HIGHER PLANT PHOTOSYSTEM II LIGHT HARVESTING ANTENNA, NOT THE REACTION CENTER, DETERMINES THE EXCITED STATE LIFETIME - BOTH, THE MAXIMUM AND THE NON-PHOTOCHEMICALLY QUENCHED

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Running title: Excitation kinetics in PSII depleted from RC

Supplemental Material

MATERIALS AND METHODS

Plant growth

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (*Arabidopsis*) was grown for 10 weeks in Sanyo plant growth cabinets with a 8-h photoperiod at a light intensity of 100 μmol photons m^{-2} s^{-1} and a day/night temperature of 22/15 °C. Lincomycin (0.1 g/L, Sigma Aldrich, München, Germany) was added to the irrigation water starting from the rosette stage and continued during to the whole plant growth. All measurements were performed on leaf areas where Fv/Fm was in the range of 0.1-0.3.

SDS-PAGE and Western blot

The concentration of proteins in total extracts obtained from *Arabidopsis* leaves was determined and normalised according to Bradford (19). The proteins were resolved by SDS-PAGE in 12% acrylamide gel and transferred onto nitrocellulose membrane. Immunoblotting was performed by incubation with primary antibodies specific for *Arabidopsis* D1, Lhcb1,2,3 (LhcbII) and Lhcb4 (CP29), Lhcb5 (CP26), Lhcb6 (CP24), PsaA and Lhcba2, and PsbS protein (Agrisera, Vännäs, Sweden). TROL antibody was a kind gift from prof. H. Fulgosi. Detection was performed using ECL Plus Western

Blotting Detection System (GE Healthcare Life Sciences, Little Chalfont, UK). Densitometry of the immunosignals was done using NIH ImageJ software and associated plugins (http://rsb.info.nih.gov/ij/) taking the wild-type level as 100% and by using anti-TROL antibody as a control for the lane loading. The percentages reported in the Result section are the average of between 5 and 10 different samples.

FPLC Analysis of Thylakoid Membranes

FPLC analysis was performed on detergent-solubilized, freshly prepared stacked thylakoid membranes as described previously (20). Thylakoids were suspended to a final chlorophyll concentration of 1.0 mg/mL and partially solubilized by the addition of n-dodecyl α-D-maltoside to a final concentration of 1% and incubated for 1 min at room temperature. Unsolubilized material was removed by 1 min of centrifugation at 16,000 g. The supernatant was then filtered through a 0.45-µm filter and subjected to gel filtration chromatography using an Amersham-Pharmacia Äcta purifier system (GE Healthcare Life Sciences, Little Chalfont, UK), including a Superdex 200 HR 10/30 column.

Pigment analysis

Total pigments were extracted from leaves after incubation on 80% ice cold acetone and 5 minutes centrifugation in bench centrifuge at maximum speed. Absorbance at 646.6, 663.6 and 750 nm was detected to determine total chlorophyll concentration and chlorophyll *a/b* ratio, calculated according to Porra et al. (21). Zeaxanthin and de-epoxidation state (Zeaxantin +0.5*Antheraxantin)/(Violaxantin+Antheraxantin+Zeaxanthin) were determined by reversed phase HPLC using a LiChrospher 100 RP-18 column (Merck) and a Dionex Summit chromatography system (Dionex Ltd., Leeds, UK) as previously described (22).

Chlorophyll fluorescence induction

Chlorophyll fluorescence was measured with a Dual PAM 100 chlorophyll fluorescence photosynthesis analyzer (Heinz Walz, Effeltrich, Germany). Plants were adapted in the dark for 30 min prior to measurement. Actinic illumination of 200 and 700 µmol photons m^{-2} s^{-1}, respectively (see the Result section) was provided by arrays of 635-nm LEDs illuminating both the adaxial and abaxial surfaces of the leaf. Fo (the fluorescence level with open PSII reaction centers) was measured in the presence of the 10 µmol photons m^{-2} s^{-1} measuring beam. Maximum fluorescence in the dark-adapted state (Fm), during the course of actinic illumination (Fm') and in the subsequent dark relaxation periods was determined using a 0.8 s saturating light pulse (1000 µmol photons m^{-2} s^{-1}). The quantum yield of PSII (Fv/Fm) was defined as ((Fm– Fo)/Fm); NPQ as ((Fm – Fm')/Fm'); qP as ((Fm' – Fs)/Fo), where Fs is the steady-state fluorescence level.

Fitting NPQ kinetics

NPQ formation data was fitted with a Hill function $NPQ(t) = NPQ_{max} t^b/(t_{0.5}{}^b + t^b)$ using SigmaPlot software (SPSS,Chicago, IL, USA), where NPQ_{max} is the maximum NPQ amplitude, $t_{0.5}$ is the t value at which NPQ = 50% of NPQ_{max} and b is the sigmoidicity parameter, thus $(1/ t_{0.5})$ is the rate of NPQ formation. NPQ relaxation data was fitted with a hyperbolic decay $NPQ(t) = NPQ_0 + NPQ_{max}\, t'_{0.5}/(t'_{0.5} + t)$ where NPQ_{max} is the starting NPQ amplitude, $t'_{0.5}$ is the t value at which NPQ = 50% of NPQ_{max} and NPQ_0 is the

amplitude of NPQ that does not relax within 300 s, thus $1/t'_{0.5}$ is the rate of NPQ relaxation.

Measurements of chlorophyll fluorescence lifetime

Time-correlated single photon counting (TCSPC) measurements were performed using a FluoTime 200 picosecond fluorometer (PicoQuant, Berlin, Germany). Detached leaves were vacuum-infiltrated with 50 μM nigericin to completely inhibit NPQ. Excitation at the 10 MHz repetition rate was provided by the 470 nm laser diode, which was carefully adjusted to completely close all PSII reaction centers without causing photoinhibitory quenching of Fm and to be far below the onset of singlet–singlet exciton annihilation. For the measurements of Fm' values, NPQ was induced in leaves infiltrated with water with 200 (lincomycin-treated) or 700 (untreated) μmol photons m^{-2} s^{-1} by arrays of 635 nm LEDs. Fluorescence was detected at 682 nm on leaves with the 2 nm slit width. The instrumental response function was in the range of 50 ps. For lifetime analysis, FluoFit software (PicoQuant) was used. The quality of the fits was judged by the $\chi 2$ parameter. In addition, the autocorrelation function of the weighted residual data was obtained as a measure of the correlation between the residuals in distinct channels separated by various times. Since the residual values should be normally distributed in the good fit (not correlated), the autocorrelation data were randomly distributed around zero and their fluctuations were small. A support plane analysis was also performed by calculating the $\chi 2$ as a function of each single parameter in order to estimate the region of the function below the tolerance level. Intensity weighted average lifetimes were calculated as $\sum (A*\tau)/ \sum A$, while amplitude average were calculated as $\sum (A*\tau^2)/ \sum (A \tau)$.

Steady state fluorescence spectroscopy

For all fluorescence measurements leaf homogenates were carefully prepared and diluted in order to avoid reabsorption (23) in 10 mM Hepes pH 7.6 buffer. Low temperature (77 K) emission spectra were recorded using a Jobin Yvon FluoroMax-3 spectrophotometer equipped with a liquid nitrogen cooled cryostat. Excitation was defined at 435 nm with the 5 nm spectral bandwidth. The fluorescence spectral resolution was 1 nm. Spectra were normalized at their absolute maximum. The second derivative analysis was calculated after a smoothing of the spectrum (FFT-filter of the Originlab program) with a wavelength interval of 3 nm.

LHCII isolation

Spinach trimeric LHCIIb was isolated as previously described (22). LHCII was desalted 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 (*β*-DM). Aggregated LHCII was prepared by removal of detergent by SM-2 bioabsorbent beads (Bio-Rad, Hemel Hempstead, UK) producing reduction in fluorescence average lifetime (see below) from 4 ns to 2 ns.

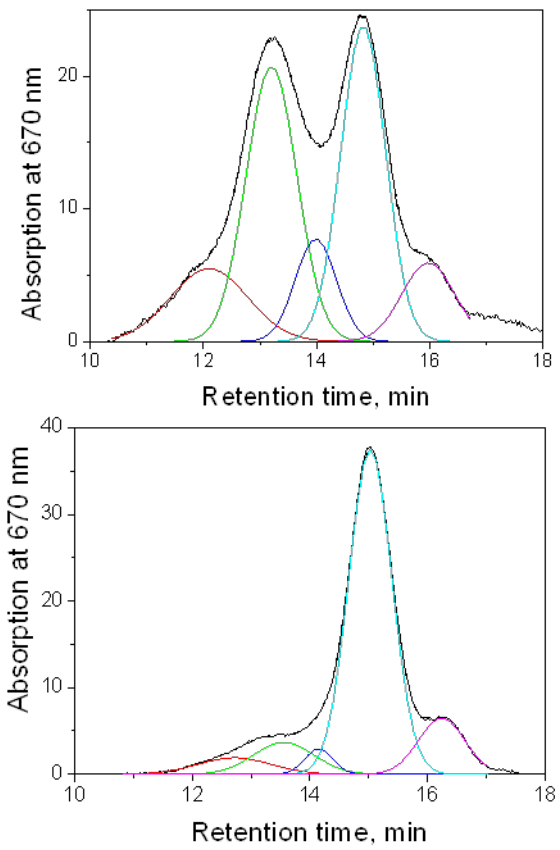
Isolation of PSI

PSI complex was isolated by sucrose gradient ultracentrifugation as previously described (20). Briefly, unstacked thylakoids were treated on ice for 30 min with 1% *β*-DM with a ratio of *β*-DM/chl of 10. Sucrose gradients were seven-step exponential gradients from 0.15 to 1.0 M sucrose dissolved in 20 mM HEPES buffer containing 20 μM *β*-DM. The run time was 18 h at 200,000 x g in a SW41 rotor at 4 °C.

Supplemental Data

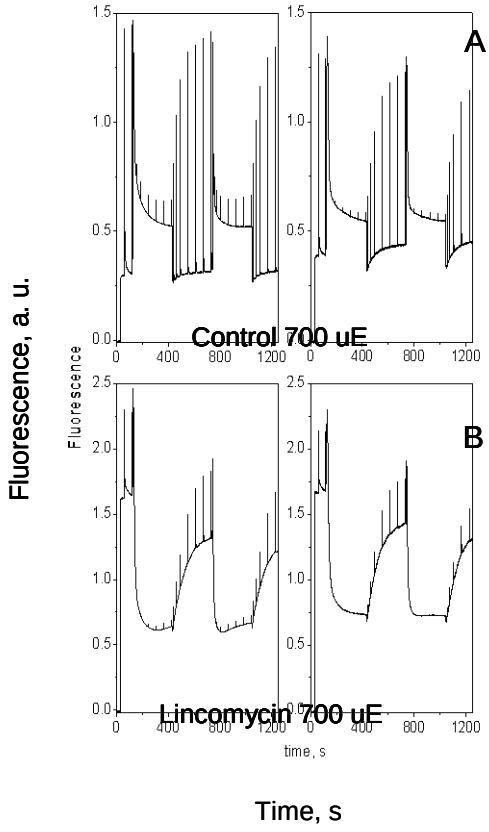
	Zeaxanthin (% of total xanthophyll)	**DEPs (**%)	**XC** (%)	**chlorophyll a/b** (molar ratio)	**chlorophyll per leaf area** (µg/cm^2)
control dark	0	0	7.8±0.5	3±0.1	42±3
treated dark	0	15.5±3.2	10.0±0.5	2±0.1	26±1
control light	2.5±0.5	40±5	7.3±0.7	3±0.2	42±3
treated light	2.5±0.5	37±2	10.0±0.4	2±0.1	26±1

Supplemental Table 1. Pigment composition of *Arabidopsis* leaves before and after ('dark' and 'light' respectively). Illumination was for 30 min at 700 µmol photons m^{-2} s^{-1}. DEPs: de-epoxidation state = (Z + 0.5A)/(V + A + Z); XC: xantophyll pool (Z+A+V)/(Z+A+V+N+L+ β-car) where Z=zeaxanthin, V=violaxanthin, A=anteraxanthin, N=neaxanthin, L=lutein, β-car=β-caroten. Data presented as means ±SEM from three to six replicates.

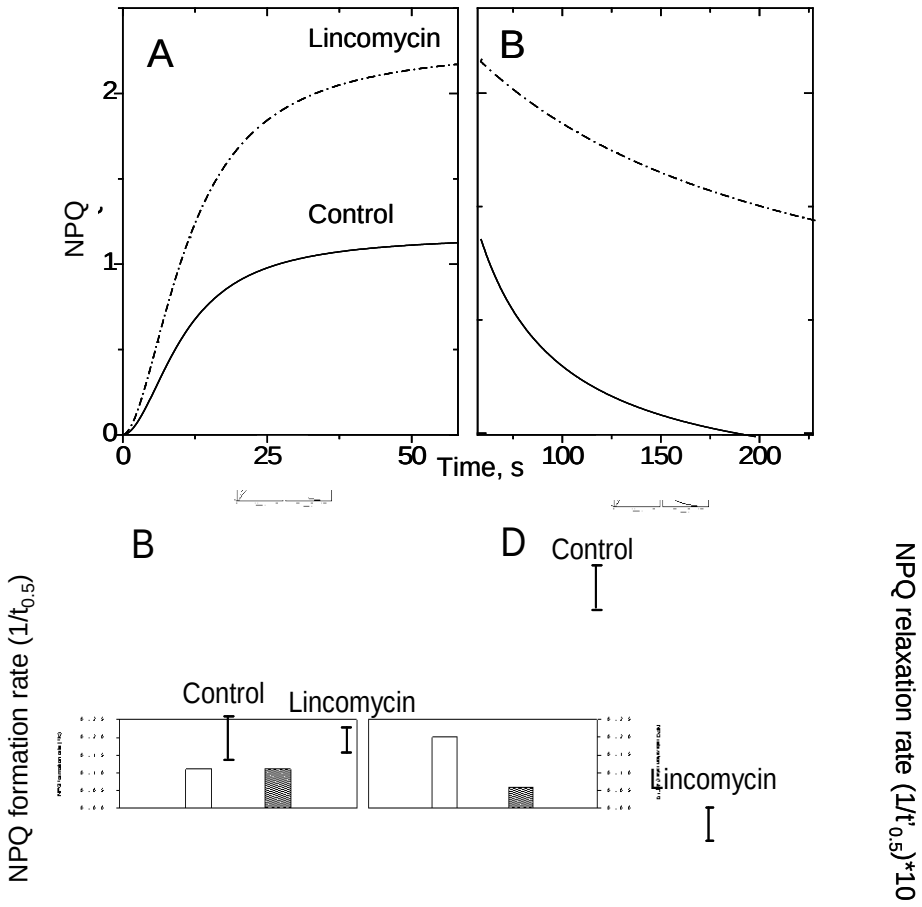


Supplemental Figure 1.

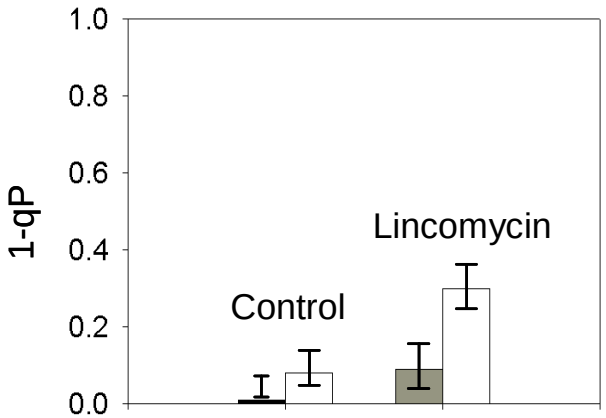
Decomposition of the FPLC elution profies shown in Fig. 2 and reported here in solid black line. Top: control membranes; bottom: treated membranes. The fit (dashed black line) was obtained with the Gaussian fit multi-peak option of Originlab program providing the number and positions of the peaks to fit the spectrum. Red: PSII supercomplex and unsolubilised materials; green: PSI complex; blue: PSII core; cyan: LHCII trimers; pink: LHCII monomers and minor complexes.



Supplemental Figure 2. Pulse amplitude modulated chlorophyll fluorescence analysis of *Arabidopsis* leaves. Typical chlorophyll fluorescence induction curves recorded during 5 min light exposure (700 μmol photons m^{-2} sec^{-1}) followed by 5 min of dark relaxation of leaves untreated (top) and treated (bottom) with lincomycin.



Supplemental Figure 3. Analysis of NPQ kinetics. (A) Kinetics of NPQ formation in control (solid line) and lincomycin-treated leaves (dashed line) illuminated with 700 and 200 μmol photons m^{-2} sec^{-1}, respectively. Leaves were illuminated for 5 min and then given a period of dark recovery prior to measurement to allow synthesis of zeaxanthin and light activation of electron transport. (B) Rates of NPQ formation. Data are average of three independent experiments ±SE. (C) Kinetics of NPQ relaxation in control (solid line), and treated leaves (dashed line) following a 5 min illumination. (D) Rates of NPQ relaxation. Data are average of three independent experiments ±SE.



Supplemental Figure 4. Average proportion of photosynthetically inactive photosystem II (PSII) reaction centres calculated as (1-qP) in the dark after illumination of leaves with 200 (grey bar) and 700 μmol photons m^{-2} sec^{-1} (white bar) as displayed in Figures 3 and S3.