Supplementary Information

Molecular Mechanisms of Light Harvesting in the Minor Antenna CP29 in Near-Native Membrane Lipidic Environment

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MATERIALS AND METHODS

A. Sample Preparation

MPS 1E3D1 overexpression, purification and TEV cleavage: Plasmid MSP1E3D1 was kindly provided by Prof. G. Schlau-Cohen (Massachusetts Institute of Technology, Cambridge). Protein expression was made as described in Pandit et al.[1] with few modifications. Upon induction, 1 litre of cell culture was centrifuged for 10 min, 5000 *g*, 4°C; phenylmethylsulfonyl fluoride (PMSF, 2 mM) was added as protease inhibitor in the storage buffer; disrupted cells were pelleted by centrifugation (20000 *g*, 10 min, 4° C); imidazole was added to supernatant before incubation with beads (Sepharose Fast Flow, GE Healthcare) to a final concentration of 10 mM. During the Immobilized Metal Affinity Chromatography (IMAC), the washing step with buffer D was extended to 10 beads volume. Purity of MSP was assayed with Coomassie R staining on SDS-PAGE. To remove the imidazole, MSP was dialyzed over-night at 4°C using a 20 kDa dialysis membrane submerged in 1 L of dialysis buffer (DB) (20 mM Tris pH 8.0, 0.15 M NaCl). Finally, protein was quantified measuring the absorbance at 280 nm (NanoDrop UV-Vis Spectrophotometer, Thermo Fischer) (ε=29,900 Mol^{-1} cm^{-1}; MW: 32.6 kDa).

His-TAG was removed exploiting the TEV cleavage site in between 7xHis-TAG and MSP amino acidic sequence. Protein was diluted to 2 mg/mL using DB buffer. Digestion was made over-night at RT under slow rotation, adding 10 mM TCEP-HCl as reducing compound and a home-made His-tagged TEV enzyme (mg of MSP/mg of TEV ratio equal to 20). The day after, TEV was removed by reverse IMAC exploiting the His-TAG of TEV protein. Ni-NTA column was pre-equilibrated with DB supplemented with 20 mM imidazole. Imidazole 20 mM was added to the digestion mixture, then it was incubated for 2h at 4°C with beads under slow rotation. Digested MSP was eluted with 5 extra beads volumes of DB supplemented with 20 mM imidazole,

and the TEV was removed from the column with DB supplemented with 400 mM imidazole. Digestion efficiency was verified with Coomassie R staining on SDS-PAGE. Protein was quantified measuring the absorbance at 280 nm (ε=26.930 Mol^{-1} cm^{-1}; MW: 29.982 kDa).

CP29-Vio and CP29-Zea expression and purification: The plasmid pBI121 carrying the gene *Lhcb4.1* from *A. thaliana*[2] was manipulated to add a 6xHis-TAG at the C-terminus. Upstream the stop codon, two glycines and six histidines were added using Q5 site-directed mutagenesis kit (New England Biolabs). Primers were designed using the appropriate Tool NEBaseChanger (primers sequences in Supplementary Table T1), then insertion was confirmed using Sanger sequencing. The new plasmid was amplified in *E. coli* TOP10 strain and used to transform *A. tumefaciens* strain GV3101. *Arabidopsis thaliana* Col-0 triple knock-out mutant (*koLhcb4*) was transformed[3] and T1 seeds population was plated in MS-agar containing kanamycin 100 µg/mL as selection marker. 86 independent lines were selected, and a semi-quantitative immune titration was performed to identify the high-expressing lines. T3 homozygous plants were obtained through the analysis of segregation based on antibiotic resistance.

The CP29-TAG-expressing line of *A. thaliana* was genome-edited to knock-out zeaxanthin epoxidase (ZEP) encoding gene (AT5G67030). A construct bringing CRISPR-Cas9 system with four sgRNAs was assembled according to Ordon et al.[4,5], see Supplementary Table T1 for primers sequences. The correct assembly was tested through PCR and Sanger sequencing. Plasmid was amplified in *E. coli* TOP10 strain and used to transform *A. tumefaciens* strain GV3101. T3 *A. thaliana* plants expressing CP29-TAG were transformed by *Agrobacterium*-mediated transformation.[3] T1 seeds were plated in MS-agar medium with Hygromycin 35 µg/mL as selection antibiotic. Seedling plants were transplanted into the soil , lines devoid of ZEP were selected by immune titration using α-ZEP primary antibody (Agrisera AS15 3092) and by HPLC[6].

Supplementary Table T1: Primer sequences used to insert the Histidine-TAG to the Lhcb4.1 gene (AT5G01530) and primer sequences used to create sgRNAs of the CRISPR-Cas9 system to knockout ZEP encoding gene (AT5G67030).

