Recycling of energy dissipated as heat accounts for high activity of Photosystem II

Monika Zubik[1,3], Rafal Luchowski[1,3], Dariusz Kluczyk[1], Wojciech Grudzinski[1], Magdalena Maksim[1,2], Artur Nosalewicz[2] and Wieslaw I. Gruszecki[1*]

[1] Department of Biophysics, Institute of Physics, Maria Curie-Sklodowska University, 20-031 Lublin, Poland

[2] Institute of Agrophysics, Polish Academy of Sciences, Doswiadczalna 4, 20-290 Lublin, Poland

[3]- These authors contributed equally to this work

[2]corresponding author: wieslaw.gruszecki@umcs.pl

ORCID ID: 0000-0002-8245-3913 (W.I.G.)

Abstract

Photosystem II (PSII) converts light into chemical energy powering almost entire life on Earth. The primary photovoltaic reaction in the PSII reaction centre requires energy corresponding to 680 nm that is higher than in the case of low-energy states in complexes involved in the harvesting of excitations driving PSII. This promotes the process of out of the center energy migration and thermal dissipation, reducing photosynthesis efficiency. Here we show that part of energy dissipated as heat is used to drive PSII thanks to the thermally-driven up-conversion. We demonstrate the operation of this mechanism both in isolated antenna complex LHCII and in leaves. A mechanism is proposed, according to which the utilization of thermal energy in the photosynthetic apparatus is possible owing to the formation of LHCII supramolecular structures, leading to the coupled energy levels, corresponding to approx. 680 nm and 700 nm, capable of exchanging excitation energy through the spontaneous relaxation and the thermal up-conversion.

Introduction

Life on Earth is powered by sunlight and photosynthesis is practically a sole process able to convert the energy of electromagnetic radiation to the forms which can be directly utilized to drive biochemical reactions in living organisms [1]. Importantly for life in our biosphere, oxygenic photosynthesis supplies molecular oxygen to the atmosphere, which most of the organisms use for respiration [2]. Efficient and fluent operation of photosynthesis is assured by the activity of pigment-protein complexes, called antenna, collecting photons and transferring excitation energy towards the reaction centres responsible for the primary electric charge separation [3,4]. The largest light-harvesting pigment-protein complex of plants, referred to as LHCII, is particularly well suited to play a photosynthetic antenna function, owing to the relatively high concentration of chlorophylls, the presence of xanthophylls (effectively protecting the complex against photo-damage) and internal pathways of extremely efficient excitation energy transfer within the network of the protein-embedded chromophores [3,4]. A consequence of exceptionally high protein crowding in the thylakoid membranes is the clustering of antenna complexes, potentially resulting in excitation quenching [5-10]. On the one hand, such a quenching leading to thermal energy dissipation may be advantageous under overexcitation conditions associated with high light intensity. On the other hand, energy dissipation decreases the efficiency of photosynthesis, particularly undesirable at low light. The results of the recent studies demonstrated that physiologically-controlled phosphorylation of LHCII could effectively regulate the formation of supramolecular structures of the complex and resultant nonradiative energy dissipation [11]. Interestingly, fluorescence lifetime imaging microscopy (FLIM) analyses of single chloroplasts revealed that photoprotective chlorophyll excitation quenching, manifested by shortening of fluorescence lifetimes, operating at high light intensities, was also highly effective at very low light intensities [12]. An intriguing phenomenon of very effective thermal energy dissipation at very low light intensities, far below the photosynthesis saturation level, raises the question whether such a process has any physiological meaning or rather it is just an example of the unavoidable energy losses in natural processes?

Results

Fig. 1 presents the FLIM images of *Arabidopsis thaliana* chloroplasts, recorded with increasing laser intensities. As can be seen, light intensity influences chlorophyll *a* (Chl *a*) fluorescence lifetimes. Relatively strong scanning light activates the photoprotective mechanisms consisting in Chl *a* excitation quenching manifested by shortening of

fluorescence lifetimes. The threshold for such a photoprotective quenching (~1400 µmol photons m^{-2} s^{-1}) corresponds to the saturation of photosynthesis (see Fig. 1b).

