Supporting Information

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SI Materials and Methods

Cloning, Overexpression, and Refolding of Recombinant PsbS. The cDNA for the photosystem II subunit S (*psbS*) gene from *Arabidopsis thaliana* (clone U13410) was provided by the Arabidopsis Biological Resource Center at Ohio State University. The coding region was amplified by PCR, and primers contained human rhinovirus 3C protease cleavage site/ligation- independent cloning (3C/LIC) specific sequences for cloning into the pET-52 3C/LIC vector (Novagen) fusing an N-terminal Strep-II tag and a C-terminal 10-histidine tag to the protein: PsbS_CAGGGACC CGGTGCTCCTAAAAAGGTTGAGAAGCCG and PsbS_Cterm_ GGCACCAGAGCGTTGCTTTCTTCACCATCATCGG.

PsbS was overexpressed in *Escherichia coli* BL21 (DE3) (1). Cells were disrupted with a microfluidizer (Microfluidics), and inclusion bodies were purified as described by Paulsen et al. (2).

Refolding of PsbS. Inclusion bodies were solubilized in 50 mM Hepes, 2% (wt/vol) lithium dodecyl sulfate (LDS), and 8 M urea (pH 8) and then centrifuged for 5 min at 20, 000 × *g*. The supernatant was loaded onto His-select Nickel Affinity Gel (Sigma). After washing with 50 mM Hepes and 0.1% LDS (pH 8), PsbS was eluted by lowering the pH to 5.3. The eluted protein (~0.8 mg/mL) was mixed with an equal volume of 0.1 M Hepes (pH 7.5), 4% (wt/vol) LDS, and 25% (wt/vol) sucrose, and it was heated to 100 °C for 1 min. Detergent was exchanged by addition of 1% *n*-octyl-β-D-glucopyranoside (OG; Glycon Biochemicals GmbH) and precipitation of LDS with 200 mM KCl. Samples were centrifuged for 10 min at 20,000 × *g*. The supernatant was further concentrated by ultrafiltration [Vivaspin (Sartorius); molecular mass cutoff = 50 kDa] and repeatedly washed with 50 mM Hepes (pH 7.5)/1% OG.

Analysis of Light-Harvesting Complex II Pigment Composition by HPLC. Pigments in 2-butanol were injected for analysis. The mobile phase consisted of a dual solvent system: solvent A was composed of acetonitrile/methanol/Tris·HCl (pH 9) in a volume ratio of 75:15:4, and solvent B was composed of methanol/hexane in a volume ratio of 4:1. The program for recording the chromatograms was as follows: Solvent A was run isocratically for 2 min, followed by a linear gradient to 50% (vol/vol) solvent B for 15 min. The program was set to an additional 2 min of isocratic flow, and a linear gradient to 100% (vol/vol) solvent B then followed for 6 min. The flow rate was set to 1.5 mL/min, and chromatograms were recorded at 440 nm at room temperature. Pigments were quantified by integration of the detected elution peaks and specific retention factors determined by pigment standards.

Gel Electrophoresis and Western Blotting. SDS/PAGE was performed with 12.5% (wt/vol) acrylamide gels according to the method of Laemmli (3). Proteoliposomes were dissolved in 2.5% (wt/vol) SDS and mixed at a 4:1 ratio with 5× Laemmli sample buffer containing 10 mM β-mercaptoethanol. Coomassie Blue R250 was used for staining. For Western blot analysis, proteins were transferred to PVDF membranes (Millipore), which were probed with either anti-PsbS (1:1,000 ratio; kindly provided by C. Büchel) or anti-Lhcb1 (1:2,000 ratio; Agrisera) antibody. Anti-rabbit IgG conjugated to HRP (Sigma) was used as the secondary antibody. Proteins were detected by enhanced chemiluminescence using ECL Western blotting detection reagents and Hyperfilm (both from Amersham).