Primer name	Primer sequence
CP29-GG-6xHis_FW	5' - catcaccaccacTAAAATCATTATCATGTGAGATTC - 3'
CP29-GG-6xHis_RV	5' - atgatgtcctccAGATGAGGAGAAGGTATC - 3'
sgRNA_ZEP_1_FW	5’ - attgTCGTTTTCCGGAAAACCCGG - 3’
sgRNA_ZEP_1_RV	5’ - aaacCCGGGTTTTCCGGAAAACGA - 3’
sgRNA_ZEP_2_FW	5’ - attgTCATCGTTTTCCGGAAAACC - 3’
sgRNA_ZEP_2_RV	5’ - aaacGGTTTTCCGGAAAACGATGA - 3’
sgRNA_ZEP_3_FW	5’ - attgTTACTCCGAGCAAAGCTCGA - 3’
sgRNA_ZEP_3_RV	5’ - aaacTCGAGCTTTGCTCGGAGTAA - 3’
sgRNA_ZEP_4_FW	5’ - attgCTGGGTGTATCACTGGTGAT - 3’
sgRNA_ZEP_4_RV	5’ - aaacATCACCAGTGATACACCCAG - 3’

70 seedlings for each *A. thaliana* genetic background was grown under 100 µmol photons m^{-2} s^{-1}, 24 °C, 60% humidity and 8h/16h light/dark photoperiod. After 8 weeks, leaves were harvested before the daylight, placed 1h at 4° C in the dark, then thylakoids extracted[7] Chl content was measured as described in Porra et al.[8] Thylakoids were pelleted (20000 *g*, 15 min, 4 °C) and resuspended at 1 µg of Chl/mL in Hepes 10 mM pH 7.5. An equal volume of solubilisation buffer containing Hepes 10 mM pH 7.5, n-Dodecyl α-D-maltoside (α-DM) 1.6%, was added. Mixture was vortexed for 1 min and placed 2h at 4° C under gentle stirring. Samples were centrifuged at 20000 *g* to remove insolubilized membranes and starch. Supernatants were diluted to decrease α-DM concentration to 0.5%, and supplemented with NaCl 0.15 M and imidazole 10 mM. Thylakoids were incubated with Ni-derivatized Sepharose beads pre-equilibrated with buffer A (Hepes 10 mM pH 7.5, NaCl 0.15 M, imidazole 10 mM, α-DM 0.03%). Washing was made with

10 resin volume (RV) of buffer A, 6 RV of buffer A with 20 mM imidazole, 0.5 RV of buffer A with 50 mM imidazole, then CP29 was eluted several times with 0.5 RV of buffer A with 250 mM imidazole. Fractions eluted with 50 mM and 250 mM imidazole were concentrated (VIVASPIN, Sartorius, 30 kDa cut-off) and loaded on sucrose gradients (Hepes 10 mM pH 7.5, sucrose 0.5 M, α-DM 0.03%). Samples were ultra-centrifuged 26h at 180000 *g*, 4° C to separate CP29 monomer from CP29 assembled in complexes and supercomplexes. The same protocol was followed for purification of CP29 from *npq2* background.

Nanodisc-CP29 assembly: Nanodisc assembly was performed following the method reported by Son et al.[9] Soy phospholipids were quantified[10] and phospholipids 20 mM were solubilized in buffer containing Na-cholate 40 mM, Hepes 50 mM pH 7.5, NaCl 100 mM. The excess of sucrose was removed from CP29 samples using VIVASPIN (30 kDa cut-off). CP29 was concentrated to 0.7 mg/mL and MSP to 4 mg/mL, and then CP29, MSP and lipids were mixed in a molar ratio of 0.125:1:120. The mixture was maintained for 1h at 4° C under slow rotation, and then 0.8 volumes of Bio-Beads SM-2 (Biorad) were added. Samples were then placed further 5h at 4° C under slow rotation. Supernatants were recovered by centrifugation; empty nanodiscs were removed exploiting the His-TAG on CP29. Assembled nanodiscs were incubated overnight at 4° C with Ni-column pre-equilibrated with buffer containing 10 mM Hepes pH 7.5, NaCl 20 mM. Resin was washed with 10 RV of the same buffer and loaded nanodiscs were eluted with 200 mM imidazole. To remove aggregates, samples where further purified using size-exclusion chromatography and eluted at 0.5 mL/min with Hepes 50 mM pH 7.5, NaCl 0.15 M. CP29-nanodisc assembly was evaluated with Coomassie R staining on SDS-PAGE and fluorescence emission exciting at 470 nm to assess the lack of free Chl *b*.