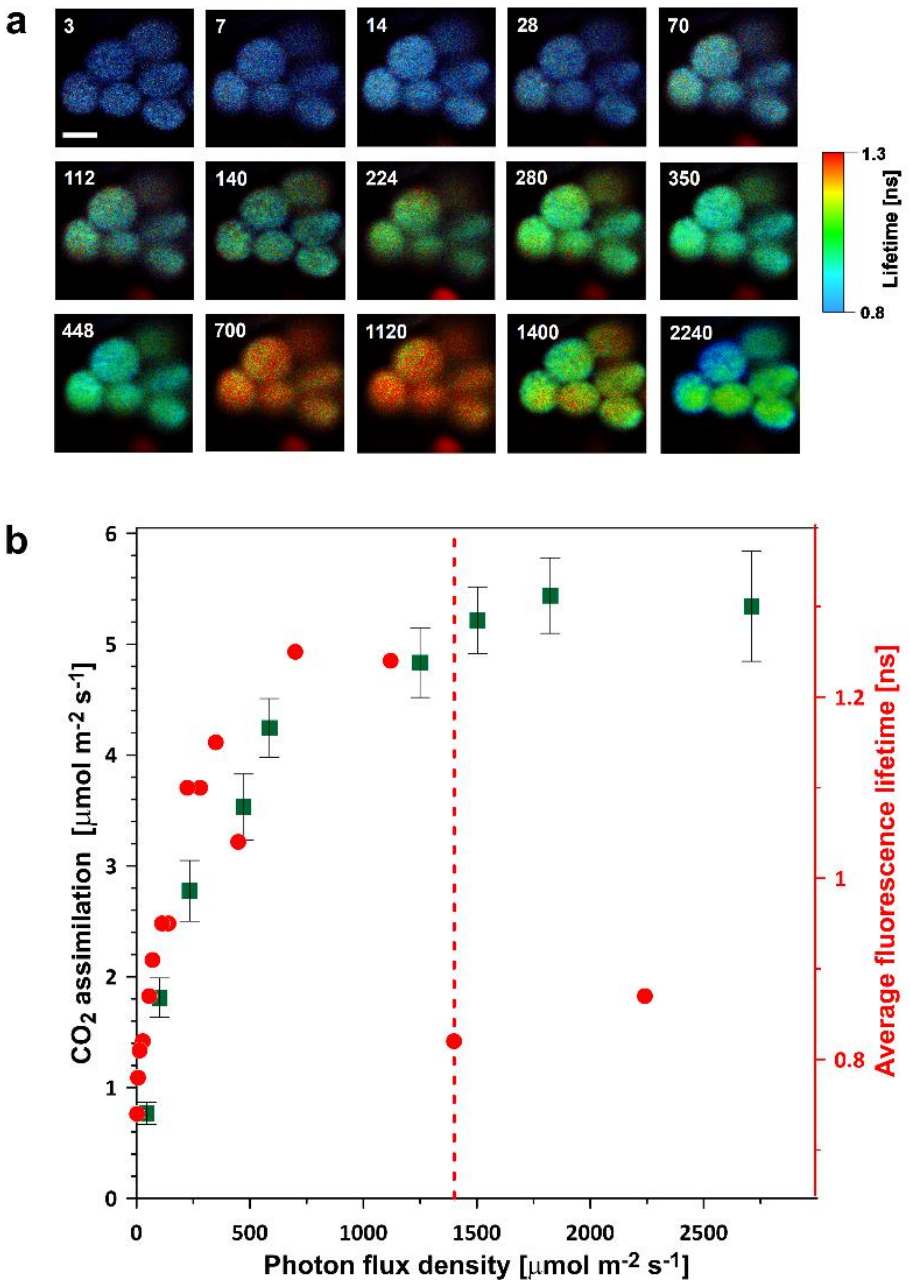

Fig. 1. Analysis of fluorescence lifetimes of chloroplasts. (a) FLIM images of *A. thaliana* chloroplasts *in situ* scanned with different laser light intensities. Light intensity, indicated in images, is expressed in the equivalent of µmol photon m^{-2} s^{-1} flux density of white light absorbed by chloroplasts, estimated according to ref. [11,12]. Scale bar 4 µm. (b) Overlaid light intensity dependencies of the photosynthetic activity in *Arabidopsis thaliana* leaves (green squares), monitored by assimilation of CO_2, and the intensity-averaged fluorescence lifetime values (red dots), determined based on the total number of photons emitted in course of the imaging of the chloroplasts shown in panel a. In the case of CO_2 measurements, each point represents an average of 5 different leaves ± S.D. The dotted line points the drop of average fluorescence lifetime observed at 1400 µmol photon m^{-2} s^{-1}.

Interestingly, a pronounced excitation quenching leading to thermal energy dissipation and manifested by shortening of fluorescence lifetimes can also be observed at relatively low light intensities, in accordance with the previous reports [12]. As can be seen from the comparison of the profiles of intensity-averaged fluorescence lifetimes and CO_2 assimilation (Fig. 1b), the lifetime dependency practically follows the photosynthetic activity, until the saturation level. This means that the thermal energy dissipation is inversely proportional to the photosynthetic activity at a lower light intensity and can be considered as an indication of the importance of heat emission for the operation of photosynthesis. A shortening of the chlorophyll fluorescence lifetimes *in vivo* can be modelled in the experimental system composed of isolated LHCII embedded into the lipid membranes formed with chloroplast lipids [8,11,12]. A spontaneous self-association of the antenna complexes in such a system gives rise to the low-energy band in a low-temperature Chl *a* fluorescence emission spectrum, centered in the region of 700 nm, accompanying the principal band centered in the region of 680 nm (Fig. 2a), in accordance with the previous reports [9,13-15]. The long-wavelength band in this particular spectral region (~700 nm) has been assigned to LHCII clusters in the natural thylakoid membranes [16]. There are several lines of evidence for the formation of such supramolecular structures of LHCII in the thylakoid membranes of plant chloroplasts, including the one based on the circular dichroism analyses [17], low-temperature fluorescence spectroscopy [16] and direct imaging based on electron microscopy [6]. Noteworthy is that the 695 nm band was also identified in the low-temperature fluorescence emission spectra recorded from isolated CP47 complexes of PSII [18]. The long-wavelength band centred close to 700 nm can be also resolved at 77 K in isolated thylakoid membranes [19] and leaves [20] (see also Fig. 2b). The "700 nm band" is a hallmark of aggregated LHCII since it is not present in the emission spectra recorded from the trimeric complex [13-15]. Importantly, the "700 nm band" (referred to as E700) can be resolved exclusively in the fluorescence emission spectra of aggregated LHCII recorded at low temperatures but not at physiological temperatures [9,13-16].

