On the regulation of photosynthesis by excitonic interactions between carotenoids and chlorophylls

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Selective 2-photon excitation (TPE) of carotenoid dark states, Car S_1, shows that in the major light-harvesting complex of photosystem II (LHCII), the extent of electronic interactions between carotenoid dark states (Car S_1) and chlorophyll (Chl) states, $\phi_{Coupling}^{Car\ S_1-Chl}$, correlates linearly with chlorophyll fluorescence quenching under different experimental conditions. Simultaneously, a linear correlation between both Chl fluorescence quenching and $\phi_{Coupling}^{Car\ S_1-Chl}$ with the intensity of red-shifted bands in the Chl Q_y and carotenoid absorption was also observed. These results suggest quenching excitonic Car S_1−Chl states as origin for the observed effects. Furthermore, real time measurements of the light-dependent down- and up-regulation of the photosynthetic activity and $\phi_{Coupling}^{Car\ S_1-Chl}$ in wild-type and mutant (*npq1*, *npq2*, *npq4*, *lut2* and *WT+PsbS*) *Arabidopsis thaliana* plants reveal that also in vivo the quenching parameter NPQ correlates always linearly with the extent of electronic Car S_1–Chl interactions in any adaptation status. Our in vivo measurements with *Arabidopsis* variants show that during high light illumination, $\phi_{Coupling}^{Car\ S_1-Chl}$ depends on the presence of PsbS and zeaxanthin (Zea) in an almost identical way as NPQ. In summary, these results provide clear evidence for a very close link between electronic Car S_1–Chl interactions and the regulation of photosynthesis. These findings support a photophysical mechanism in which short-living, low excitonic carotenoid–chlorophyll states serve as traps and dissipation valves for excess excitation energy.

Arabidopsis | LHCII | NPQ | two-photon excitation

Plants are exposed to sunlight intensities varying over several orders of magnitude during a typical day (1). Under low light conditions, almost all absorbed sunlight photons are used for the primary photosynthetic reaction steps. However, under high light conditions the photosynthetic apparatus must be protected from excess excitation energy, because it may lead to deleterious side-effects. Balancing between efficient utilization of solar energy under restrictive light conditions and dissipation of excess energy when the absorbed light exceeds the photosynthetic capacity is therefore essential for the survival and fitness of plants (2). It is known that light-induced increase of the pH gradient across the thylakoid membrane (3, 4) and the presence of the protein PsbS (5) are necessary for the down-regulation of the photosynthetic activity under excess light and that Zea is simultaneously formed from violaxanthin (Vio) through the enzymatic xanthophyll cycle (6). However, although many different studies have been undertaken to elucidate the details of this important regulation, a complete picture of its mechanisms is still missing. Several different regulation models have been proposed and indeed it cannot be excluded that different mechanisms contribute more or less to plants adaptation to varying light conditions. However, at present even the regulation site and photophysical mechanisms are unresolved, because the models are at least partly contradicting each other (5, 7–15).

The most important measurable signature of plants regulation activity is its varying residual Chl fluorescence intensity (16), which is proportional to the regulated amount of excitation energy in the photosynthetic apparatus. The actual extent of adaptation-dependent quenching of Chl singlet excited state energy, known as nonphotochemical quenching (NPQ), is typically quantified by the parameter

$$\mathrm{NPQ} = \frac{F_m}{F'_m} - 1 \qquad [1]$$

which reflects the reduction in the residual Chl fluorescence of plants, F'_m, brought about by the unknown excitation energy dissipation mechanisms, in comparison to the residual Chl fluorescence observable from the completely dark adapted plant, F_m, in which no photoprotective energy dissipation is operating. F'_m and F_m are usually measured using short, intense light flashes that saturate the photosynthetic reaction center chemistry. This guarantees that the observed differences in F'_m and F_m reflect only the extent of energy dissipation through photoprotective channels, without affecting the adaptation status of the plant (16).