SUPPLEMENTARY FIGURES

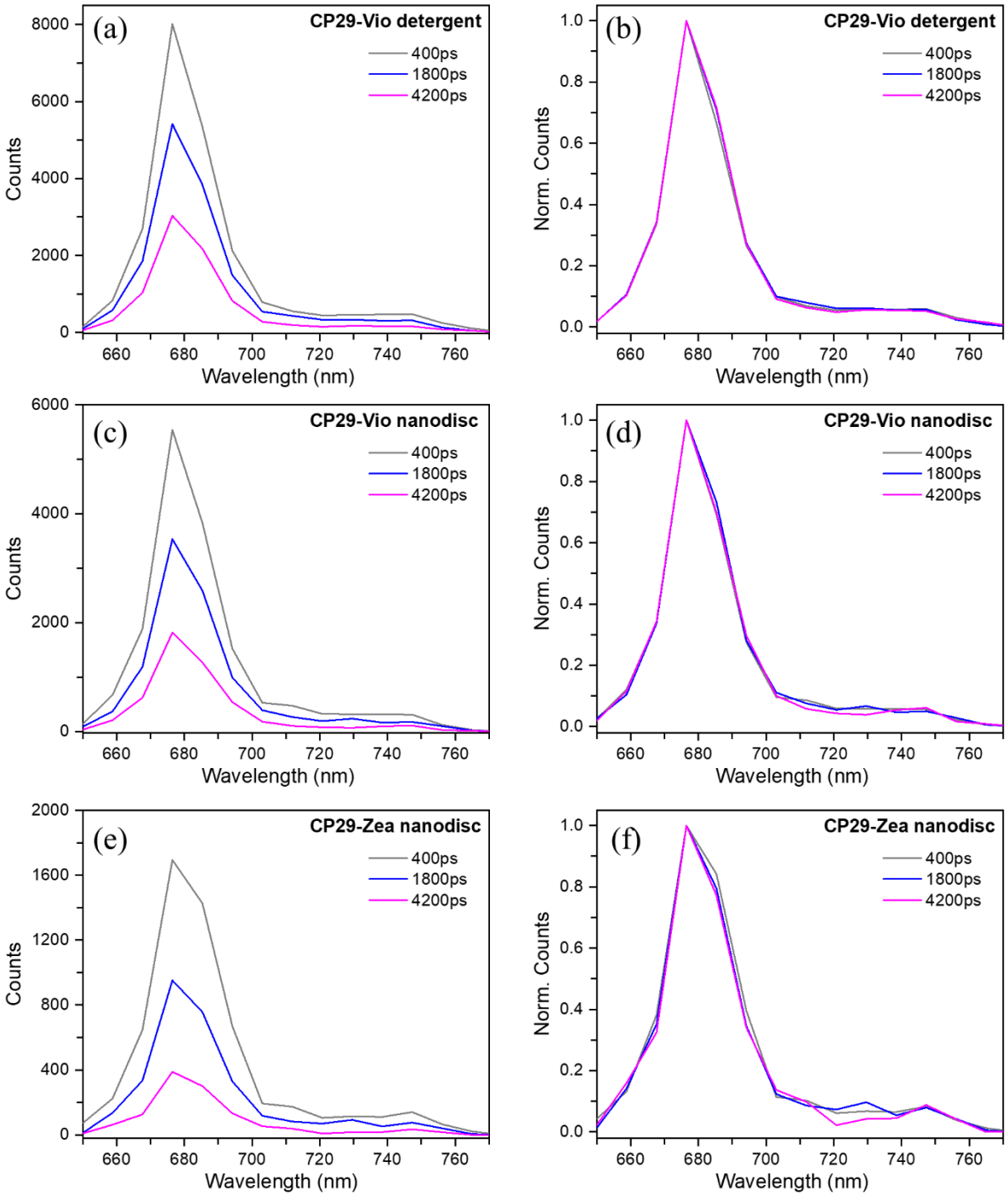

Figure S1: Time-gated fluorescence spectra of (a) CP29-Vio in detergent, (c) CP29-Vio in nanodisc and (e) CP29-Zea in nanodisc upon excitation at 630 nm. The right panel shows the corresponding normalized spectra for (b) CP29-Vio in detergent, (d) CP29-Vio in nanodisc and (f) CP29-Zea in nanodisc.

