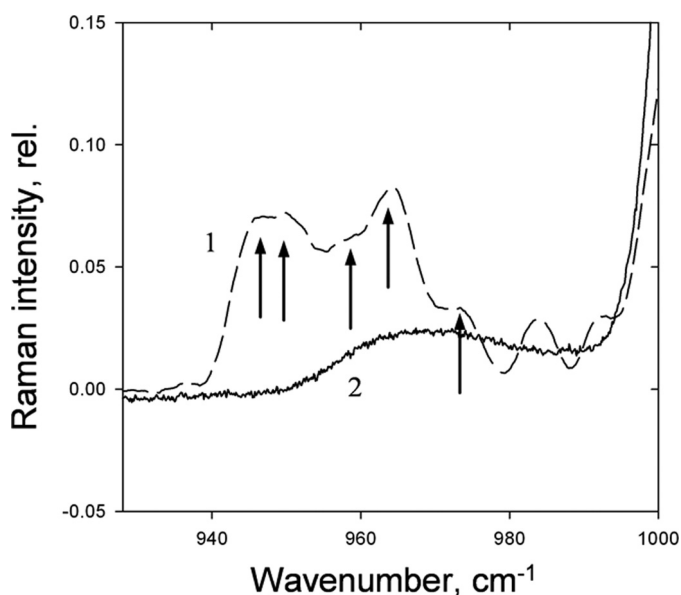


FIGURE 5. ν_4 region of the qE-associated Raman spectrum (difference spectrum of light-treated-minus-dark recovery) induced by 528.7 nm excitation in *Arabidopsis* leaves from L17 plants. The ν_4 region of the Raman spectra induced by 528.7 nm excitation (difference spectrum of wild-type dark adapted-minus-dark recovered leaves) (trace 1) is presented in comparison with the spectrum of isolated violaxanthin dissolved in lipid micelles (trace 2). *rel.*, relative.

TABLE 2

Pigment composition of isolated LHCII antenna proteins

LHCII trimers enriched in either violaxanthin or zeaxanthin were obtained from sucrose gradients following solubilization of BBY membranes in *n*-dodecyl β -D-maltoside (see "Experimental Procedures"). De-epoxidation of violaxanthin to antheraxanthin and zeaxanthin was achieved by incubating the thylakoids (from which BBYs were derived) for 30 minutes in 0.33 M sorbitol, 1 mM EDTA, 30 mM HEPES, 20 mM MES, 40 mM ascorbate at pH 5.5 at 20 °C (de-epoxidation state of thylakoids following this procedure was ~80%). Neo, Vio, Ant, Lut, Zea, DEPs, and Chl *a/b*: neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, de-epoxidation state (Z + 0.5A)/(V + A + Z), and Chl *a/b* ratio. Carotenoid data are presented normalized to 14 molecules of Chl *a* + *b* \pm S.E. from four replicates, DEPs (Zea + 0.5Ant)/total Vio + Zea + Ant)% and Chl *a/b* presented as molar ratio; ND, none detected.

Complex	Neo	Vio	Ant	Lut	Zea	DEPs	Chl <i>a/b</i>
LHCII V	1.04 \pm 0.1	0.69 \pm 0.1	ND	2.1 \pm 0.1	ND	0	1.33 \pm 0.1
LHCII Z	1.05 \pm 0.1	0.08 \pm 0.03	0.08 \pm 0.04	2.1 \pm 0.1	0.58 \pm 0.1	84%	1.33 \pm 0.1

additional modes appearing (Fig. 5, arrows). The data therefore indicate that the red-shifted violaxanthin responsible for ΔA_{525} is selectively distorted through protein binding.

Around 75% of PSII-associated xanthophyll cycle carotenoids have been shown by numerous biochemical studies to be bound to the major trimeric LHCII complex (49). To investigate whether this complex could represent the source of the ΔA_{525} and ΔA_{535} change, we compared the Raman spectra of aggregated and trimeric LHCII binding violaxanthin and zeaxanthin in the V1 sites (Table 2). Remarkably, the same selective resonance enhancements in the aggregated-minus-trimeric LHCII Raman difference spectrum were observed as those associated with ΔA_{525} and ΔA_{535} *in vivo* for both the ν_1 and the ν_3 regions, providing a strong link between the two phenomena (Fig. 6, A and B).