It is long known that carotenoids play an important role in the regulation mechanisms and several different types of electronic interactions between carotenoids and Chls have been proposed to play a key role as dissipation valves for excess excitation energy (9, 10, 12). However, so far it was difficult to quantify the extent of these interactions and to investigate directly their involvement in the flow of excitation energy and its regulation, especially in living plants. One obstacle in the investigation of the role of the carotenoids is that transitions between the carotenoid electronic ground state and their first excited state, Car S_1, are optically forbidden, preventing a direct observation by conventional absorption or fluorescence spectroscopy within photosynthetic protein complexes or organisms. However, it was shown that 2-photon excitation (TPE), using the correct excitation wavelengths and pulse intensities, enables a selective excitation of these carotenoid states in pigment-protein complexes and even in intact plants (Fig. 1*B*) (17–21, 35, 36). The detection of fluorescence from the Chl states upon selective 2-photon excitation (Fl^{TPE}) of the carotenoid dark states, Car S_1, provides direct evidence for electronic interactions between Car S_1 and Chl states. A quantitative comparison of the Chl fluorescence

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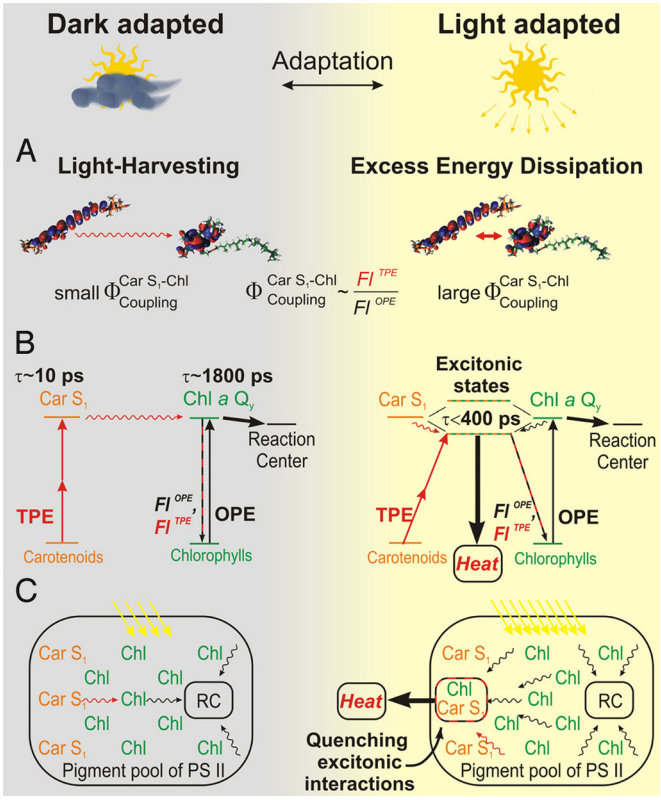

Fig. 1. Excitonic model for the regulation of photosynthesis and principle of measuring the electronic interactions between carotenoid dark states and chlorophylls, $\phi^{Car\ S_1-Chl}_{Coupling}$. (*A* and *B*) A quantitative comparison of the chlorophyll fluorescence intensity detected after selective 2-photon excitation (TPE) of the carotenoid dark states Car S_1, Fl^{TPE}, and direct 1-photon excitation (OPE) of the chlorophyll states Chl *a* Q_y, Fl^{OPE}, allows quantifying the current extent in the electronic interactions, $\phi^{Car\ S_1-Chl}_{Coupling}$, between these states even during the regulation of in intact plants (17, 18). When the energy levels of Car S_1 and Chl *a* Q_y are similar, increased electronic interactions lead to the formation of excitonic states that are delocalized over both molecules. (*B* and *C*) The lower and short-lived excitonic Car S_1-Chl *a* Q_y state serves as an energy sink and dissipation valve for a large amount of excess excitation energy in the photosynthetic pigment pool, enabling regulation of plant photosynthesis.

intensities detected upon selective excitation of the carotenoid dark states, Fl^{TPE}, and direct 1-photon excitation of the Chl, Fl^{OPE}, allows quantification of the current extent of these interactions, $\phi^{Car\ S_1-Chl}_{Coupling}$ (see SI for more details):

$$\Phi^{CarS_1-Chl}_{Coupling} \propto \frac{Fl^{TPE}}{Fl^{OPE}}. \quad [2]$$

This coupling parameter, $\phi^{Car\ S_1-Chl}_{Coupling}$, can be determined for isolated pigment-protein complexes under various conditions and in real time for plants during their photosynthetic regulation. When there is increased energy transfer from Car S_1 to the Chl molecules or when the states of Car S_1 and Chl *a* Q_y couple excitonically, a selective excitation of Car S_1 results simultaneously in an increased excitation of Chl, with the consequence that $\phi^{Car\ S_1-Chl}_{Coupling}$ increases.