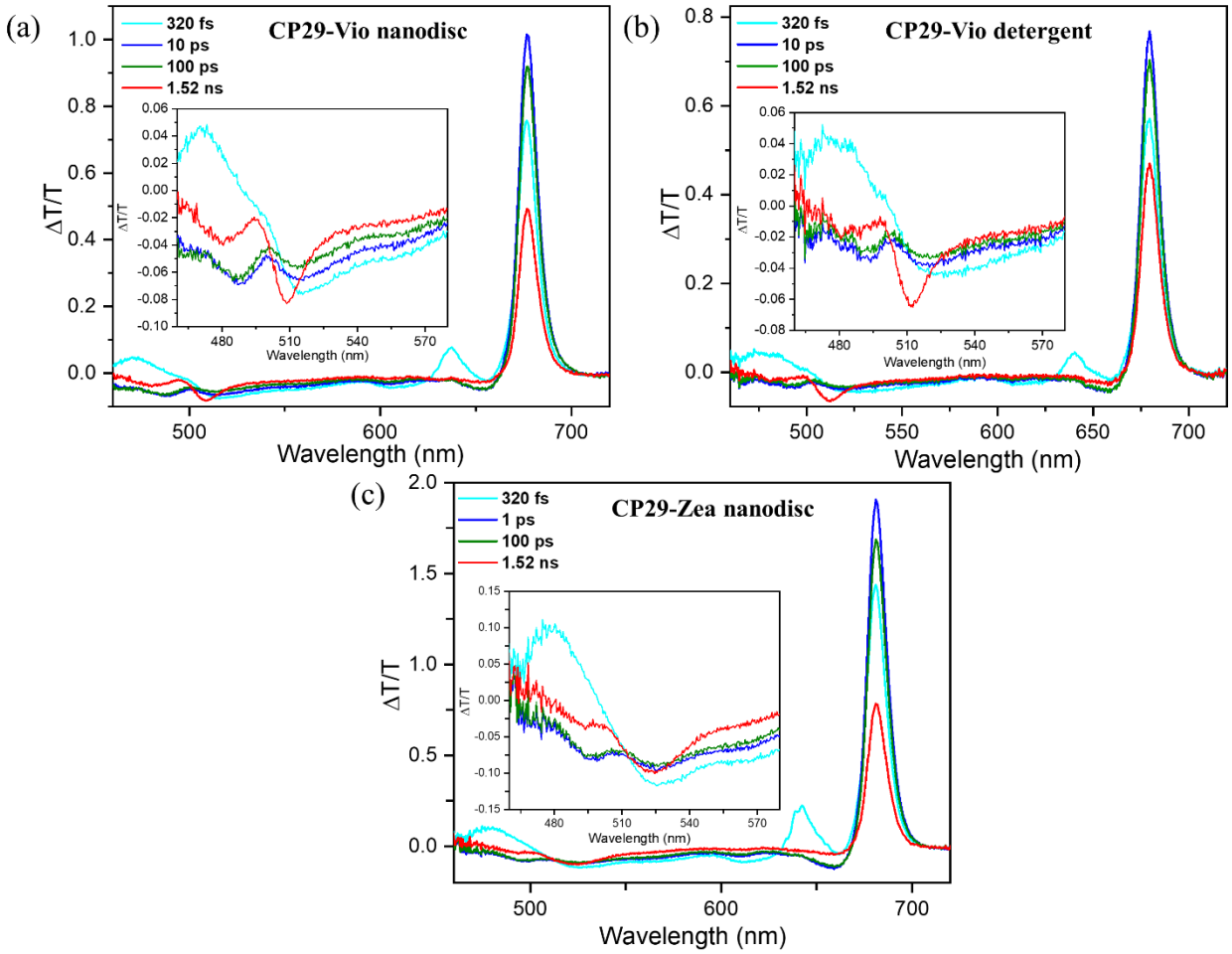

Figure S2: TA spectra upon excitation at 630 nm. TA spectra at selected time delays for (a) CP29-Vio in nanodiscs, (b) CP29-Vio in detergent and (c) CP29-Zea in nanodiscs. Inset shows the zoomed spectra in the range 470-580 nm range. The pump fluence used was 3.6 μJ/cm^2, 5.3 μJ/cm^2 and 4.7 μJ/cm^2 for CP29-Vio in detergent, CP29-Vio in nanodiscs and CP29-Zea in nanodiscs, respectively.