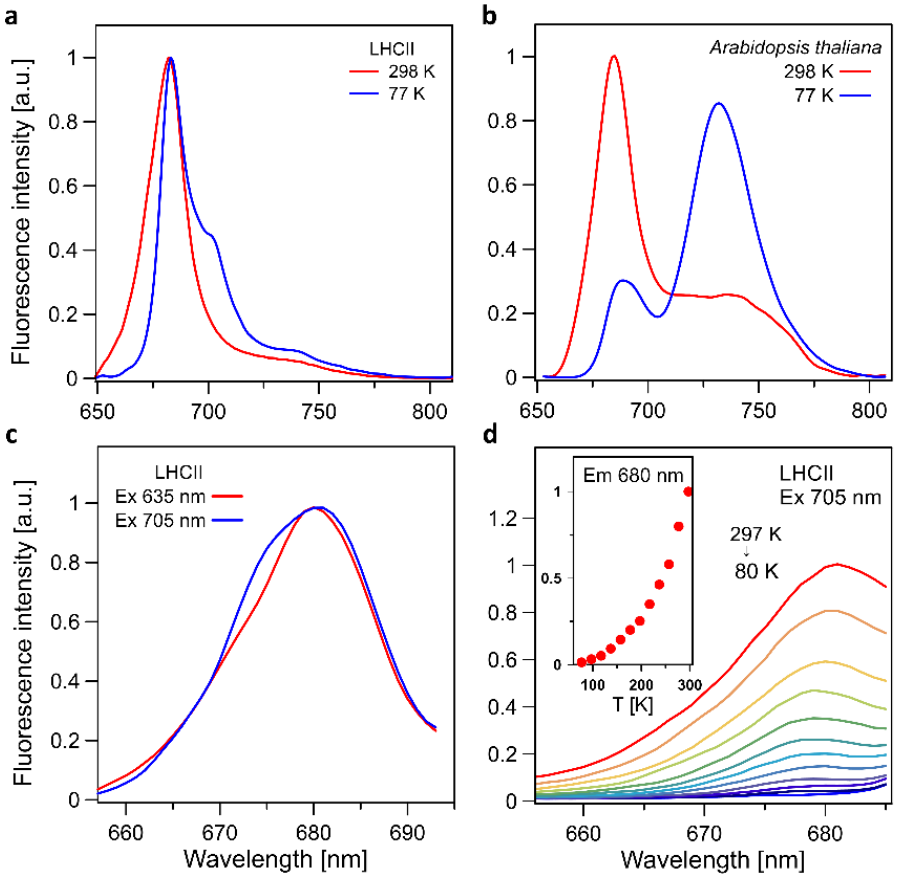

Fig. 2. Chlorophyll *a* fluorescence emission spectra recorded from LHCII and leaves. (a) Fluorescence emission spectra recorded from the sample containing supramolecular structures of LHCII formed spontaneously in the environment of the lipid membrane. Excitation at 635 nm. The spectra normalized at the maximum. (b) Fluorescence emission spectra recorded from *A. thaliana* leaves. Excitation at 635 nm. The spectra were recorded at 77 K and at 298 K, indicated. The spectra were area-normalized and presented on the arbitrary scale. (c) Fluorescence emission spectra recorded from the LHCII sample as in panel a, at 298 K with the laser excitations set at 635 nm or at 705 nm, indicated. (d) Fluorescence emission spectra recorded from the LHCII sample as in panel a, recorded at different temperatures in the range from 297 K to 80 K. Excitation was set in the lower energy spectral region, at 705 nm. The inset shows the temperature dependence of fluorescence intensity at 680 nm, based on the spectra presented. The set of the spectra is presented on the arbitrary scale.

This observation suggests possible depopulation of the E700 state by an uphill energy transfer, e.g. to the E680 energy level. Direct evidence for the operation of such a process is presented in Fig. 2c showing a comparison of the fluorescence emission spectra recorded from the same LHCII sample excited in the higher and in the lower energy regions (at 635 nm and at 705 nm) with respect to the emission spectral window. Almost identical shapes of both the emission spectra recorded are consistent with the interpretation according to which both the fluorescence emissions originate from the same energy level (Fig. 2c). Comparison of the quantum yields of fluorescence excited at 635 nm and at 705 nm shows that the quantum

yield of the emission excited at 705 nm is lower only by a factor of 6.3 than the fluorescence excited in the higher energy region (at 635 nm). In principle, no emission of fluorescence should be observed in the spectral region of 655-695 nm, with excitation at 705 nm, for energy reasons. The fact that the fluorescence spectrum can be recorded and that the quantum yield is relatively high is an irrefutable demonstration of the activity of the highly efficient up-conversion process. Fluorescence intensity in such a system, recorded at 680 nm and excited at lower wavelengths, drops down with the temperature decrease (see Fig. 2d) and with increasing a distance between the excitation and observation wavelengths (see Supplementary Fig. 1).