Here, we present evidence based on real-time 2-photon measurements in living plants that the parameter quantifying the electronic Car S_1–Chl interactions, $\phi^{Car\ S_1-Chl}_{Coupling}$, is always linearly correlated with NPQ in any adaptation status of *Arabidopsis thaliana* wild-type plants, and the most important mutants within the scope of NPQ: *WT* + *PsbS* (overexpressing PsbS) (22), *npq2* (no Vio), *npq1* (no Zea), *npq4* (no PsbS) (5) and *lut2* (no lutein). In addition, the same parameter also correlates linearly with the fluorescence quenching of isolated major light-harvesting complex of photosystem II, LHCII, under different conditions that are known to influence the quantum efficiency of LHCII fluorescence such as the pH value and/or aggregation and the content in the carotenoid Zea. Also, the occurrence of red shifted chlorophyll Q_y and carotenoid absorption bands correlate linearly with the fluorescence quenching and $\phi^{Car\ S_1-Chl}_{Coupling}$, providing strong indication that all effects are based on the formation of excitonic carotenoid– chlorophyll states. It has been proposed in refs. 23 and 24 that excitonic mixing between Car S_1 and Chl Q_y in LHCII is closely related to the phenomenon of nonphotochemical quenching in green plants. Such excitonic interactions generally lead to 2 new electronic states that are more or less delocalized over the 2 molecules and have characteristics of both original monomeric states (Fig. 1 *A* and *B*). One state is lower than the energies of the original monomeric states, the other is higher. In a pigment pool, such low lying excitonic states act as local energy trap for the entire pool (Fig. 1*C*). Because the excited state lifetime of the original, monomeric Car S_1 states is with ≈10–30 ps (25) orders of magnitude shorter than the fluorescence lifetime of the original Chl *a* Q_y state (≈1,800 ps) (26) even small excitonic mixing lead to a drastic reduction in the fluorescence lifetime of the involved Chl molecule and consequently to very effective dissipation of excitation energy (23). These changes in the Chl fluorescence quantum yield and lifetime correspond exactly to the changes in the Chl fluorescence of plants observed during the photosynthetic regulation (26).

The results presented here strongly support the formation of excitonic Car S_1–Chl interactions and are in very good agreement with the mechanism described above. Excitonic Car S_1-Chl interactions provide a simple but very powerful dissipation mechanism for excessive excitation energy and are together with the results presented here supported by many experimental observations reported in the literature.

Results

LHCII is the most important light-harvesting pigment-protein complex in the photosynthetic apparatus of plants. It has long been known, that LHCII fluorescence quenching in vitro, for example upon acidification, has similarities with nonphotochemical quenching of Chl fluorescence observed in entire plants under high-light conditions (27). Also similarly as in plants, the extent of fluorescence quenching in vitro is influenced by the presence of the carotenoid Zea (28). We therefore first determined $\phi^{Car\ S_1-Chl}_{Coupling}$ for LHCII as a function of different conditions that can lead to fluorescence quenching. In Fig. 2 the corresponding results for LHCII and LHCII enriched in Zea are shown. Under the chosen conditions, the fluorescence quantum yield observed after direct 1-photon excitation of the chlorophylls, Fl^{OPE}, decreases by ≈50% at low pH and when Zea is present (blue data in Fig. 2*A*). Simultaneously, also a significant increase in $\phi^{Car\ S_1-Chl}_{Coupling}$ can be observed (red data in Fig. 2*A*). In the Zea-enriched samples $\phi^{Car\ S_1-Chl}_{Coupling}$ increases by ≈40% when the pH value is decreased down to 5.5. To quantify the fluorescence quenching in a similar manner as for plants we calculated a NPQ parameter from the fluorescence intensities, Fl^{OPE}, analogous to Eq. **1** by using the maximum fluorescence, Fl^{OPE}, observed at pH 7.5 for the value of F_m and the quenched fluorescence intensities, Fl^{OPE}, for F'_m. Fig. 2*B* demonstrates the direct, linear correlation between $\phi^{Car\ S_1-Chl}_{Coupling}$ and fluorescence quenching (NPQ), as quantified in this manner. For LHCII preparations that do not contain Zea significantly less fluorescence quenching can be observed in otherwise identical experimental conditions (28). It is, however, important to note, that Zea is not absolutely mandatory to achieve quenching. The data obtained from both Zea-less and Zea-enriched LHCII samples