DISCUSSION

In this study, the origin of the ΔA_{525} absorption change that is characteristic for qE in the absence of zeaxanthin was studied by resonance Raman difference spectroscopy using 528.7 nm excitation. Using the same technique, the ΔA_{535} change was previously identified as belonging to a subpool of red-absorbing zeaxanthin molecules (31). Here, evidence was provided that the ΔA_{525} change is associated with a similar red-shifted pool of violaxanthin that is formed under qE conditions. A resonance enhancement of a xanthophyll species with ν_1 and ν_3 maxima consistent with that of violaxanthin and significantly different from that of lutein and the other xanthophylls was observed. The similarities in the absorption difference spectra below 500 nm and above 600 nm between qE *in vivo* and that of LHCII aggregation *in vitro* are well documented (11, 37). However, previously, the origin of the ΔA_{525} and ΔA_{535} bands has remained a mystery because they were absent in LHCII aggregates (37). However, the LHCII aggregates previously studied, unlike the ones used here, lacked peripheral xanthophyll cycle carotenoids. In this study, the same Raman signature associated with ΔA_{525} in leaves, and indeed that previously associated with ΔA_{535} , was observed in LHCII aggregates binding xanthophyll cycle carotenoids at the peripheral V1 site, providing further evidence of a common origin for quenching in both systems.

It is known that xanthophyll absorption is sensitive to the solvent environment. The 0-0 transition for violaxanthin *in vivo* peaks at ~490 nm (52), suggesting an ~35-nm red shift upon ΔA_{525} formation, similar to that associated with ΔA_{535} attributable to zeaxanthin (45). To produce such large red shifts, a dramatic change in xanthophyll environment would

Photoprotective Energy Dissipation-associated Absorption

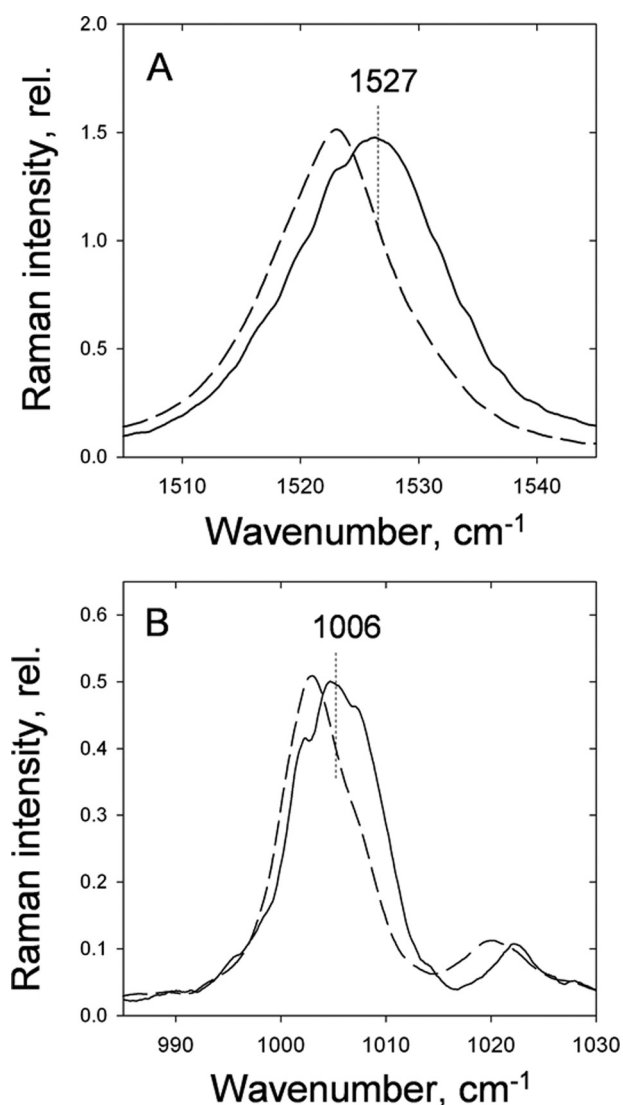


FIGURE 6. LHCII aggregation-associated Raman spectra (difference spectrum of aggregated-minus-trimeric) for LHCII binding zeaxanthin (dashed line) and violaxanthin (solid line) in the peripheral V1 binding site in the ν_1 (A) and the ν_3 (B) regions. Excitation is at 528.7 nm, rel. relative.

be required, such as the appearance of a static dipole or point charge near the pigment responsible (41, 42).

An alternative explanation for a large red shift would be an excitonic splitting of the S_2 excited state of the xanthophyll (46). This so-called *Davydov* splitting is a result of strong Coulombic coupling between the excited states of closely associated molecules. Such Coulomb coupling causes a mixing of the excited states of the individual molecules to form new excited states (excitonic states) that are delocalized across the whole molecular ensemble (53). The result of this quantum mechanical mixing is that the original single-molecule excited states move apart in energy (split).