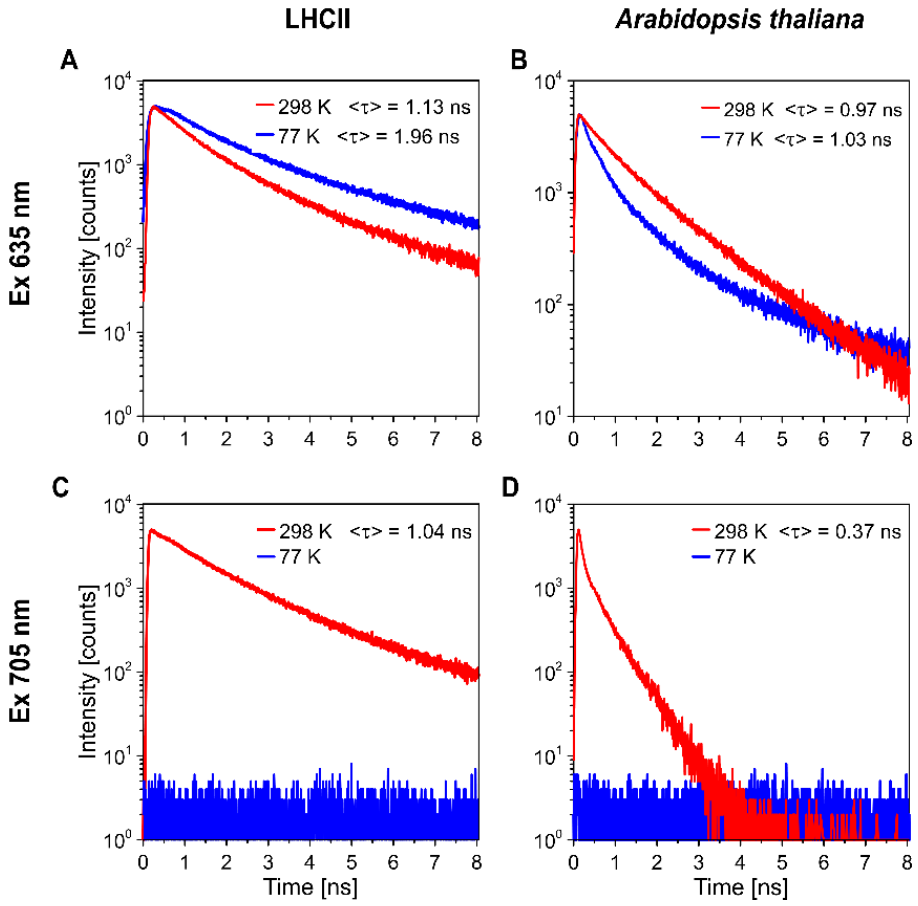

Fig. 3. Chlorophyll *a* fluorescence decay kinetics recorded from LHCII samples and from intact leaves. The kinetics were recorded from the samples containing supramolecular structures of LHCII formed spontaneously in the environment of lipid membrane (a and c) and from intact leaves of *A. thaliana* (b and d), at 77 K and at 298 K, indicated. Two combinations of the excitation and fluorescence observation wavelengths were applied: Ex 635 nm/Em 680 nm (panels a and b) and Ex 705/Em 680 nm (panels c and d). Displayed are amplitude weighted average fluorescence lifetimes (<τ>) calculated for each system.

Another kind of evidence for the operation of the thermally-driven up-conversion is shown in Fig. 3c presenting fluorescence emission kinetics of aggregated LHCII, excited at 705 nm and detected in the higher energy region, at 680 nm. The process of a thermally-driven up-conversion can be observed at room temperature but it is not possible to operate at 77 K, for energy reasons (a lack of a thermally-driven up-conversion at 77 K, see Fig. 3c). Importantly, the uphill excitation energy transfer in the same spectral region operates very efficiently in intact *A. thaliana* leaves (Fig. 3d). The difference between the E680 and E700 states (420 cm^{-1}) expressed in the kT units corresponds to the temperature of 627 K that is substantially higher than the room temperature (298 K). This means that the observed thermally-driven up-conversion has to be combined with a thermal deactivation of chromophores electronically-excited and localized in the close neighbourhood. Such a process is highly probable in the photosynthetic apparatus, owing to the fact that the overall energy conversion efficiency in photosynthesis does not exceed 6 % and most of the energy of absorbed light quanta is dissipated as heat [21,22]. It should be emphasized that the illuminated LHCII proved to be a very efficient emitter of heat that can be transmitted over long distances in the supramolecular structures of the protein [23]. The fact that fluorescence emission from the E700 can be detected at low temperatures, enables to determine fluorescence lifetime of this state (reported in the diagram, Fig. 4). It appears that the average lifetime of the E700 is substantially longer than that of the E680 state: 4.14 ns versus 1.96 ns, very close to the previous determinations of similar LHCII systems [15]. This suggests that the presence of the E700 energy level, below the E680 state, creates conditions for effective excitation quenching from this latter state, by a downhill energy transfer. Most probably, due to such a quenching, the fluorescence lifetime of E680 is even shorter at the physiological temperatures (1.13 ns, see Fig. 3a). Coexistence of the two-direction energy transfer pathways between the E680 and E700 states which are present in supramolecular structures of LHCII, namely the spontaneous down-conversion from E680 to E700 and the thermally-driven up-conversion from E700 to E680, can be discussed in the context of overall excitation energy flows in the photosynthetic apparatus of plants (see the model presented in Fig. 5).