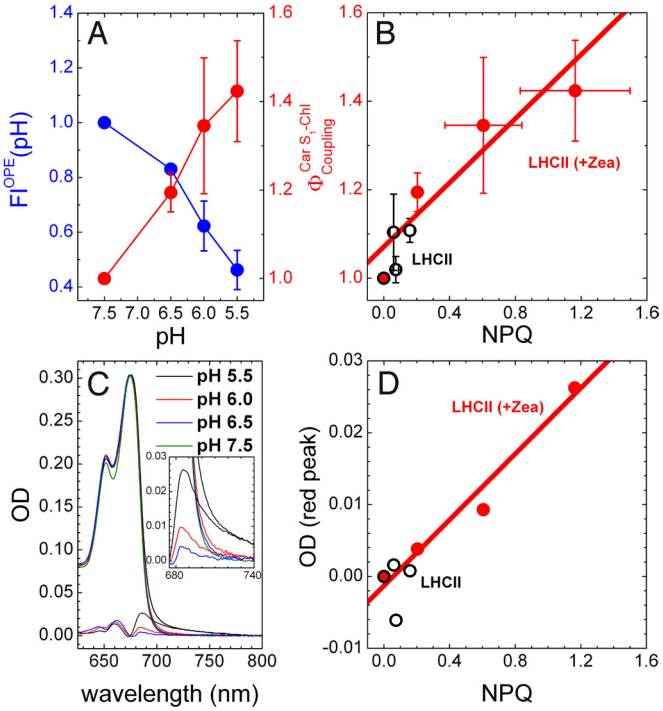

Fig. 2. Correlations between the fluorescence quenching, the electronic interactions, $\phi_{Coupling}^{Car\ S_1-Chl}$, and the red-shifted absorption band of isolated LHC II. (*A*) pH dependence of the relative chlorophyll fluorescence intensities, FI^{OPE}, observed with LHCII enriched in Zea (blue), and corresponding values in the Car S_1-Chl *a* Q_y interactions, (red). (*B*) Correlation of $\phi_{Coupling}^{Car\ S_1-Chl}$ with the fluorescence quenching, NPQ, calculated from the LHCII fluorescence as described in the text (Eq. **1**) for LHCII enriched in Zea (red) and LHCII containing only Vio (black, open circles). (*C*) Absorption spectra of LHCII enriched in Zea observed at various pH values along with corresponding difference spectra (spectra subtracted from the spectrum observed at pH 7.5). (*Inset*) Cut-out of the spectral region showing the additional absorption ≈686 nm in the difference spectra. (*D*) Correlation between the red peak intensity at 686 nm and fluorescence quenching, NPQ for LHCII enriched in Zea (red) and LHCII containing only Vio (black). The data shown in *A*, *B*, and *D* are averages from 3 independent measurements.

fit perfectly a linear correlation between $\phi_{Coupling}^{Car\ S_1-Chl}$ and fluorescence quenching (Fig. 2*B*).

Because excitonic interactions lead to lower state energies, evidence for their presence is typically provided by the occurrence of red-shifted absorption bands (compare also with Fig. 1*B*). Indeed, such additional red-shifted absorption bands are observed in the spectral region of Chl *a* Q_y (and also Car S_2) of the quenched LHCII samples. In Fig. 2*C* pH-dependent absorption spectra of the Zea-enriched LHCII samples are shown along with corresponding difference spectra calculated by subtracting the spectrum observed at pH 7.5 from the spectra of the quenched samples. In the difference spectra a new absorption band arises at ≈686 nm (Fig. 2*C Inset*), whose intensity is again linearly proportional to NPQ (Fig. 2*D*). The occurrence of such red shifted bands in LHCII is also related to oligomerization (28). It might be surprising that the combination of a relatively small amount of 0.14 Zea molecules per monomer of LHCII with the acidification leads to a reduction of the fluorescence of LHCII by >50% in comparison to the LHC II samples without Zea. However, it has already been reported that just by adding Zea to solutions containing LHCII monomers can have a very similar effect (29). Likewise, a very close correlation with an additional absorption band at 683 nm was reported.

To investigate whether similar observations can also be found in vivo in plants under excess light illumination, we determined $\phi_{Coupling}^{Car\ S_1-Chl}$ for wild-type and mutant *A. thaliana* plants during the adaptation to high- and low light conditions. We compared the relative values of $\phi_{Coupling}^{Car\ S_1-Chl}$ during the regulation of wild-type plants with various mutants with increased or decreased content of pigments or proteins that are known to be of high relevance in the regulation of plant photosynthesis. In contrast to previous studies (17), here we focused on changes in $\phi_{Coupling}^{Car\ S_1-Chl}$ occurring during high light illumination, when the transmembrane pH gradient is largest. We therefore plotted the $\phi_{Coupling}^{Car\ S_1-Chl}$ values for all plant variants relative to the values observed at the beginning of the measurements of each plant variant $\phi_{Coupling}^{Car\ S_1-Chl}$ (~0*s*) in the dark adapted state.