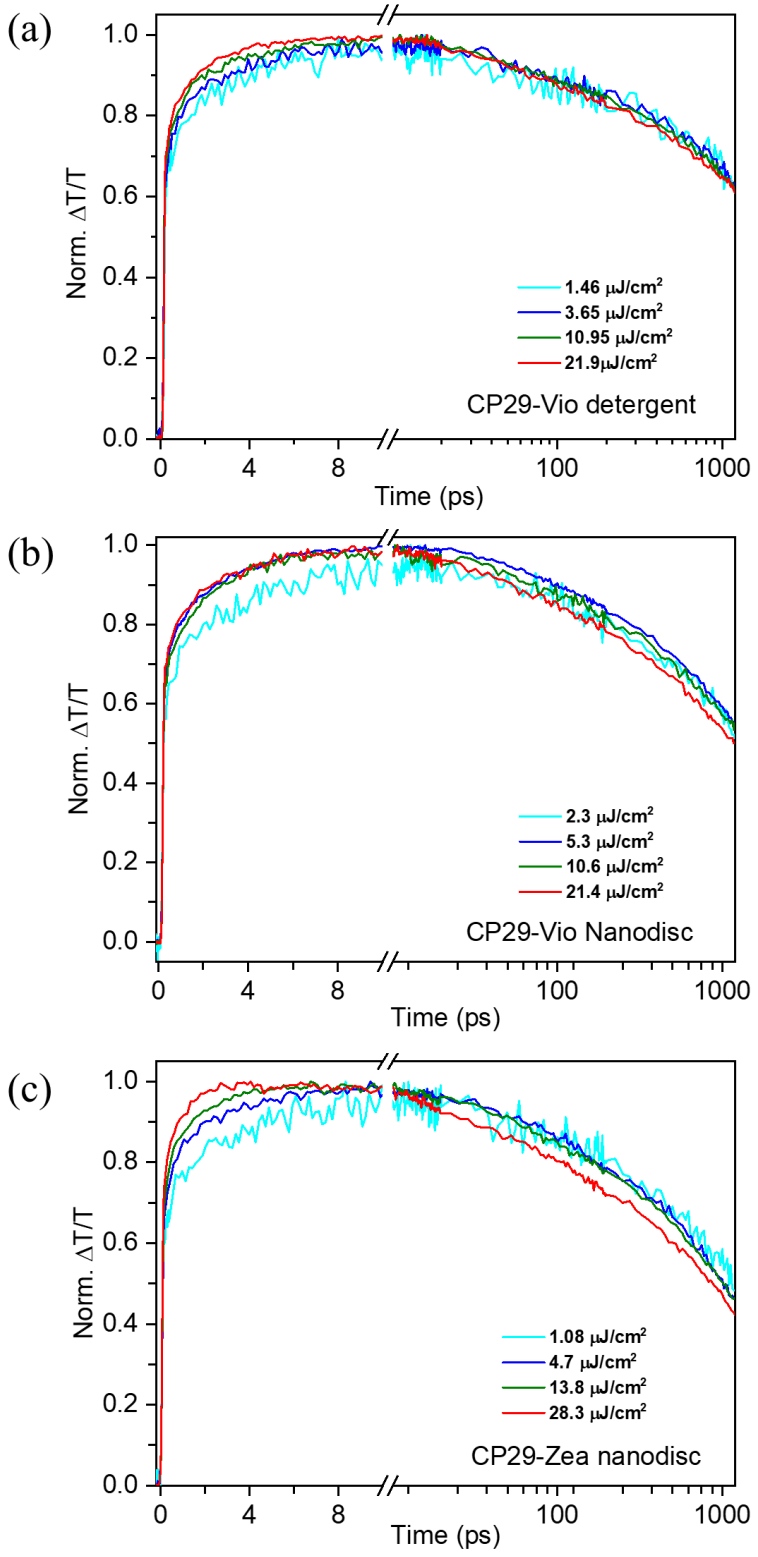

Figure S3: TA kinetics at 680 nm upon excitation at 630 nm for (a) CP29-Vio in detergent, (b) CP29-Vio in nanodiscs and (c) CP29-Zea in nanodiscs at different fluences.

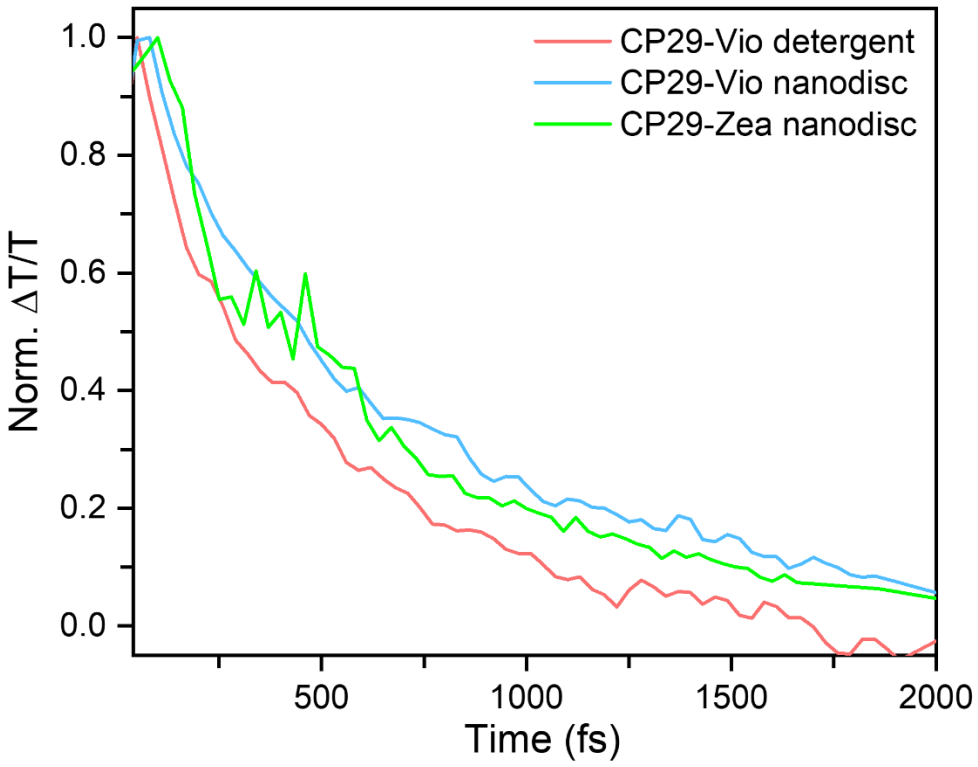

Figure S4: Chl *b* to Chl *a* energy transfer upon excitation at 630 nm. TA kinetics at 640 nm.