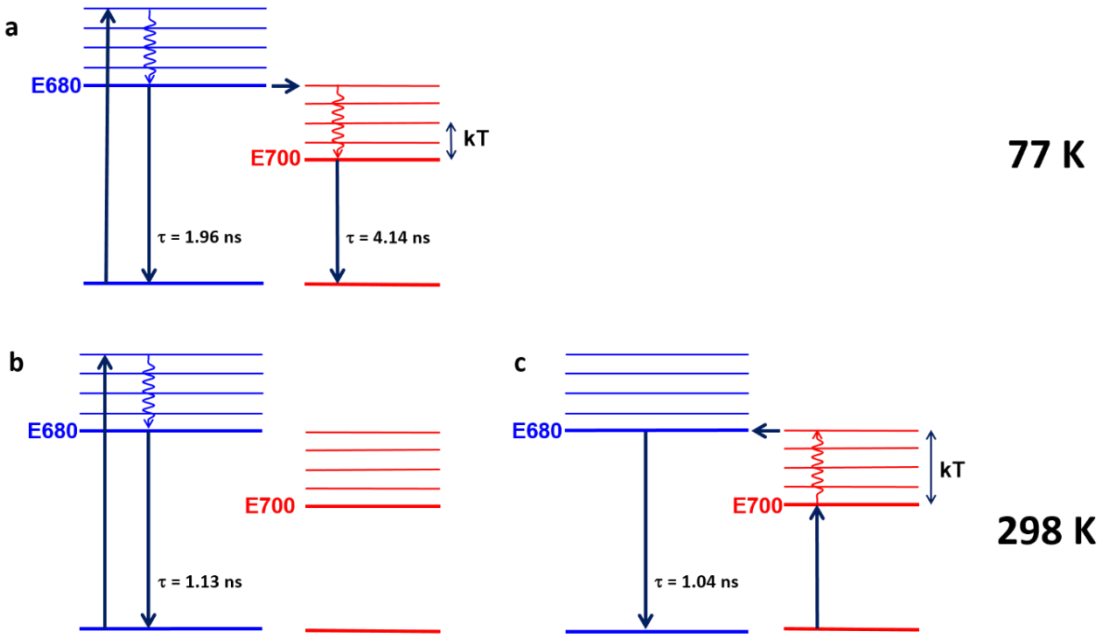

Fig. 4. Energy level diagrams representing selected electronic states and transitions in supramolecular structures of LHCII. The average fluorescence lifetime values are also reported, determined on the basis of the decay kinetics shown in Fig. 3. Vertical arrows up represent light absorption, vertical arrows down represent fluorescence, wavy and horizontal arrows represent nonradiative processes.

Discussion

As can be seen from Fig. 1, the excitation energy dissipation rate expressed by quenching of the chlorophyll fluorescence, observed at very low light intensities, is comparable or even higher than that observed at the light intensities saturating photosynthesis. Many studies have been devoted to the identification of mechanisms responsible for the photoprotective energy dissipation operating under overexcitation conditions (see [24] for a review). In principle, physical mechanisms leading to the thermal energy dissipation at low light intensities can be very similar to those responsible for the excitation quenching at high light. One of such mechanisms is based upon the formation of supramolecular structures of LHCII, characterized by a pronounced chlorophyll excitation quenching manifested by a considerable shortening of fluorescence lifetimes [13]. It has been demonstrated that LHCII phosphorylation influences the formation of LHCII supramolecular structures and therefore can be involved in the regulation of thermal energy dissipation [11]. On the other hand, a key factor in overall thermal energy dissipation in the antenna system associated with PSII would be not only the phosphorylation of LHCII alone but also the phosphorylation of the PSII core

proteins (e.g. D1), taking into consideration the fact that the light-intensity-dependencies of these two processes are very different [25]. It is also possible that a regulatory interplay with the Calvin cycle reactions modulates usage of electronic excitations for photochemical reactions and, in consequence, excitation quenching manifested by shortening of chlorophyll fluorescence lifetimes. Identification of the exact molecular mechanism(s) responsible for the thermal energy dissipation observed at very low light intensities, described herein, seems to be a scientific challenge that merits especially dedicated research. In the present work, the model system based on supramolecular structures of LHCII, formed in the lipid membrane environment, has been applied. The system has numerous advantages, discussed previously [11], as well as several limitations. One of the weaknesses of this model is the possibility of incorporation of the protein in two opposite orientations, i.e. the N- and C-termini facing to both sides of the lipid bilayers, in contrast to the native thylakoid membranes. On the other hand, very close agreement of the spectral shapes representing the aggregated structures of LHCII formed in the natural thylakoid membranes and under laboratory conditions justifies the application of such a system for model study. In particular, both the prominent spectral bands cantered in the region of 680 nm and 700 nm, are present both in the spectra of thylakoid membranes and LHCII aggregates [16]. Nevertheless, for the sake of caution, the conclusions drawn on the basis of the experiments carried out on model membranes were confirmed in the present study by the results of experiments carried out on *A. thaliana* leaves. The main of such findings is a thermally-driven up-conversion of the low-energy electronic excitations.