During high light illumination (time period under the gray bar in Fig. 3) of wild-type plants, $\phi_{Coupling}^{Car\ S_1-Chl}$ (black data in the lower row, Fig. 3*a*) is indeed significantly larger than during the dark period. In contrast, mutants lacking the protein PsbS, which is known to be essential for plants short-term regulation, do not show any increase in $\phi_{Coupling}^{Car\ S_1-Chl}$ during high light illumination (*npq4*, green data in the lower row, Fig. 3*b*), whereas mutants overexpressing PsbS (*WT* + *PsbS*, green data in the lower row, Fig. 3*b*) show an even larger increase in $\phi_{Coupling}^{Car\ S_1-Chl}$ than wild-type. Similarly, the Zea-lacking plant mutants *npq1* (Fig. 3*d*) show significantly smaller $\phi_{Coupling}^{Car\ S_1-Chl}$ values than wild-type, whereas mutant plants that accumulate Zea (*npq2*, Fig. 3*e*) show a larger $\phi_{Coupling}^{Car\ S_1-Chl}$ under light illumination. Also the lutein-lacking plant mutant *lut2* (Fig. 3*f*) shows somewhat smaller $\phi_{Coupling}^{Car\ S_1-Chl}$ values than wild-type.

Intriguingly, for all plant variants, wild-type or mutants, the observed magnitude of $\phi_{Coupling}^{Car\ S_1-Chl}$ correlates in any adaptation status directly and linearly with the quenching parameter NPQ (blue data in Fig. 3 *A–F* and Fig. 4). Here, NPQ was calculated as usually from the maximum fluorescence observed during the first saturating 1-photon excitation pulse before high light adaptation, F_m, and during saturating pulses applied in the subsequent down- or up-regulation of the plants, F'_m. Also plants grown under short day or under long day conditions fit very well the linear relationship (Fig. 4), demonstrating the general principle that the extent of electronic Car S_1–Chll interactions, $\phi_{Coupling}^{Car\ S_1-Chl}$, is invariably and directly correlated to the current extent of NPQ, regardless of the current light intensities, adaptation status or growth conditions.

Discussion

In contrast to the present work, previous studies on the changing interactions between Car and Chl during the regulation of photosynthesis typically detected Car excitation after selective Chl excitation. It was usually found in those studies an increased Car excitation under conditions in which photosynthesis is down-regulated. The method presented here gives therefore complementary information, because it detects Chl excitation upon selective Car S_1 excitation. When increased energy transfer is detected in both directions (Car S_1 → Chl and Car S_1 ← Chl), strong indication is produced that both molecules actually share excitation energy under quenching conditions, i.e., that excitonic Car S_1–Chl states are formed as proposed in ref. 23.

In LHCII, the simultaneous increase of Car S_1–Chl interactions and appearance of a red shifted Chl *a* Q_y absorption band that is proportional to Chl *a* Q_y fluorescence quenching provides evidence for the formation of Car S_1–Chl *a* Q_y excitonic states under conditions in which the LHCII fluorescence is quenched (Fig. 2) (24). *In planta*, the occurrence of increased Car S_1–Chl interactions, $\phi_{Coupling}^{Car\ S_1-Chl}$ (Fig. 4), which are always linearly correlated with NPQ, provides strong indication that also here similar excitonic interactions are connected to the down-regulation of photosynthesis. In vivo, the formation of excitonic

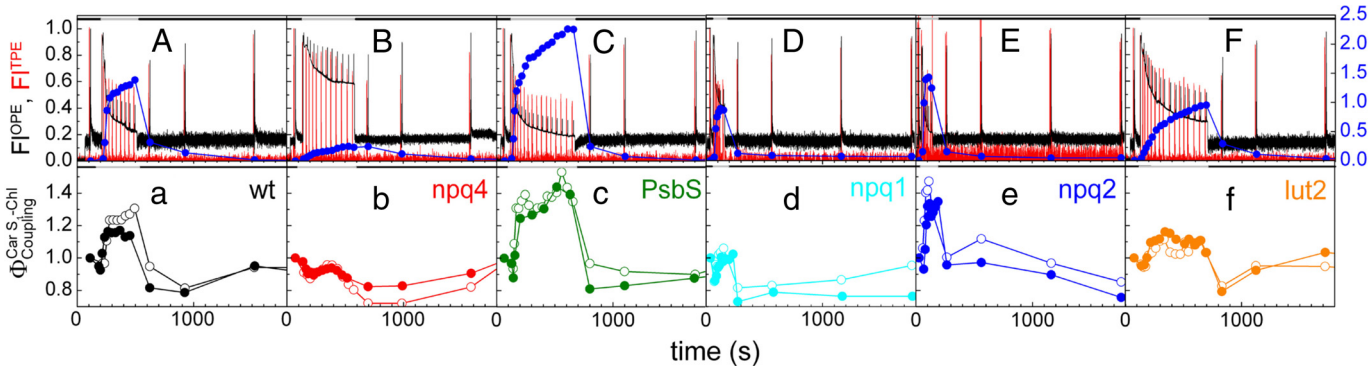

Fig. 3. Simultaneous real-time measurements of NPQ (blue data in *A–F*) and the electronic interactions, $\phi_{Coupling}^{Car\ S_1-Chl}$ (*a–f*), during the regulation of plants. Typical raw data for FI^{OPE} (black lines) and FI^{TPE} (red lines) for different *A. thaliana* variants during high light adaptation (gray bar on top) and dark adaptation (black bar on top) along with NPQ values calculated from the pulses in the OPE data according to Eq. **1** (blue data). The OPE and TPE pulses corresponded to saturating conditions. (*a–f*) *Car S*$_1$-Chl *a* Q_y interactions, $\phi_{Coupling}^{Car\ S_1-Chl}$, calculated from FI^{TPE} and FI^{OPE} that are observed during the saturating TPE and OPE pulses according to Eq. **1**. The open circles are $\phi_{Coupling}^{Car\ S_1-Chl}$ values calculated from the TPE and OPE pulses of the example data shown in *A–F* and the close circles are mean values for averaged from at least 3 independent plant measurements. The wild-type plants and mutants *npq4*, *WT + PsbS*, and *lut2* were grown under short day and the mutants *npq1* and *npq2* under long day conditions, correspondingly (see *Materials and Methods* for further details).

Car S_1–Chl interactions under quenched conditions is additionally supported by results from other independent studies. One is a previous study by Ma et al., in which in down-regulated thylakoids the occurrence of instantaneous signals in the spectral region of the Car S_1 transient absorption after excitation of the lowest chlorophyll states were observed (11). Another study is a recent study by Ruban and coworkers in which it was possible to construct fluorescence spectra of the fast-decaying fluorescence component in intact chloroplasts under quenching conditions that were shifted to a very similar extent to red wavelengths as the red shifted absorption band correlated to the fluorescence quenching and $\phi_{Coupling}^{Car\ S_1-Chl}$ here presented (Fig. 2 *C* and *D*). As mentioned in the introduction, excitonic Car S_1–Chl interactions immediately lead to low lying states that are capable to effectively trap a larger fraction of the excitation energy in the pigment pool (Fig. 1 *B* and *C*) (23) and to dissipate the trapped energy on a short time scale due to the short-living (≈10–30 ps) carotenoid state contribution. Although it cannot be expected that the decrease in lifetime is a linear function of the extent in electronic coupling, $\phi_{Coupling}^{Car\ S_1-Chl}$, the formation of excitonic Car S_1–Chl *a* Q_y interactions easily explain the short decay components observed in the Chl fluorescence of plants and its red shift under conditions in which the photosynthetic light-harvesting efficiency is down-regulated (26). In summary, our results together with the observations reported in the literature provide a solid experimental support for the presence of low lying, short-living excitonic Car S_1–Chl states as a primary photophysical origin for the dissipation of excess excitation energy in plants (Fig. 1).

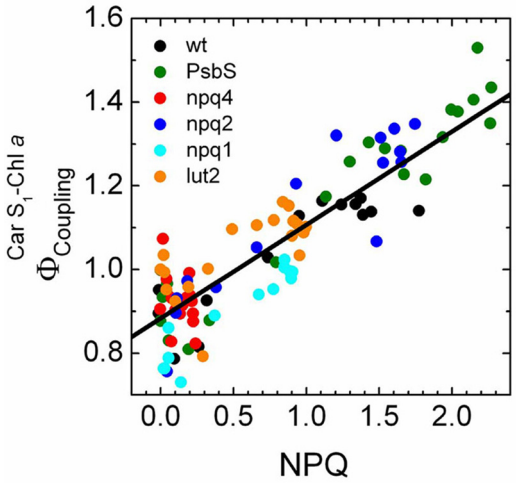

Fig. 4. Correlation between the Car S_1-Chl *a* Q_y interactions, $\phi_{Coupling}^{Car\ S_1-Chl}$ and the actual regulation status, NPQ, during light and dark adaptation for all plants from Fig. 3. The points correspond to the adaptation-dependent $\phi_{Coupling}^{Car\ S_1-Chl}$ values shown in Fig. 3 and the corresponding NPQ values calculated from the FI^{OPE} (*Fm*) intensities observed during saturating OPE pulses. For example, the maximum NPQ and $\phi_{Coupling}^{Car\ S_1-Chl}$ values of ≈1.8 and ≈1.2 observed for WT plants (black dots) correspond to the data measured at ≈500 s in Fig. 3*A* (blue data) and *a* (black data), respectively, after ≈300 s of high light illumination. In contrast, the minimum values NPQ and $\phi_{Coupling}^{Car\ S_1-Chl}$ values of ≈0 and ≈0.9–1.0 for WT are observed during low-light (≈0–200 and ≈500–1,800 s in Fig. 3). Intermediate values correspond to measurements during the adaptation of the plants to high- or low-light conditions. The color code for all plants are the same as in Fig. 3 *a–f*: WT plants (black), *WT + PsbS* mutants (dark green), *npq4* mutants (red), *npq2* mutants (dark blue), *npq1* (light blue) and *lut2* mutants (orange).

However, also other possible explanations for our observations must be discussed. For example, it cannot be entirely excluded that the observed increased Car S_1–Chl coupling upon increasing NPQ is not directly related to the quenching process, but only reflecting, for example, general pH-induced conformational changes in PSII antenna complexes. In contrast, in a study by Barros et al. (15) it was concluded, that in LHCII conformational changes are rather unlikely based on a comparison between currently available X-ray structures of LHCII (30) and structural rigidity parameters. Nevertheless, the changes in the Car S_1–Chl interactions observed in the present study and the suggested excitonic Car S_1–Chl model can be brought well into accordance with other photophysical quenching models, such as the formation of carotenoid radical cations or quenching by lutein 1 or neoxanthin, and probably even provides a link between these models. It is not unlikely, for example, that Car S_1–Chl excitonic states are direct precursors of the observed Car S_1–Chl radical ion pairs (7, 10). It is also feasible, that lutein 1 or neoxanthin is participating in the formation of the excitonic states and that Zea is rather regulating this process, although it can also directly participate in the formation Car S_1–Chl excitonic states.

It is not unlikely that different NPQ mechanisms are acting in parallel under in vivo conditions. Because the coupling parameter $\phi_{Coupling}^{Car\ S_1-Chl}$determined in this work reflects the average Car S_1–Chl interactions involving all carotenoids in the samples, currently only indications can be derived regarding which type of

carotenoid contributes in what way to the observed effects. For example, Fig. 3 shows, that in both the lutein deficient mutant *lut2* and the Zea deficient mutant *npq1*, the maximum $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ value is decreased to a similar extent as the maximum NPQ value (13) compared with WT plants. Because the quenching and correlated Car S_1–Chl interactions are not entirely vanishing in any mutant that is deficient in a certain carotenoid, it can be concluded that replacement of the missing pigment by other carotenoids can, at least partially, compensate for the formation of quenching excitonic Car S_1–Chl states. An exact assignment of the site responsible for the observed effects requires future studies investigating pigment-protein complexes in which specific chlorophyll binding sites neighboring certain carotenoids are genetically deleted.

The similar increase of $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ in plants (Fig. 4) and LHCII (Fig. 2*B*) under quenching conditions suggests that LHCII is at least one very important site for this type of excess excitation-energy dissipation. It is important to note, however, that our findings are in line with the study in ref. 31, which found that no additional radical cations are formed in LHCII when Zea replaces Vio. As reported in ref. 17, also no difference in the electronic Car S_1–Chl interactions are found in isolated LHCII samples with and without Zea. Only if the LHCII samples are investigated under quenching conditions by lowering the pH and/or aggregation the direct correlation between $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ and the quenching becomes obvious.

Because the PsbS lacking mutant *npq4* is the only variant that almost completely lacks additional Car S_1–Chl couplings during high light illumination it can be concluded that PsbS is essential for the formation of these interactions *in planta*. The question why PsbS is not necessary to observe quenching in isolated LHCII remains open but one possible explanation is that in plants a highly regulated environment in the thylakoid membranes requires PsbS to switch on these interactions whereas in pure LHCII samples these interactions can be brought about by simpler mechanisms such as aggregation and/or a change in the pH.

Conclusions

In summary, our results clearly show that electronic Car S_1–Chl interactions are directly correlated to quenching conditions in LHCII and entire plants. This discovery of a quantitative, linear correlation of Car S_1–Chl *a* Q_y interactions in LHCII with NPQ constitutes a significant step toward a complete understanding of the photophysical regulation-mechanism of photosynthesis. All experimental data and the quenching mechanism can be explained by a model in which the formation of excitonic Car S_1–Chl *a* Q_y interactions lead to short-living carotenoid–chlorophyll states that serve as traps and dissipation valves for excess excitation energy. The new possibility to quantify the Car S_1–Chl interactions in real time in intact plants will allow identification of the exact site of these regulating interactions, using plant mutants in which specific chlorophyll and carotenoid binding sites are disrupted.

Materials and Methods

The TPE light was generated by an ultrafast laser system. For OPE the light of a standard PAM fluorimeter was used. Both devices were arranged in a confocal set-up to allow the measurement of the fluorescences, Fl^{OPE} and Fl^{TPE}, at the same spot. Both signals, Fl^{OPE} and Fl^{TPE}, can be detected simultaneously by different signal modulation techniques. The detection scheme thus allows determining changes in the Car S_1–Chl interactions, $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$, in plants in real-time and in isolated pigment-protein complexes under identical conditions. The values $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ for the LHCII measurements shown in Fig. 2 were calculated from steady state fluorescence intensities, Fl^{TPE} and Fl^{OPE}, observed after TPE at (λ_{Exc}/2–594 nm (see SI) and OPE at 594 nm using Eq. **2**. Similarly, the values $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ for the plant measurements shown in Fig. 3 were calculated from the fluorescence intensities, $Fl^{TPE}(t)$ and $Fl^{OPE}(t)$, observed during saturating pulses of TPE and OPE at same wavelengths using Eq. **2**. Only relative values for $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ are given in Fig. 2 and 3. The values calculated for LHCII were scaled so that $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ at pH 7.5 was 1. Similarly, the values calculated for the plants were scaled that $\phi_{\text{Coupling}}^{\text{Car S}_1-\text{Chl}}$ before high-light illumination was 1. For further details see SI. Absorption spectra were recorded on a UV/VIS spectrometer from Perkin–Elmer Lambda.

Sample Preparation. ***Proteins.*** Native LHCII proteins were isolated from spinach as described in ref. 32. The monomer composition was determined by HPLC and has been listed with the HPLC signal intensity in *SI*. Pigment content was analyzed by HPLC by a method adapted from Gilmore and Yamamoto (33) using a Synergi Hydro-RP column (Phenomenex). For the measurements from pH 5.5 to pH 6.5 the LHCII samples were diluted with an aqueous buffer of 50 mM Mes and 0.3% NG (β-nonyl β-D-glucopyranoside). At pH 7.5 a 50 mM Tris buffer was used instead of Mes. The desired pH level was titrated using NaOH under the control of a pH meter (Hanna Instruments; HI991001). Great care was taken that the diluted samples have the same optical densities and that concentrations were as low as possible to avoid potential artifacts like fluorescence reabsorption.

Plants. *A. thaliana Col-0* wild-type plants and *lut2*, *npq4* and *WT + PsbS* mutants were kindly offered by the lab of K. Niyogi (University of California, Berkeley) (34, 35). Plants were grown in potting soil at light intensities of 80 μmol of photons·m^{-2}·s^{-1}, a constant temperature of 21 °C and at a relative humidity of 50%. Most plants were typically grown under short day conditions (10 h light, 14 h dark). *npq1* and *npq2* mutants purchased from NASC (European Arabidopsis Stock Center) were grown equally but under long day conditions (14 h light, 10 h dark) and at light intensities of 200 μmol of photons·m^{-2}·s^{-1}. For the averaged results presented, several independently grown plant charges were used.

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