A characteristic feature of excitonic splitting is that the new states have greatly different oscillator strengths when compared with the original single-molecule states, arising from different superpositions of the single-molecule transition dipole moments. If we consider the simple case of excitonic interactions between two identical xanthophyll models, then

the excitonic splitting will be dominated by the highly dipole-allowed S_2 states. The lower lying S_1 state does not play as significant a role because it has no dipole connection with the ground state, meaning that the Coulomb coupling between S_1 states will be much weaker than that between S_2 states. The mixing of the two S_2 states will result in the appearance of two new states, one strongly dipole-allowed, or “bright,” and the other only weakly dipole-allowed, or “dark”. Both of these states are split symmetrically about the original S_2 state, and the energy ordering depends entirely on the relative geometry of the two molecules. An H-type (parallel or “card-packed”) geometry yields a dark state lower in energy than the bright state, whereas a J-type (collinear or top-to-tail) geometry results in the bright state being the higher in energy. It is clear that the formation of an H-aggregate is associated with an overall blue shift of the S_2 absorption peak because the bright state lies above the original S_2 state. By the same logic, the formation of a J-aggregate is associated with a corresponding red shift. We therefore propose that both ΔA_{535} and ΔA_{525} are the result of the formation of xanthophyll J-aggregates (zeaxanthin in the case of ΔA_{535} and violaxanthin for ΔA_{525}) within the membrane.

Indeed, it has previously been shown that isolated zeaxanthin can mimic ΔA_{535} upon formation of J-type aggregates *in vitro* (45). A casual formation of J-type aggregates without protein interaction can be excluded for both ΔA_{525} and ΔA_{535} *in vivo* because the structure of the ν_4 region of the Raman difference spectrum indicates that the pigments remain bound to protein. The distinct lack of structure of the ν_4 region in J-type aggregates formed between zeaxanthin molecules isolated *in vitro* when compared with *in vivo* supports this view (32, 34). The data are rather more consistent with the hypothesis made recently, that upon LHCII aggregation, pairs of either zeaxanthin or violaxanthin, depending upon the de-epoxidation state, may come into contact in such a way that a J-type aggregate is formed at the trimer-trimer interface (43).

In vitro, zeaxanthin enhances LHCII aggregation and violaxanthin restricts it, consistent with an enhancement of the qE-related structural changes by the former (22, 54). A notable feature regarding both ΔA_{525} and ΔA_{535} is that neither depends upon the presence of a specific LHCII, minor antenna complex, or PsbS; rather they are features of the ensemble of LHC(-like) proteins in the membrane, just like NPQ (25, 36). This would be anticipated given the high level of homology between LHCII and the minor antenna proteins (55). Indeed, CP26 and possibly CP29 are predicted to have V1 sites occupied by xanthophyll cycle carotenoids, according to biochemical studies (49). Thus, involvement of the V1 site in both ΔA_{525} and ΔA_{535} formation could explain why a signal is observed at ΔA_{530} in leaves of the *Arabidopsis szll1* mutant, which lacks zeaxanthin but compensates by greatly increasing levels of lutein at the expense of violaxanthin (56). In this mutant, we predict that the extra lutein would replace violaxanthin in the V1 site, providing an explanation of the slight red shift of ΔA_{525} to ΔA_{530} in these plants (56).

The correlation between $\Delta A_{525}/\Delta A_{535}$ and qE has led to the suggestion that this change may monitor directly the forma-

tion of the quencher (5). An excitonic interaction between xanthophylls that lowers the energy of the S_2 excited state may also affect the energy of the S_1 state. Because the dissipation of energy during qE *in vivo* has been linked to the population of a carotenoid S_1 state (12, 13, 16), a red-shifted xanthophyll species could indeed be involved. However, several lines of evidence indicate that ΔA_{525} and ΔA_{535} are not quenchers. Firstly, the xanthophylls responsible for these changes do not appear in the qE-related fluorescence excitation difference spectrum, suggesting that the S_2 (and by inference S_1) states of these xanthophylls are not strongly energetically coupled to Chl (11), as concluded previously for the bulk xanthophyll cycle pool (57) and consistent with their peripheral location and proposed role in the allosteric structural regulation of qE (58). Secondly, quenching still occurs in LHCII aggregates *in vitro* even in the absence of xanthophyll cycle carotenoids (37). Thirdly, the *lut2npq2* mutant of *Arabidopsis*, which possesses zeaxanthin as the only xanthophyll, has a similar level of ΔA_{535} to the wild-type but significantly less qE (25). Finally, the S_1 energy level of all carotenoids reconstituted within the LHCII complex has already been demonstrated experimentally to be lower than that of the lowest Chl Qy transition (59). Thus, it is likely that other factors, such as distance, are more important should carotenoids be quenchers. Rather, we suggest that the ΔA_{525} and ΔA_{535} monitors the tighter interaction between LHCII units formed upon aggregation both *in vivo* and *in vitro*. The fact that all four LHCII xanthophylls undergo some change in their absorption, excitation, and Raman spectra upon formation of quenching (12, 25, 31) indicates that the associated structural changes are felt across the protein complex in each xanthophyll-binding domain.

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