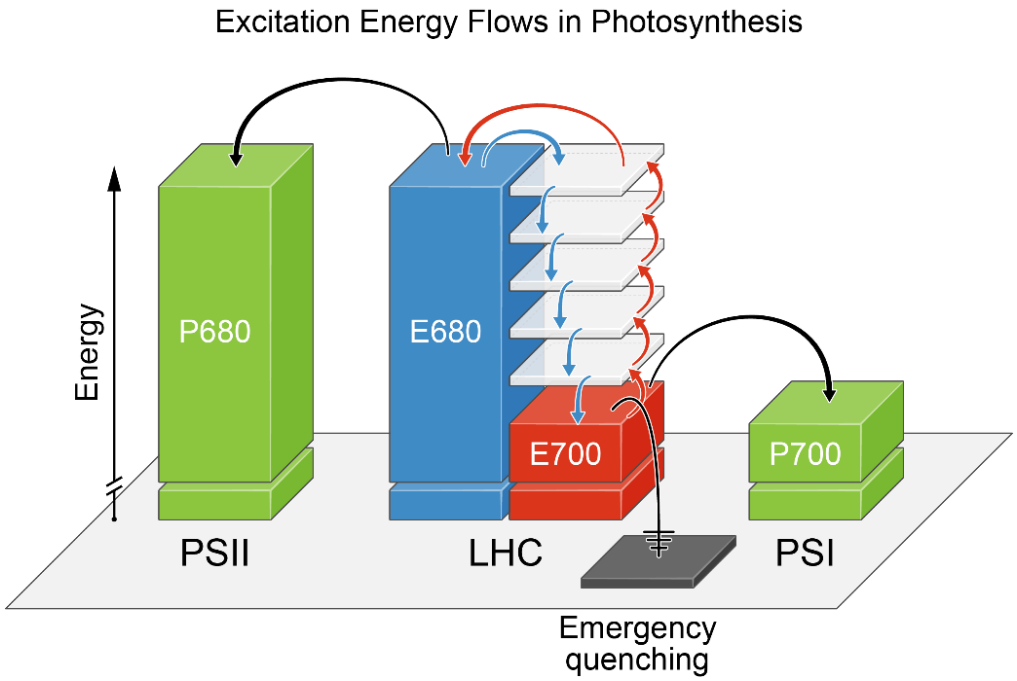

Fig. 5. A simplified "energy recycling" model representing the excitation energy flows in the photosynthetic apparatus of plants. The scheme presents the excitation energy flows between the light-harvesting antenna complexes (LHC) and the reaction centres of Photosystem II (P680) and Photosystem I (P700). Two experimentally identified, energetically coupled excitation energy states of supramolecular structures formed by LHCII are marked as E680 and E700. Energy circulation between these states is represented by arrows: the quenching of E680 by E700 and the thermally-driven up-conversion from E700 to E680. An emergency excitation quenching channel leading to thermal dissipation of excess excitation energy is also shown.

Numerous consequences of the operation of the molecular system based on supramolecular structures of LHCII (referred to as an "energy recycler", see Fig. 5), can be envisaged, important for the activity of PSII and for the efficiency of photosynthetic energy conversion in plants. The main consequence seems to be the ability to use low-energy excitations (corresponding to wavelengths longer than 680 nm) to drive the photo-physical processes in the PSII reaction centre, thanks to the utilization of a fraction of the excitation energy dissipated as heat in the pigment-protein antenna systems. On the one hand, recovering a certain fraction of the excitation energy already dissipated as heat shall increase the overall energy efficiency of PSII and therefore of photosynthesis. On the other hand, operation of this mechanism extends the action spectrum of PSII, towards the far-red spectral region, beyond the limit defined by the energy of the PSII reaction centre. A steep decrease in light absorption at wavelengths greater than 680 nm is associated with a very sharp decrease in the photosynthetic activity, referred to as the "red drop" [22]. Importantly, a significant photosynthetic activity of light from the far-red spectral region has been demonstrated by

direct measurements of photosynthetic oxygen evolution [26,27] and by means of EPR spectroscopy [28]. This effect has been observed with a single wavelength excitation [27,28] and with isolated PSII particles [28] and therefore does not correspond directly to the classical Emerson effect [29]. The photo-physical mechanism, reported in the present work, of thermally-driven up-conversion of the low-energy excitations to the energy level sufficiently high to drive the photochemical activity of PSII provides a direct explanation for the photosynthetic activity of light beyond the "red drop", long-wavelength threshold. In our opinion, the mechanism of the thermally-driven up-conversion proved here to operate both in model systems and *in vivo*, is more realistic than involvement of unidentified "long-wavelength chlorophylls" [27] or hypothetical excited X^* states [28]. Importantly, the uphill activation energies for the far-red light-driven oxygen evolution in sunflower, determined based on the temperature dependencies, have been found to correspond directly to the energy gaps between the level attributed to PSII (680 nm) and the energies of light representing excitations in the long-wavelength spectral region [27]. Interestingly, the quantum yield levels of PSII activity corresponding to 680 nm and to the far-red region differ only by the factor of ~6, despite pronounced differences in light absorption in the corresponding spectral regions [27]. A very close factor (6.3) has been determined in the present work for the chlorophyll fluorescence quantum yield in LHCII, in the direct, downhill fluorescence excitation and in the process of the uphill fluorescence excitation, mediated by the processes of thermally-driven up-conversion. This implies the potential role of such a photo-physical mechanism in shaping the photo-activity of PSII. In our opinion, this effect can be clearly seen from the comparison of the fluorescence emission spectra of Chl *a* in leaves (Fig. 2b). As can be seen, at the low temperature, the fluorescence emission from Chl *a* molecules associated with PSI (maximum of the emission band at ca. 735 nm [30]) is substantially higher than from the pool associated with PSII (maximum at ca. 680 nm). The opposite proportion can be observed at room temperature, in accordance with the numerous previous reports [20]. Determined at 77 K, the fluorescence yield of PSII core complex is about 2 times higher and the fluorescence yield of PSI about 20 times higher, as compared to room temperature [20,31]. The integration of the Chl *a* fluorescence emission spectra in leaves in the spectral region representing mostly PSII (wavelengths below 703 nm) and in the spectral region representing largely PSI (wavelengths higher than 703 nm) gives the ratio of photons emitted by PSII and PSI as high as 1.16 ± 0.22 at 298 K but only 0.20 ± 0.02 at 77 K (a mean value from three different leaves ± S.D., Fig. 2b). This indicates directly that the process of thermally-driven up-conversion to the energy states corresponding to P680 is necessary for effective excitation supply to PSII. The fact that