Freeze-Fracture EM. Proteoliposome samples (2–3 μL) without cryoprotectant were placed between thin copper plates and rapidly frozen in ethane cooled by liquid nitrogen. Frozen samples were transferred to a BAF060 freeze-fracture apparatus (Balzers), with the specimen stage precooled to −130 °C, and fractured. Platinum/carbon was evaporated at an angle of 45°, and carbon reinforcing was performed at 90°. Organic material was removed from replicas by chromosulfuric acid treatment and subsequent washes in water. Replicas were deposited on Formvar (AGAR Scientific)-coated copper grids and examined in an EM208S electron microscope (FEI) equipped with a 1,024 × 1,024-pixel Tietz Video and Imaging Processing Systems CCD-camera.

Fluorescence Correlation Spectroscopy and Lifetime Measurements. For fluorescence lifetime and fluorescence correlation spectroscopy measurements, time-correlated single-photon counting (TCSPC) with two-photon excitation (TPE) was performed. The excitation light from a titanium-sapphire laser (830 nm, 90 MHz) was expanded and coupled into an IX50 inverted microscope with a UPlanSApo 60×/1.2-W water immersion objective (Olympus Europa Holding GmbH). A dichroic mirror (715 DCSPXR; AHF) was used to separate emission and excitation beams. The emission beam was further cleaned with a band-pass filter with a 50-nm bandwidth for sampling the 650- to 700-nm range (HQ 675/50; AHF) and then focused onto an avalanche photodiode (APD) (SPCM-AQR-13; PerkinElmer). The signals from the APDs were recorded using a TCSPC card (DPC-230; Becker & Hickel). Data acquisition, correlation, and lifetime histogramming were performed using in-house software. The acquisition time for a single measurement was 20 s, with an excitation power of 20 mW.

TPE Spectroscopy. An ultrafast laser system composed of a Vitesse Duo, RegA 9000, and OPA 9450 (all components from Coherent, Inc.) was used. The idler beam of 1,188 nm was used for the TPE of carotenoids. A 1,100-nm long-pass filter (FEL 1100; Thorlabs) and a hot mirror (L46-386; Edmund Optics) were used to remove the visible light of the signal beam thoroughly to prevent any undesired one-photon excitation (OPE) of chlorophylls. The TPE beam was steered to a home-built confocal setup allowing beam focus at the samples and collection of the chlorophyll fluorescence after TPE (F^{TPE}). Fluorescence was detected by a photodiode (designed by D. Schwarzer, Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany) connected to a lock-in amplifier (EG&G). The lock-in amplifier was synchronized with a mechanical chopper positioned in the idler beam path. OPE of chlorophylls was carried out using a conventional pulse-amplitude modulated (PAM) fluorometer (FMS1; Hansatech) integrated into the confocal setup. A 594-nm modulation beam was used, and the chlorophyll fluorescence after OPE (F^{OPE}) was detected by the fluorometer. The confocal setup enabled measurement of F^{TPE} and F^{OPE} at the same spot.

Samples were adjusted to the same absorbance at 670 nm and placed in a quartz cuvette with a path length of 1 mm. During data acquisition, samples were constantly moved back and forth by means of a peristaltic pump.

Determination of Carotenoid-Chlorophyll Energy Flow by Measuring $\Phi_{Coupling}^{CarS_1-Chl}$. The chlorophyll fluorescence observed after direct chlorophyll one-photon excitation, F^{OPE}, is proportional to the actual chlorophyll fluorescence quantum yield, including the effects of nonphotochemical quenching (NPQ), Φ_{Fl}:

$$F^{OPE} \propto \phi_{Fl}$$

The chlorophyll fluorescence observed after selective carotenoid TPE, F^{TPE}, is additionally proportional to the quantum yield of energy flow from carotenoids to chlorophyll that requires the presence of electronic interactions between the pigments: $\Phi_{\text{Coupling}}^{CarS_1-Chl}$:

$$F^{TPE} \propto \phi_{Fl} \cdot \phi_{\text{Coupling}}^{CarS_1-Chl}$$

Therefore, a direct comparison of both values measured under identical conditions is directly proportional to the energy flow from carotenoids to chlorophyll, $\Phi_{\text{Coupling}}^{CarS_1-Chl}$:

$$\frac{F^{TPE}}{F^{OPE}} \propto \phi_{\text{Coupling}}^{CarS_1-Chl}$$

The determination of Φ^{CarS_1-Chl} allows a quantitative comparison of the energy flow between the first excited state of carotenoids, *Car S_1*, and chlorophylls for different samples. For this comparison, it is important that the samples be measured under identical conditions, but the determination $\Phi_{\text{Coupling}}^{CarS_1-Chl}$ itself is independent of changes in the chlorophyll fluorescence quantum yield, for example, due to NPQ. Further details are provided in the study by Bode et al. (4).

1. Studier FW, Moffatt BA (1986) Use of bacteriophage T7RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 189(1):113–130.
2. Paulsen H, Rümler U, Rüdiger W (1990) Reconstitution of pigment-containing complexes from light-harvesting chlorophyll-a/b-binding protein overexpressed in Escherichia coli. *Planta* 181(2):204–211.
3. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259):680–685.
4. Bode S, et al. (2009) On the regulation of photosynthesis by excitonic interactions between carotenoids and chlorophylls. *Proc Natl Acad Sci USA* 106(30):12311–12316.

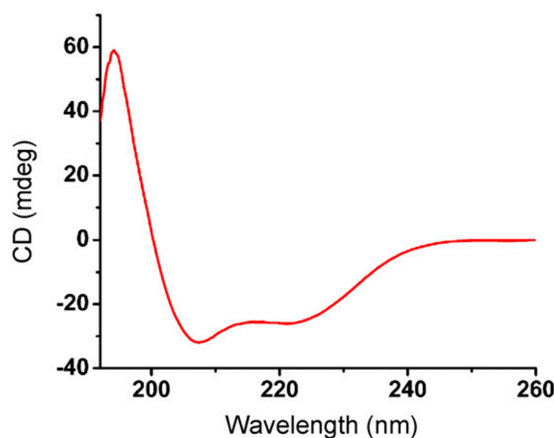
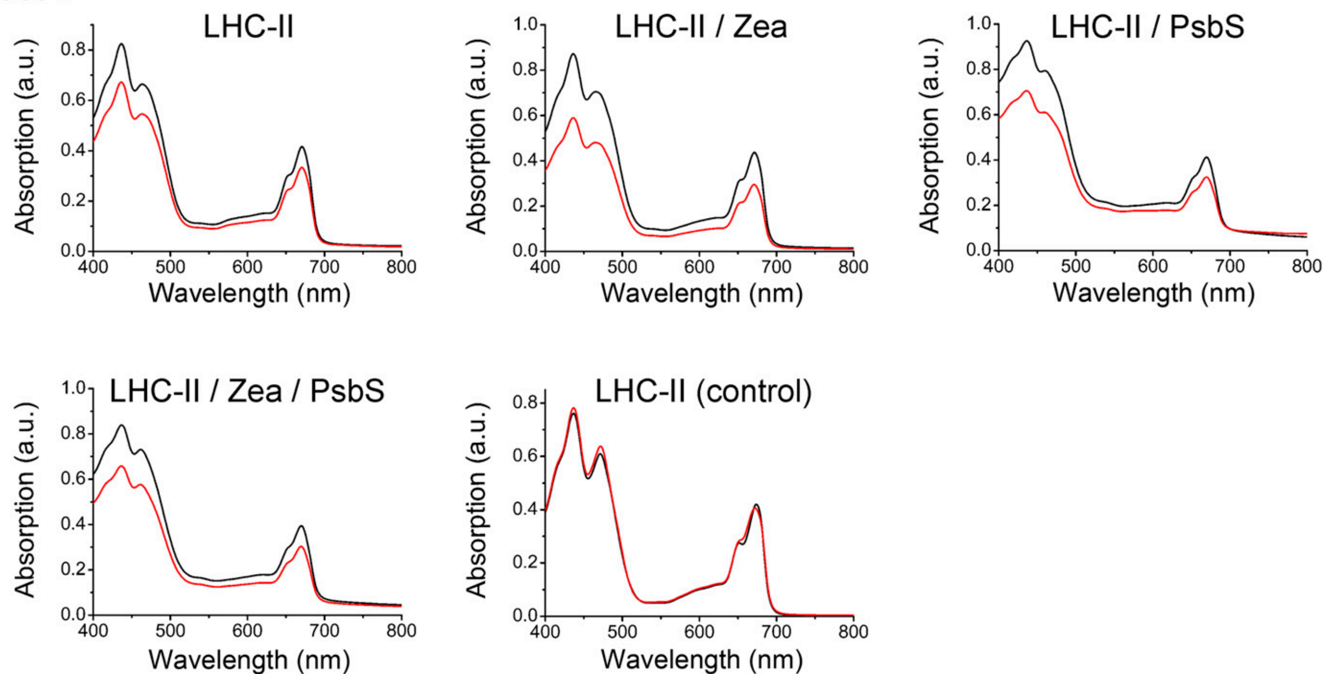


Fig. S1. CD spectrum of refolded PsbS in detergent solution. Refolding and CD measurement were performed in phosphate buffer. CD spectra were recorded on a Jasco J-810 spectropolarimeter in the UV region with PsbS in 100 mM sodium phosphate and 1% OG. The CD difference spectrum was calculated by subtraction of the buffered detergent solution spectrum. mdeg, millidegrees.



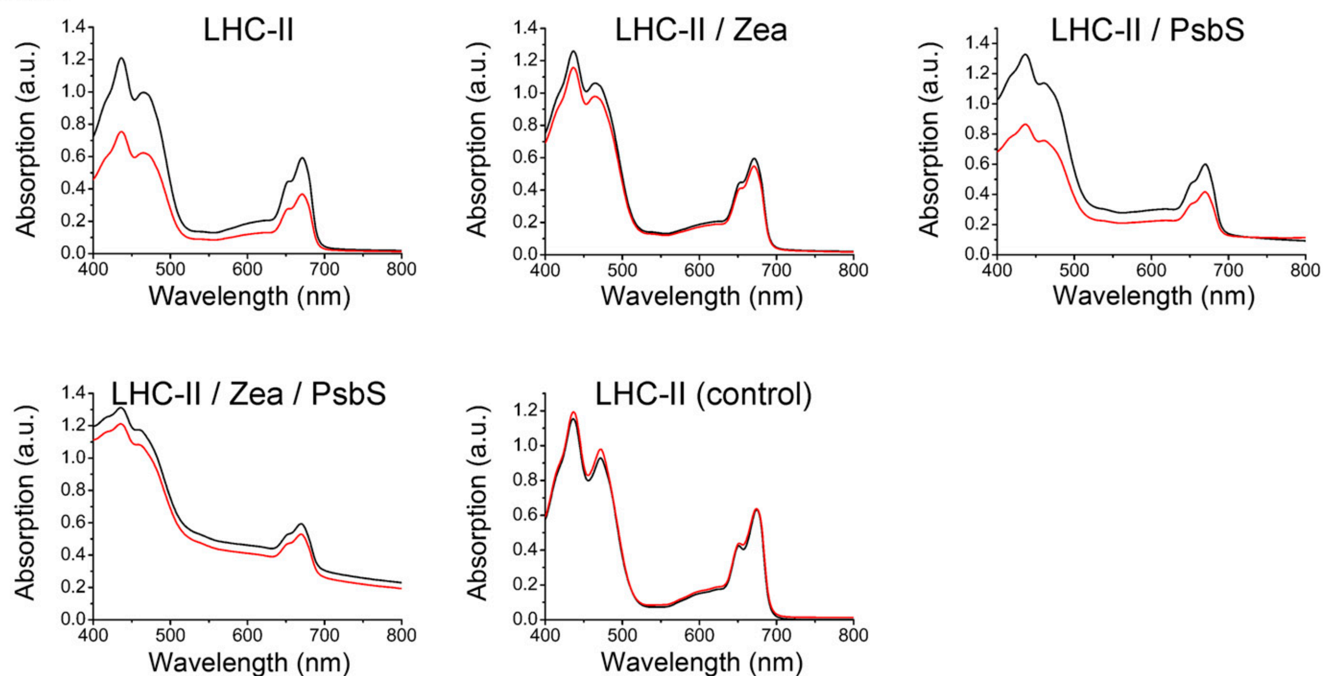
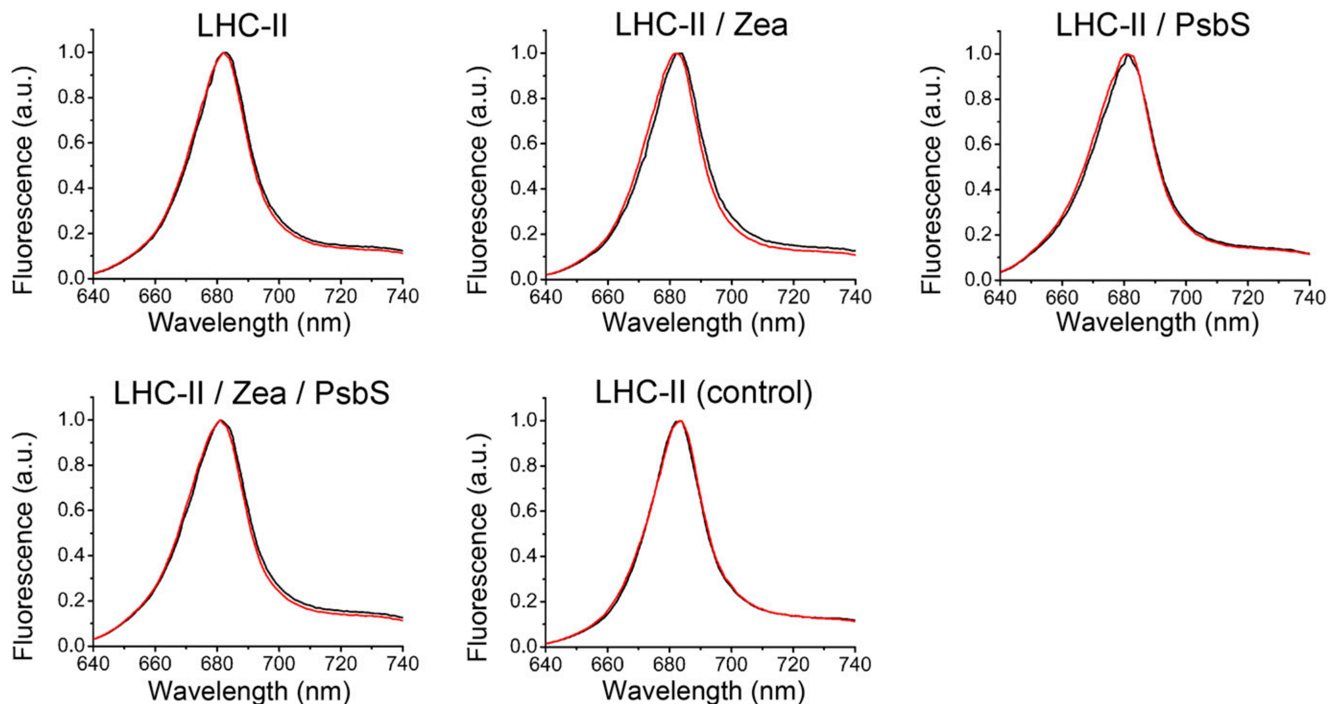


Fig. S2. Absorption spectra of both proteoliposome sets (set 1 and set 2) before (black) and after (red) OPE and TPE measurements. Light-harvesting complex II (LHC-II) in detergent solution was used for comparison. All spectra were recorded at pH 7.5. The heterogeneous absorption intensity of some samples is due to transfer losses between cuvettes after OPE and TPE measurements. a.u., arbitrary units.



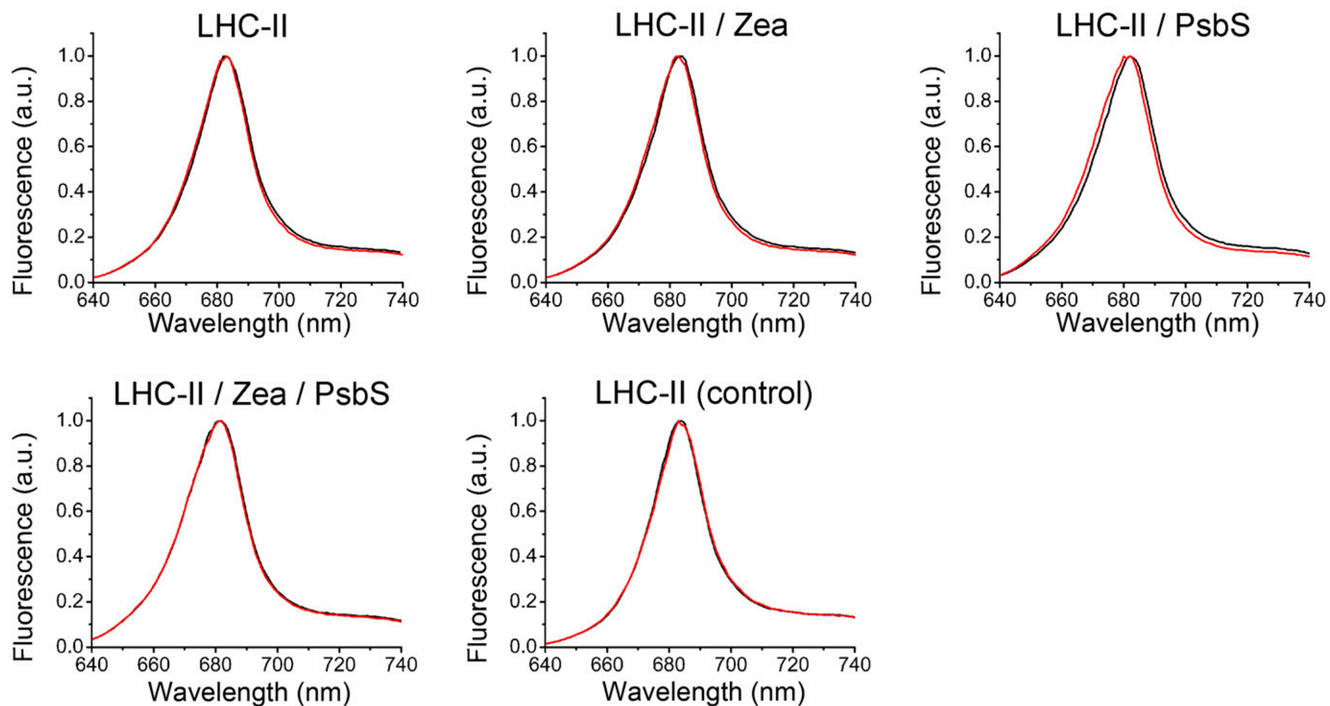


Fig. S3. Fluorescence emission spectra before and after OPE and TPE measurements for both sets (set 1 and set 2) of proteoliposomes and LHC-II in detergent solution (control). All measurements were performed at pH 7.5. The excitation wavelength was set to 441 nm, and emission spectra were recorded between 640 and 740 nm. All emission spectra were normalized to 1.

Table S1. Correction factors for PAM fluorescence and corresponding NPQ values

Proteoliposomes	F^{TPE}	F^{OPE}	Correction factor	NPQ	$\Phi_{Coupling}^{CarS_1-Chl}$
Set 1					
LHC-II	1.00 ± 0.02	1.00 ± 0.01	0.38	0.00	1.00 ± 0.02
LHC-II/Zea	1.03 ± 0.03	0.85 ± 0.01	0.40	0.26	1.21 ± 0.03
LHC-II/PsbS	0.82 ± 0.03	0.58 ± 0.01	0.32	0.44	1.40 ± 0.06
LHC-II/Zea/PsbS	0.76 ± 0.04	0.45 ± 0.01	0.32	0.89	1.69 ± 0.09
Set 2					
LHC-II	1.00 ± 0.01	1.00 ± 0.00	0.55	0.00	1.00 ± 0.02
LHC-II/Zea	1.05 ± 0.03	0.96 ± 0.02	0.55	0.04	1.09 ± 0.04
LHC-II/PsbS	0.81 ± 0.01	0.64 ± 0.00	0.46	0.31	1.27 ± 0.02
LHC-II/Zea/PsbS	0.45 ± 0.01	0.22 ± 0.01	0.29	1.39	2.06 ± 0.10

Chlorophyll (Chl) fluorescence after OPE and TPE (F^{OPE} and F^{TPE}, respectively) was measured from two sets of proteoliposome samples (set 1 and set 2). The absorption and fluorescence emission spectra of these sets are shown in Figs. S2 and S3. For the calculation of NPQ (Eq. 2), the F^{OPE} values were corrected for differing light-harvesting complex II (LHC-II) concentrations as deduced from Chl absorption. The correction factors were determined by subtraction of the 700-nm absorption from the 680-nm absorption. Fluorescence from LHC-II proteoliposomes was set as F_m. Electronic carotenoid (Car) S_1-Chl coupling, $\Phi_{Coupling}^{CarS_1-Chl}$, was calculated according to Eq. 1. (Here, it makes no difference whether both values, F^{OPE} and F^{TPE}, are corrected for differing LHC-II concentrations or not.) Zea, zeaxanthin.

Table S2. Average diffusion times determined by fluorescence correlation spectroscopy

	Average diffusion time, s	
Proteoliposomes	pH 7.5	pH 5.5
LHC-II	0.01088 ± 0.00537	0.01184 ± 0.00546
LHC-II/Zea	0.01219 ± 0.005	0.01383 ± 0.00485
LHC-II/PsbS	0.03797 ± 0.03769	0.03815 ± 0.04368
LHC-II/Zea/PsbS	0.04314 ± 0.03677	0.03333 ± 0.02925
LHC-II (in solution as control)	0.00477 ± 0.00824	0.01854 ± 0.00308

Average diffusion times for proteoliposomes and LHC-II in solution were determined by fluorescence correlation spectroscopy at pH 7.5 and pH 5.5. LHC-II diluted into detergent/buffer solution at pH 7.5 or into detergent-free buffer at pH 5.5 served as a control. The mean average values with the SD of three different liposome batches are given.

Table S3. Determination of hydrodynamic radii by dynamic light scattering

	Hydrodynamic radius, nm	
Proteoliposomes	pH 7.5	pH 5.5
LHC-II	38.70	37.00
LHC-II/Zea	37.20	37.40
LHC-II/PsbS	76.00	72.20
LHC-II/Zea/PsbS	87.60	90.70
LHC-II (in solution as control)	4.00	66.47

Approximate hydrodynamic radii of proteoliposomes and LHC-II in solution at pH 7.5 and pH 5.5 were determined by dynamic light scattering. As a control, LHC-II diluted into detergent/buffer solution at pH 7.5 or into detergent-free buffer at pH 5.5 was used. Dynamic light scattering measurements were performed on one batch of proteoliposomes.