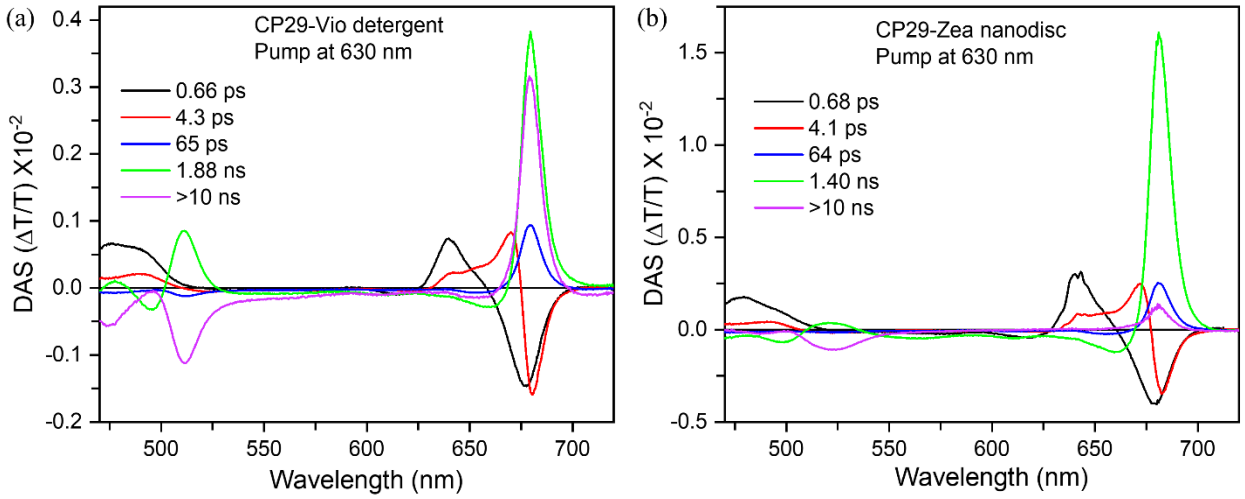

Figure S5: Global analysis of TA data upon excitation at 630 nm. Decay Associated Spectra (DAS) for (a) CP29-Vio in detergent and (b) CP29-Zea in nanodiscs.

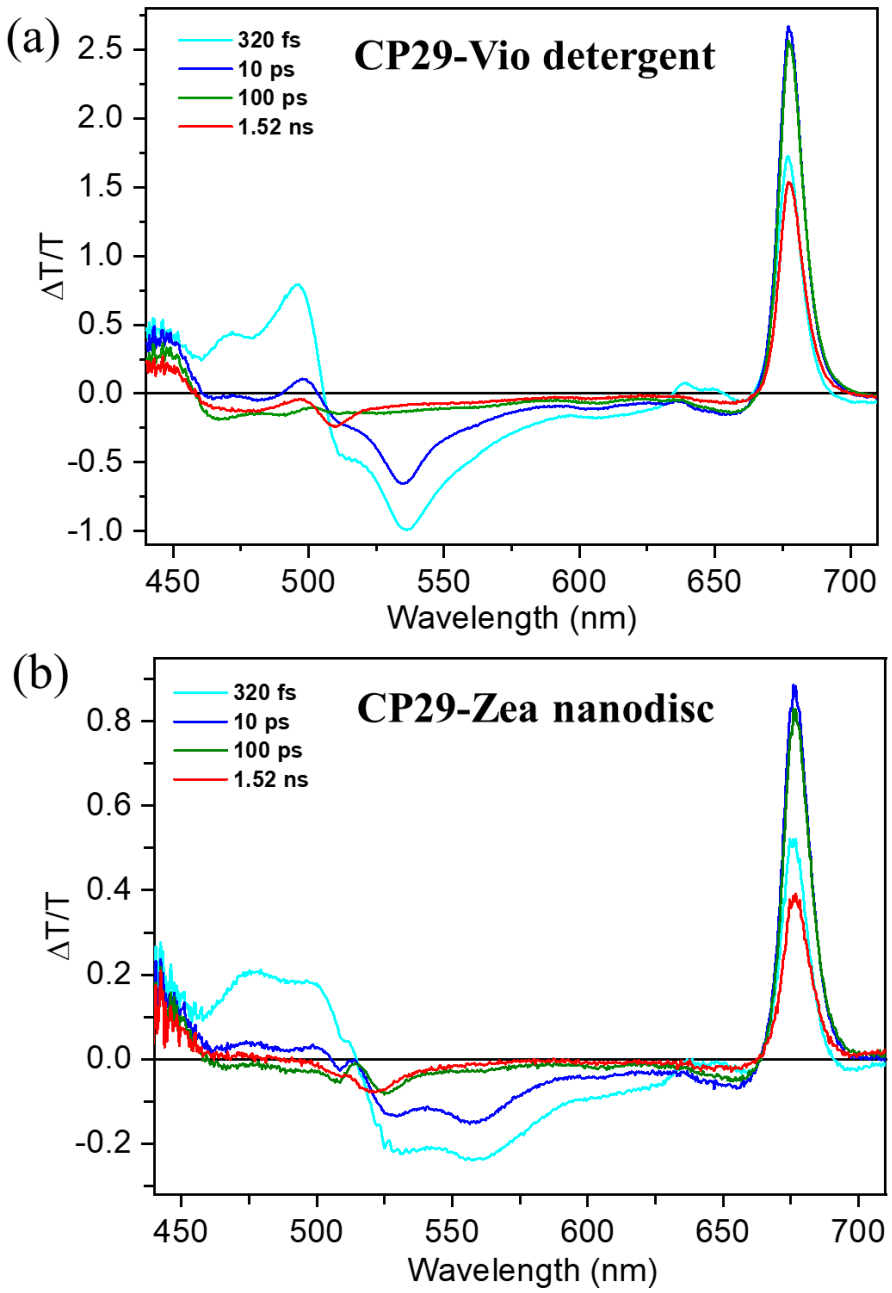

Figure S6: TA spectra upon excitation at 510 nm. TA spectra at selected time delays for (a) CP29-Vio in detergent and (b) CP29-Zea in nanodiscs. The pump fluence were at 27.1 μJ/cm^2 and 30.8 μJ/cm^2 for CP29-Vio in detergent and CP29-Zea in nanodiscs, respectively.