two energetically coupled energy states reported in the present work, E680 and E700, are apparently precisely tuned to the energies of the reaction centres of both photosystems, namely P680 and P700, provides favourable conditions for LHCII to act as a universal antenna complex. Very similar effect to that one observed in the case of LHCII, of the coexistence of the energy states giving rise to the fluorescence emission maxima close to 680 nm and to 700 nm, at room temperatures and at 77 K, have been reported for isolated PSII core complexes [18]. It is, therefore, possible that the thermally-driven up-conversion also operates independently in this photosystem. Moreover, the thermally-driven up-conversion has been recently shown to operate efficiently in isolated PSI [32]. This can lead to a more general conclusion that thermally-driven up-conversion is a common mechanism in the photosynthetic apparatus and potentially important for the process of photosynthesis. It is generally accepted that thermal energy dissipation has biological importance in the protection of the photosynthetic apparatus against overexcitation and photo-damage [13,33]. In light of the results of the present study it can be concluded that heat emission observed at all light intensities, and in particular, at dim light, has another important physiological significance, namely in "upgrading" low-energy excitations to the level sufficiently high to power the photo-physical reactions in PSII (P680). It is therefore very likely that direct utilization of thermal energy to drive photosynthesis is one of the reasons why the increase in light intensity does not cause an increase in temperature of illuminated leaves, in the light intensity region below the photosynthesis saturation level (see Supplementary Fig. 2).

In summary, we demonstrated that not only light but also thermal energy is essential to evoke excitations powering photosynthesis in plants, in particular, those able to drive PSII. Importance of this mechanism is associated with the fact that PSII is directly responsible for the process of water splitting, which is a direct source of electrons ("reducing power") for numerous metabolic reactions. Moreover, the photosynthetic water splitting is directly responsible for emission to the atmosphere of molecular oxygen used for respiration for most living organisms. Finally, it can be concluded that reabsorption of a fraction of excitation energy dissipated as heat, demonstrated in this work, potentiates utilization of low-energy excitations thus increasing the efficiency of photosynthetic energy conversion in plants.

Methods

Plant material and treatment

Arabidopsis thaliana (L.) wild-type Columbia 406 (Col-0) seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Plants used in the experiments were grown in a growth chamber under controlled environmental conditions with air humidity at 60 %, with day and night temperatures of 22°C and 18°C, respectively. Plants were grown in a mixture of soil and sand of optimum soil water availability controlled by daily weighing and watering. The average light intensity at the level of rosette was maintained at 150 µmol photons m^{-2} s^{-1} during an 8-hr light cycle. Measurements were carried out on mature leaves of 8-week-old plants.

In order to obtain intact cells with chloroplasts, the bottom epidermis from *Arabidopsis thaliana* leaves was peeled off using Scotch tape (3M). The sample was placed onto non-fluorescent glass slides (Menzel-Glaser), soaked in a buffer (20 mM Tricine, 0.4 M Sorbitol, pH 7.8) and subjected to measurements.

Measurements of photosynthesis light responses

The measurements of CO_2 assimilation was carried out using a gas exchange system consisting of GFS300, Dual PAM 100 and 3010-Dual cuvette (Walz GmbH, Germany). The youngest fully expanded leaves of *Arabidopsis thaliana* were used for the measurements. During the measurements, leaf temperature was maintained within the range 22 ± 0.5 ^{o}C, air relative humidity and CO_2 concentrations were 60 % and 400 ppm respectively. Light from red and blue LEDs (60:40), was increased from 45 to 2710 µmol photons $m^{-2}s^{-1}$, each of the steps lasted until constant readings of photosynthesis were obtained.

Measurements of leaf temperature

Thermal images of *Arabidopsis thaliana* leaves were recorded using a FLIR T55901-T620 thermal imaging camera (ThermaCAM-FLIR Systems, USA). Leaves were illuminated with each light intensity for 3 min, using an LED light source (BVP 105 LED 45/840 PSUVWB 100 Philips). Temperature image for each light intensity was recorded directly after turning light off. The thermal camera was positioned nearly perpendicular to the plant surface and the distance between the objective and the leaves was approximately 30 cm. The specified temperature resolution was below 0.1˚C, and images were directly analyzed on a camera for temperature determination using the image analysis software provided by the manufacturer.

Preparation of lipid-LHCII membranes

LHCII was isolated according to [34] with slight modifications [35]. The purity of preparations was controlled using HPLC and electrophoretic methods [35]. Lipid-LHCII membranes were prepared according to [11]. MGDG and DGDG (Avanti Polar Lipids Inc., USA) were mixed in chloroform:methanol (2:1, v:v) solution in a molar ratio of 2:1. Next, the mixture was dried under a stream of nitrogen to obtain a thin film in a glass tube. Obtained samples were placed in a vacuum (10^{-5} bar) for 40 min in order to remove traces of organic solvents. For incorporation of LHCII into lipid membranes, LHCII complexes suspended in tricine buffer (20 mM Tricine, 10 mM KCl, pH 7.6) containing 0.1% n-dodecyl-β-D-maltoside (DM) were transferred to glass tubes containing the deposited lipid film and subjected to mild sonication using an ultrasonic bath for 30 min. The molar ratio of LHCII:lipids was 1:200. Detergent (DM) was removed from the suspension by incubation with Bio-beads adsorbent (Bio-Rad Laboratories, USA) at 4℃ for 14 h. The pellet obtained by centrifugation for 5 min at 14000 x g consisted of the lipid-LHCII membranes.