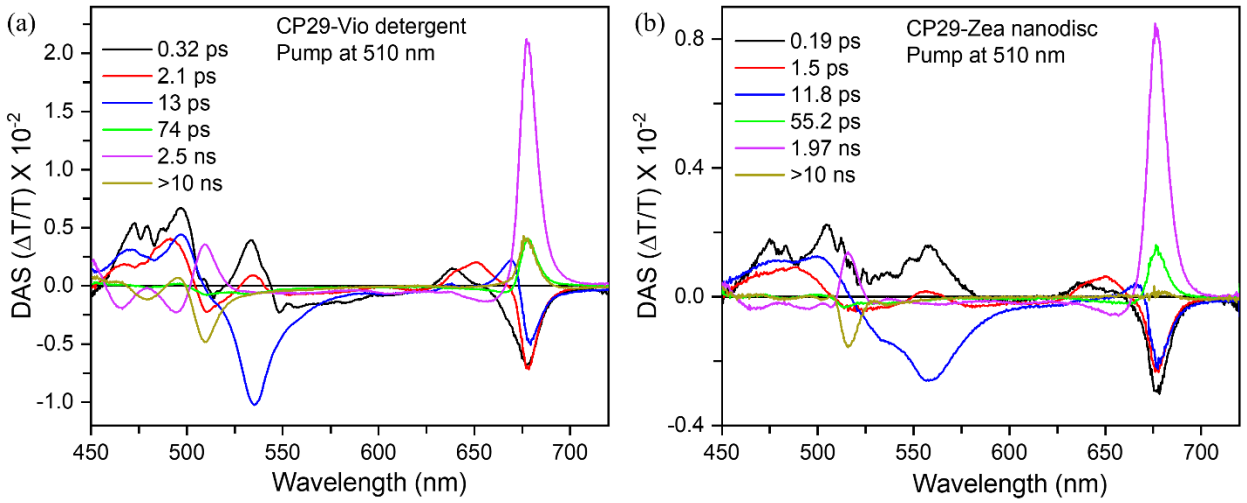

Figure S7: Global analysis of TA data upon excitation at 510 nm. Decay Associated Spectra (DAS) for (a) CP29-Vio in detergent and (b) CP29-Zea in nanodiscs.

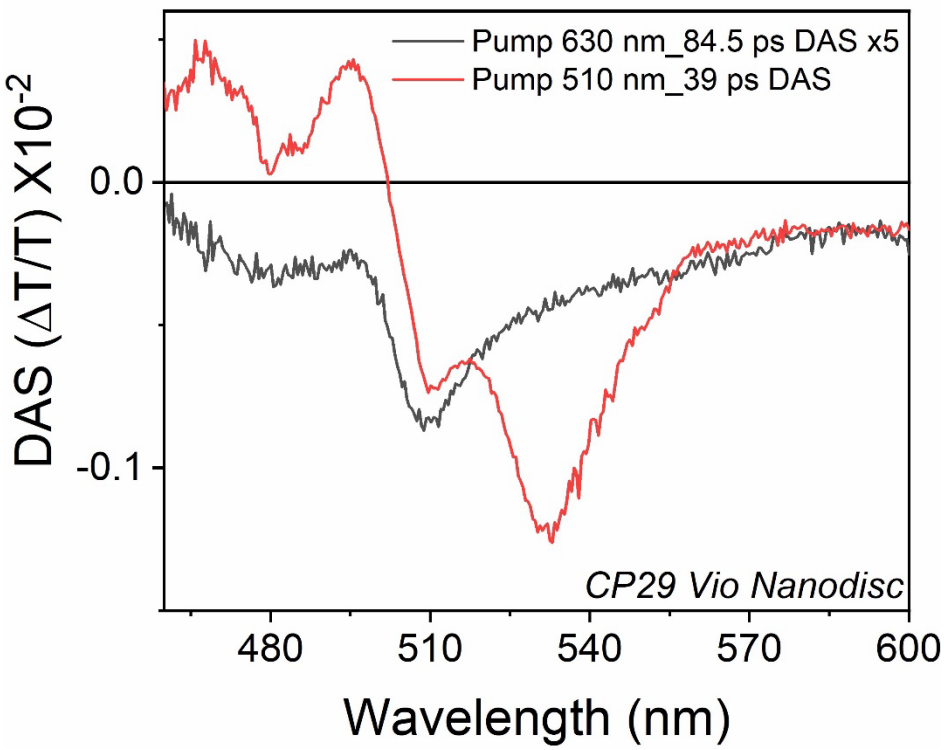

Figure S8: Comparison of Decay Associated Spectra (DAS) of CP29-Vio in nanodiscs between pump at 630 nm, 84.5 ps DAS and pump at 510 nm, 39 ps DAS.

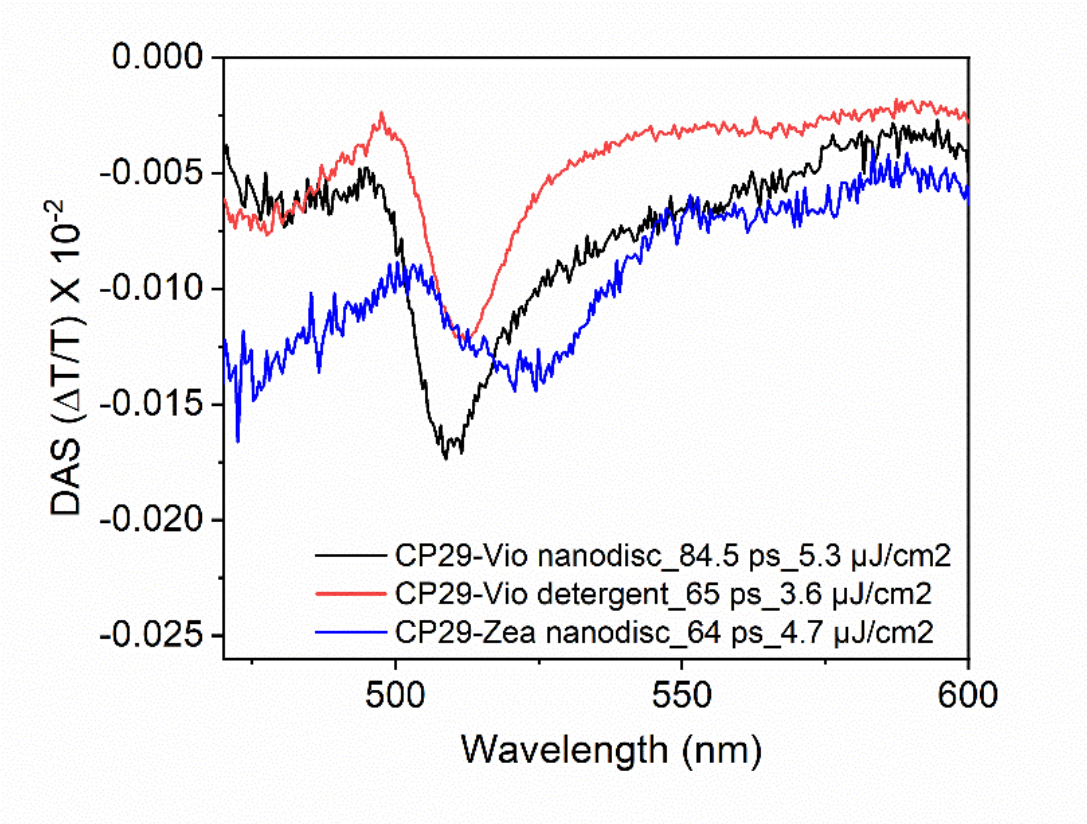

Figure S9: Comparison of Decay Associated Spectra (DAS) of CP29-Vio in detergent, CP29-Vio in nanodiscs and CP29-Zea in nanodiscs upon excitation at 630 nm obtained at 65 ps, 84.5 ps, and 64 ps, respectively.

References

[1] A. Pandit, N. Shirzad-Wasei, L.M. Wlodarczyk, H. van Roon, E.J. Boekema, J.P. Dekker, and W.J. de Grip, Biophys. J. **101**, 2507 (2011).

[2] Z. Guardini, M. Bressan, R. Caferri, R. Bassi, and L. Dall'Osto, Nat. Plants **6**, 303 (2020).

[3] X. Zhang, R. Henriques, S.-S. Lin, Q.-W. Niu, and N.-H. Chua, Nat. Protoc. **1**, 641 (2006).

[4] J. Ordon, J. Gantner, J. Kemna, L. Schwalgun, M. Reschke, J. Streubel, J. Boch, and J. Stuttmann, Plant J. **89**, 155 (2017).

[5] J. Ordon, M. Bressan, C. Kretschmer, L. Dall'Osto, S. Marillonnet, R. Bassi, and J. Stuttmann, Funct. Integr. Genomics **20**, 151 (2019).

[6] A.M. Gilmore and H.Y. Yamamoto, J. Chromatogr. A **543**, 137 (1991).

[7] R. Bassi, O. Machold, and D. Simpson, Cadsb. Res. Commun **50**, 145 (1985).

[8] R.J. Porra, W.A. Thompson, and P.E. Kriedemann, Biochim. Biophys. Acta - Bioenerg. **975**, 384 (1989).

[9] M. Son, A. Pinnola, S.C. Gordon, R. Bassi, and G.S. Schlau-Cohen, Nat. Commun. **11**, 1 (2020).

[10] T. Boldog, M. Li, and G.L. Hazelbauer, Methods Enzymol. **423**, 317 (2007).