Fluorescence spectroscopy

Fluorescence emission spectra of leaves and LHCII samples were recorded with an FS5 spectrofluorometer (Edinburgh Instruments, UK) with excitation and emission bandwidths set to 3 nm. In order to avoid spectral distortion caused by the Kautsky effect, each leaf was preilluminated with a white light LED lamp with the controlled photon flux density (150 μmol photons m^{-2} s^{-1}, 2 min) prior to the recording of fluorescence spectra.

Time-resolved fluorescence intensity decays were measured using FluoTime 300 spectrometer (PicoQuant, Germany). Excitation at 635 nm with 20 MHz frequency of pulses was from solid-state laser LDH-P-635 with a pulse width 68 ps, 214 μW. Excitation at 705 nm with 4 MHz repetition rate, 1.03 mW was accomplished by a single pulse selector (APE, GmbH) with a tunable Ti-sapphire laser Chameleon Ultra (Coherent, Inc.). The emission at 680 nm was filtered by 680/13 bandpass filter (Semrock, Inc.) and 665 glass long-wavelength pass filter (Edmund Optics, Inc.). Detection was performed with a micro-channel plate and time-correlated single-photon counting system PicoHarp 300. Fluorescence lifetime decays were fitted using FluoFit Pro software (PicoQuant, Germany). A FluoTime 300 spectrometer was also used to record fluorescence emission spectra of samples excited at 635 nm and 705 nm, with the application of the lasers specified above. The fluorescence quantum yields, excited at those wavelengths, were compared based on the integration of the emission spectra and taking into consideration the laser powers and the values of light absorption by the

sample at selected wavelengths (calculated as 1-transmission). Steady-state and time-resolved fluorescence measurements at different temperatures were conducted with the application of an Optistat DN2 Cryostat (Oxford Instruments, UK). The LHCII samples to be measured in a frozen state were diluted in buffer-glycerol (1:2, v:v). An important issue in the recording and analysis of chlorophyll fluorescence spectra *in vivo* is a proper correction for possible light reabsorption [36]. In order to minimize spectral distortions due to fluorescence reabsorption in leaves, Chl *a* fluorescence emission spectra *in vivo* were recorded from the epidermis separated from a leaf and placed in a cryostat. In order to correct such emission spectra for the effect of fluorescence reabsorption, each emission spectrum was divided by the transmission spectrum recorded from the same sample, under identical experimental conditions. Such an approach certainly does not lead to underestimation the fluorescent reabsorption effect in the spectral area characteristic of light emission by Chl *a* molecules associated with PSII.

FLIM (Fluorescence Lifetime Imaging Microscopy)

In vivo, chlorophyll fluorescence measurements were applied to study the process of excitation quenching in the photosynthetic apparatus exposed to different light intensities. In order to avoid possible artefacts related to light-induced chloroplast translocation in leaves, fluorescence lifetime analyses of single chloroplasts, imaged by a FLIM technique, was performed instead of steady-state fluorescence analyses of whole leaves. Fluorescence lifetime imaging of chloroplasts was performed according to the method described previously [12]. Measurements were conducted on a confocal MicroTime 200 (PicoQuant, Germany) system coupled to an OLYMPUS IX71 microscope. A 635 nm pulsed laser, with repetition adjusted in the range from 0.2 to 32 MHz was used as an excitation source. The full width at half height (FWHM) of the pulse response function was 68 ps (measured by PicoQuant, Germany). The time resolution was 16 ps. The laser beam was focused on samples with the use of a 60x silicon-oil immersed objective (NA 1.3, OLYMPUS). In order to select a single focal plane and reduce excitation light, a pinhole diameter of 75 μm was used. The scattered light was removed by using a Notch Filter NF633-25 (Thor Labs, Inc.), ZT 470/488/640RPC dichroic filter (Chroma Technology Corp.) and a 680/13 bandpass filter (Semrock, Inc). Fluorescence photons were collected with a single photon sensitive avalanche photodiode (APD) with processing accomplished by the Hydra Harp 400 time-correlated single-photon counting (TCSPC) mode. Laser minimum power was adjusted to 0.83×10^{-10} W at 0.2 MHz repetition frequency. Decay data analysis was performed using the SymPhoTime 64 software package. In order to eliminate fluorescence intensity changes related to Kautsky effect, before

first fluorescence imaging, chloroplasts were pre-scanned two times (2 minutes for each pre-scan) with the lowest laser repetition frequency. Each epidermis area containing chloroplasts was scanned for several times, each scan (realized within 3 minutes) with increased repetition frequency. Laser repetition frequency is related linearly to a photon flux density (μmol photon m^{-2} s^{-1}) and can be expressed as equivalent of a photon flux density of white light absorbed by chloroplasts. FLIM imaging in the whole range of laser repetition frequency (0.2 – 32 MHz) was performed for more than 20 chloroplasts, from different leaves.

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Author contributions

WIG, RL, MZ, WG conceived the project and designed the experiments, MZ, RL, DK, WG, MM and AN performed measurements, all the authors interpreted the results, WIG drafted the manuscript with contribution from other authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper.