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REVIEW

LHCII – a protein like a 'Swiss Army knife' with many mechanisms and functions

E. JANIK-ZABROTOWICZ[*,+] and W.I. GRUSZECKI[**]

Department of Cell Biology, Institute of Biology and Biochemistry, Maria Curie-Sklodowska University, ul. Akademicka 19, 20-033 Lublin, Poland[*]
Department of Biophysics, Institute of Physics, Maria Curie-Sklodowska University, Pl. Marii Curie-Sklodowskiej 1, 20-031 Lublin, Poland[**]

Abstract

The review highlights the relationship between the molecular organization of the light-harvesting complex of photosystem II (LHCII) and sunlight utilization by higher plants. The molecular form of LHCII switches rapidly and reversibly during diurnal changes of light intensity, from low (*ca.* 10) to high [*ca.* 1,000 μmol(photon) m^{-2} s^{-1}], so the sensitivity of LHCII to light may control the balance between light harvesting and photoprotection state. Our understanding and concept of this mechanism are based on the knowledge of the structure and photophysics of different LHCII molecular forms: monomer, dimer, trimer, and aggregate. It is proposed that LHCII monomers, dimers, and lateral aggregates are fundamental blocks of excess light-dissipation machinery. Trimer is exceptionally well suited to play a physiological role of an antenna complex. A correlation between the LHCII molecular form and the presence of xanthophyll cycle pigment violaxanthin and zeaxanthin in the complex structure is also shown. Moreover, the role of LHCII protein phosphorylation in thylakoid membrane architecture is also discussed. The dual function of LHCII has been studied in the natural thylakoid membranes of chloroplasts, in the artificial lipid-LHCII model membranes, and by suspension of LHCII in a detergent solution.

Keywords: aggregation; antenna complex; dimer; monomer; photoprotection; thylakoid membrane; trimer; xanthophyll cycle.

Introduction

Life on our planet is powered by the radiant energy emitted generously by the Sun, and photosynthesis serves as a bridge to transform solar energy into chemical energy which is stored in the chemical bonds of sugar molecules. Both the spectrum of visible light and the absorption of light by photosynthetic organisms (higher plants, algae, and some bacteria) determine the green color of our biosphere. In addition, oxygenic photosynthesis is

Highlights

- LHCII monomerization changes grana architecture
- Monomers and high light-induced dimers are photoprotective forms of LHCII
- Violaxanthin is a molecule stabilizing trimers and n-LHCII structures

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[+]Corresponding author
e-mail: ewa.janik-zabrotowicz@mail.umcs.pl

Abbreviations: AFM – atomic force microscopy; CD – circular dichroism; Chl – chlorophyll; CLSM – confocal laser scanning microscopy; CP – chlorophyll-protein; DGDG – digalactosyldiacylglycerol; DM – n-dodecyl-β-D-maloside; FCS – fluorescence correlation spectroscopy; FLIM – fluorescence lifetime imaging microscopy; HL – high light; IR – infrared radiation; LL – low light; MGDG – monogalactosyldiacylglycerol; NPQ – nonphotochemical quenching; PG – phosphatidylglycerol; TEM – transmission electron microscopy.

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Conflict of interest: The authors declare that they have no conflict of interest.

a process that releases molecular atmospheric oxygen used in cellular respiration. For 2.5 billion years, photosynthesis is an essential biological process in the production of biomass that powers much of our modern world. Each carbon atom that builds the human body is the first substrate for photosynthesis. In higher plants, blue and red light waves are preferentially absorbed by complexes [named photosystems II (PSII) and I (PSI)] containing photosynthetic pigments (chlorophylls and xanthophylls) bound to the protein bed in the thylakoid membranes of chloroplasts. It should be noted that chlorophylls are often referred to be 'the pigments of life' (Battersby 2000). These biomolecules have been mentioned by the laureates in the Nobel award lectures (Willstätter and Stoll 1913, Woodward *et al.* 1960, Trauner 2015). The network of excitonic interactions between chlorophylls and xanthophylls in the thylakoid membranes is designed to transfer of absorbed energy (Hancock *et al.* 2021). This energy is passed from pigment to pigment until it reaches the reaction center – a special pair of Chl *a* molecules. Sunlight gathering by pigments drives electron transport during the light phase of photosynthesis followed by NADPH and ATP synthesis. These molecules are used in glucose production during the Calvin cycle. Each energy collector system consists of the core complex which is surrounded by chlorophyll–protein (CP) light-harvesting complexes (LHC). This photosynthetic basic functional unit is named as PSII–LHCII supercomplex ($C_2S_2M_2$) (Fig. 1). It is comprising dimeric core subunits (D1 and D2 proteins with CP43 and CP47 antenna) with the inner small monomeric antennae (CP24, CP26, and CP29), and the outer peripheral major component: the largest light-harvesting complex of PSII (LHCII) trimers, directly associated with the PSII core (C), two strongly (S) and two moderately (M) (Su *et al.* 2017).

LHCII is the largest, most abundant PSII protein–pigment complex, comprising roughly half of the Chl molecules in the biosphere. It may be homo- or heterotrimers composed of Lhcb1, Lhcb2, and Lhcb3 proteins (products of *lhcb 1–3* genes) combination (Dekker and Boekema 2005, Pagliano *et al.* 2014). As demonstrated by crystallographic structure analysis (Liu *et al.* 2004), each monomer of the trimer is an approximately 232 amino acid polypeptide forming three transmembrane α-helices A, B, and C and two short α-helices D and E. The polypeptide chain binds eight Chl *a* molecules, six Chl *b* molecules, xanthophylls: violaxanthin, neoxanthin, and two molecules of lutein.

One of the main factors affecting the rate of photosynthesis is light intensity. The light conditions in the natural environment of higher plants may vary highly in intensity and quality on a broad time scale, ranging from seconds to minutes up to hours, days, and even months. These changes are caused by various environmental factors, *e.g.*, the movement of clouds in the sky, weather changes, or mutual shading by neighboring plants (Owens 1994). Light intensity may fluctuate from very low [*ca.* 10 µmol(photon) m^{-2} s^{-1} to high *ca.* 1,000 µmol(photon) m^{-2} s^{-1}] values in the day-night cycle. So, the safe functioning of photosynthesis in extreme environmental conditions and different climate zones is life-giving for plants and therefore all life on Earth. It was observed that under the illumination of plants with low intensity of light, when the spectral composition of light changes, a process of phosphorylation of N-terminal tyrosine of the polypeptide of LHCII is triggered (Rintamäki *et al.* 1997). It is agreed that it induces fast regulation of energy distribution between photosystems under the low intensity of light to maximize the efficiency of light capture. This mechanism is referred to as state transition and is manifested by the relocation of the LHCII between PSI and PSII (Bellafiore *et al.* 2005, Wood and Johnson 2020). Moreover, in the shade (under long-term low-light acclimation), higher plants increase the size of the antennae complexes (Wu *et al.* 2020) and the area of grana membranes (Grinzato *et al.* 2020). More light can mean more photosynthesis, but short- and long-term

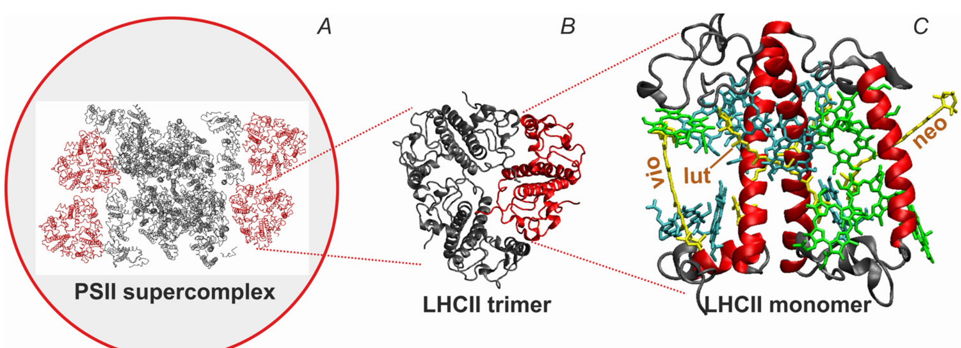

Fig. 1. Overall architecture of the $C_2S_2M_2$ PSII–LHCII supercomplex (*A*). The model is based on the PDB structure (PDB ID: 5MDX). The light-harvesting complex of PSII (LHCII) trimers was shown in *red* color. (*B*) The top view of the LHCII trimer. For clarity, one monomer is shown in *red* color. (*C*) Model of the structure of the LHCII monomer based on PDB data (ID: 2bhw). α-helices *A*, *B*, and *C* are presented in *red* color, xanthophylls: violaxanthin (vio), neoxanthin (neo), and two lutein (lut) molecules are represented by *yellow*, Chl *a* by *cyan*, and Chl *b* by *green* color. The images were created with the *VMD* (*Visual Molecular Dynamics*) software. *VMD* is developed with NIH support by the Theoretical and Computational Biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign.

illumination of plants with high intensity of light may lead to photoinhibition manifested in damage to the structure of D1 protein, photosynthetic pigments, or unsaturated fatty acids of thylakoid membrane lipids (Marshall *et al.* 2000, Liu *et al.* 2004). This is due to the photosensitizing properties of Chl molecules followed by singlet oxygen generation. Moreover, too much light can cause heat and dryness abiotic stresses which are also harmful to the physiological processes in plants.

In the course of evolution, plants have evolved photoprotective mechanisms at different levels of their organization. Protection against photoinhibition can be ensured by changing the position of leaves relative to the direction of the incidence of light rays (Koller 1990) or by phototranslocation of chloroplasts in leaf cells through cytoskeleton network (Chow *et al.* 1988, Kasahara *et al.* 2002, Gotoh *et al.* 2018, Perico and Sparkes 2018, Wada and Kong 2018). Unfortunately, these mechanisms reduce light absorption efficiency under light stress merely by approx. 10–20% (Brugnoli and Björkman 1992). Effective protection of the photosynthetic apparatus against photodamage takes place at the molecular level of the cell, *i.e.*, in the appressed grana thylakoid membranes of chloroplasts where PSII of photosynthetic apparatus is mainly located (Wood and Johnson 2020). A system of dynamic regulation of light quanta utilization consists of a core complex of PSII associated with variable numbers of antenna complexes. The antenna size of the supercomplex is adjusted in response to long-term high-light intensity (Wu *et al.* 2020). Plants' acclimation to changing light conditions may be regulated even by the expression of *lhcb* genes (Borisova-Mubarakshina *et al.* 2022). Effective plant acclimation to prolonged periods of high light is crucial for plants' survival and competitiveness. So, the leaves of a plant that grow under high-light conditions are thicker and have larger cells with plural chloroplasts. These organelles are characterized by a decrease in grana structure, high PSII/PSI and Chl *a*/*b* ratio, and high content of β-carotene and xanthophyll cycle pigments.

In optimal light conditions, the LHCII trimers form serves a function of an antenna involved in the absorption of light energy, which is subsequently utilized in the photochemical photosynthesis processes. Excess energy reaching the photosynthetic apparatus induces a fast switch of the antenna function of the LHCII complex into a photoprotective role (Ruban 2009). These changes in the function of LHCII are associated with alterations in its molecular organization (Ruban *et al.* 2012). The pool of LHCII trimers that are not permanently linked with PSII may form oligomeric structures responsible for photoprotective quenching of excess excitation energy under subsaturating light conditions (Ruban *et al.* 1995, 1997; Dekker *et al.* 1999, Gruszecki *et al.* 1999a, 2009a; Holm *et al.* 2005, Horton and Ruban 2005, Miloslavina *et al.* 2008).

A process called the xanthophyll cycle is involved in photoprotection at the molecular level. It takes place in the thylakoid membrane in high-light conditions and consists of a two-step enzymatic de-epoxidation of the LHCII xanthophyll pigment violaxanthin *via* antheraxanthin to zeaxanthin (Sapozhnikov *et al.* 1957, Yamamoto *et al.* 1962). This reaction is reversed upon illumination of plants with low-intensity light or in the dark. The role of xanthophyll cycle pigments in photoprotection has been the subject of many studies for over many years (Gruszecki and Strzalka 1991, Gruszecki *et al.* 1994, 1997a,b; 1999a,b; 2000, 2006, 2009b, 2010; Ruban *et al.* 1997, 2002; Ruban and Horton 1999, Latowski *et al.* 2002, Gruszecki 2004, Dall'Osto *et al.* 2012, Bethmann *et al.* 2019, Kaiser *et al.* 2019, Demmig-Adams *et al.* 2020, Nan *et al.* 2022). The results of these investigations showed that the presence of zeaxanthin was correlated with the activity of a process in the thylakoid membrane based on efficient nonphotochemical quenching (NPQ) of excess energy in light stress conditions. Furthermore, the physiological significance of the xanthophyll cycle is attributed to the formation of specific supramolecular structures of LHCII complexes characterized by efficient energy dissipation (Welc *et al.* 2016, Zhou *et al.* 2020).

Because the major challenge facing future generations is the sustainable production of food and fuels, studies of global biological processes such as photosynthesis are crucial. Moreover, natural photosynthesis constantly inspires technological activity (solar cells, collectors of green energy). It is generally believed that LHCII can change its double function by switching between different conformations. Therefore, detailed knowledge of the molecular organization and functioning of the LHCII antenna complex is essential for understanding the photoprotective mechanisms involved in an efficient photosynthesis process in variable light conditions (short-term acclimation to variable light intensity). Interesting seems to be the answer to the question: what exact mechanism is responsible for the involvement of LHCII complexes in a balance between light tunneling to the PSII reaction center on the one hand and its photoprotective thermal dissipation on the other hand? This knowledge is crucial because of the low thermodynamic efficiency of photosynthesis, which ranges from 1 to about 4% (Wu *et al.* 2020). Photosynthesis saturates at one-quarter of full harvested sunlight energy, followed by its loss in the ways of dissipation as heat and fluorescence.

In this article, we explore several concepts, resulting from the study using novel experimental techniques, addressing the problem of the reorganization of the LHCII molecular form in response to fluctuating light as an essential and universal mechanism of plant photoprotection. A deeper understanding of this mechanism may not only help understand photoprotective plants' behavior but ultimately maintain high productivity of food crops and maybe a future of artificial photosynthesis, which is a hot spot in a global economy (Kromdijk *et al.* 2016, Ruban 2017, Dogutan and Nocera 2019).

Do not waste it – use it. Recycling of dissipated heat energy into high PSII activity under the low light intensity – 'chloroplast wake up' mechanism

CP complexes cover approximately 80% of the thylakoid membrane surface (Kirchoff 2008). Such high density may lead to undesirable loss of absorbed light energy

due to strong thermal dissipation of excitation energy, which is typical of aggregated LHCII structures formed under subsaturating growth light conditions. This process seems to be highly unfavorable at an illumination of plants with low-intensity light when the function of the photosynthetic apparatus is focused on the most efficient energy absorption for an efficient photosynthesis process. Despite this, below the light intensity of 20 μmol(photon) m^{-2} s^{-1}, energy quenching in chloroplast was observed. The physiological significance of the absorbed energy loss has been addressed by Zubik *et al.* (2020). It was shown that a fraction of energy released from LHCII as heat could be recycled by photosynthetic apparatus and utilized to power the linear electron transfer between PSII and PSI thanks to the thermally-driven uphill energy transfer mechanism. So, thermal dissipation of energy is necessary for effective photosynthesis even under low light intensity. The energy can circulate between energetically coupled excitation energy states of LHCII structures which was proposed to be the 'energy recycling unit'. It is composed of two populations of spontaneously formed supramolecular LHCII forms able to the spontaneous relaxation and the thermal up-conversion of the energy. The recycling unit not only powers up PSII (energy collector system) but also can exist as an emergency channel reducing an excess of light energy tunneled to PSII (photoprotection function). Summarising, the presentation of the model of excitation energy flow in the photosynthetic apparatus seems to be an important step toward a deeper understanding high energetic efficiency of PSII in plants.

The low-light activation of the LHCII antenna function, named the 'chloroplast wake up' mechanism, was also analyzed in experiments on a single intact chloroplast carried out using the FLIM technique (Janik *et al.* 2017b). A chloroplast located on a fragment of the abaxial epidermis of a spinach leaf was illuminated with varied-intensity light [from 9 to 600 μmol(photon) m^{-2} s^{-1}]. When the chloroplast was illuminated with low-intensity light (below the saturation level of the light phase of photosynthesis), the average lifetime of fluorescence of Chl *a* increased, which effect was mainly visible within thylakoid grana. Additionally, the increase in the average fluorescence lifetime was correlated with the destabilization of the structure of thylakoid grana in the chloroplast observed in the cross-sections of the fluorescent signal in the FLIM images. Since the highest efficiency of phosphorylation of LHCII complexes was observed at 100 μmol(photon) m^{-2} s^{-1}, the reorganization of thylakoid membranes can be associated with the phosphorylation of these complexes. To confirm this hypothesis, analogous experiments were conducted for a single chloroplast from a leaf of *Arabidopsis thaliana* w.t. and *stn7* mutant, in which the LHCII phosphorylation process was blocked. The extension of the average fluorescence lifetime in the low-light intensity range was observed in the chloroplast of *Arabidopsis thaliana* w.t., whereas no such effect was found in the chloroplast of the *stn7* mutant.

For deeper insight into the molecular mechanism of changes in the LHCII molecular organization and the structure of thylakoid grana regulated by phosphorylation of LHCII complexes, multicomponent model systems corresponding to natural thylakoid membranes were designed. Immunodetection revealed a high degree of phosphorylation of the complexes isolated from the leaves illuminated with 100 μmol(photon) m^{-2} s^{-1}. Additionally, native electrophoresis showed that complexes isolated from darkened leaves were mainly present in the trimeric form (T/M = 5.92 ± 1.10), whereas the pool of complexes isolated from the illuminated leaves was characterized by a large amount of monomeric LHCII forms (T/M = 2.20 ± 0.28). Additionally, there were substantially higher numbers of monomers in the phosphorylated form, compared with the number of trimers. Analyses of the FLIM images of artificial lipid-protein membranes revealed an increase in the average fluorescence lifetime in the case of lipid membranes formed with phosphorylated LHCII complexes, compared with membranes with incorporated nonphosphorylated complexes. This effect indicated a lower degree of aggregation of nonphosphorylated LHCII complexes and their homogeneous distribution in the artificial lipid membrane.

Phosphorylation of LHCII complexes can lead to electrostatic repulsion between the negatively charged phosphate groups of complexes located within a single trimer and the weakening of the interaction between trimers located in adjacent thylakoid membranes (Fig. 2).

Thus, LHCII phosphorylation may lead to the monomerization of trimers. It is known that trimeric forms of the complexes stabilize the structure of thylakoid grana (Standfuss *et al.* 2005). The increase in the pool of monomers in the membrane may thus lead to destabilization of the grana and enhancement of the mobility of LHCII complexes in the membrane. *Via* this mechanism, phosphorylation of LHCII complexes induced by weak light can regulate the photosynthetic function of the complexes in low-light conditions. The disintegration of aggregated LHCII forms, characterized by efficient energy quenching, results in a homogeneous distribution of LHCII forms ensuring conditions for efficient absorption and transfer of light energy at low-intensity light.

Low light induces the monomerization of LHCII trimers. Is that physiologically relevant?

The primary physiological function of the trimeric LHCII form is the absorption of incoming solar radiation to be used in the light phase of photosynthesis. There is a somewhat puzzling question of why the presence of such an LHCII molecular form in the thylakoid membrane is beneficial to plants since other antenna complexes associated with PSII occur natively only as monomers. This problem was explored on intact chloroplasts and LHCII complexes isolated from spinach leaves (Janik *et al.* 2015).

It was found that the trimeric LHCII could serve an antenna function which is based on the energy transfer to the PSII reaction center, given the efficient transfer of excitation energy from Chl *b* to Chl *a* occurring therein. So, LHCII trimers are naturally designed as energy

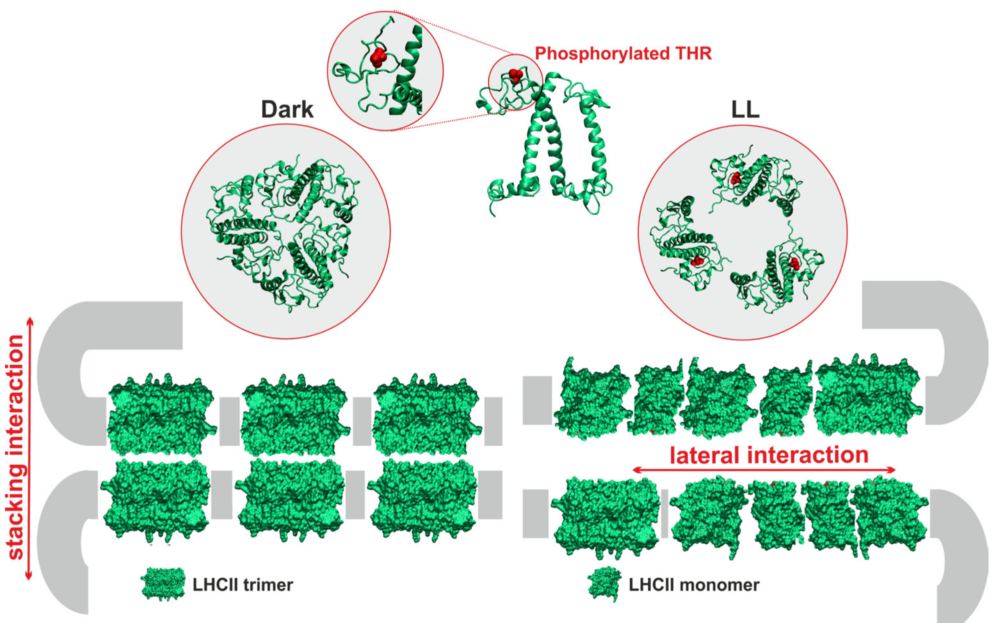

Fig. 2. A model demonstrating the molecular LHCII organization in the thylakoid membranes under dark and low (LL) light conditions. The schematic structure of the monomers and trimers of the light-harvesting complex of PSII (LHCII) are shown [top view, based on PDB data (ID: 2bhw)]. The images were created with the *VMD* (*Visual Molecular Dynamics*) software. The site of LHCII phosphorylation [threonine (THR) at the N-terminus] is presented in *red* color.

collectors to drive the light phase of photosynthesis in the delicate machinery of the plant's powerhouse. On the other hand, the weaker excitation energy transfer from Chl *b* to Chl *a* in the LHCII monomer, compared to that in the trimer, was confirmed by the presence of a band at 656 nm assigned to Chl *b* in the low-temperature fluorescence emission spectrum of this form of the complex (Fig. 3).

The efficient energy coupling between Chl *b* and Chl *a* in the trimeric structure results from the specific spatial organization of Chl molecules. Molecules of Chl *a* and *b* in the LHCII monomer are grouped, and the groups are relatively separated from each other. Only the formation of a trimeric structure by monomers enables the molecules of Chl *b* of one monomer to approximate the molecules of Chl *a* in the adjacent monomer close enough for efficient energy transfer from Chl *b* to Chl *a* (Fig. 2). This specific organization of chlorophylls explains the high fluorescence quantum yield in the trimer, compared with that in the monomer form. It was found that the fluorescence quantum yield of the LHCII monomer (for fluorescence emission excitation at 440 nm) accounted for only 0.55 ± 0.03 of the value of this parameter for the LHCII trimer. The association of monomers with the trimeric LHCII structure is also related to changes in the average fluorescence lifetime. This parameter (calculated from the intensity with fluorescence excitation at 470 nm) is 2.74 ± 0.05 ns for the LHCII monomer in a detergent solution and 3.50 ± 0.05 ns for the LHCII trimer.

The monomer-specific thermal excitation energy quenching is typical of the mechanism of plant protection against light stress. Therefore, a question arises whether the monomeric LHCII form can be directly involved in photoprotection. The process of light-induced LHCII transition from the trimeric to monomeric form was observed for the first time by Garab *et al.* (2002) in a system containing isolated LHCII complexes and whole thylakoid membranes. Moreover, the monomeric state of LHCII incorporated into liposomes was observed by Natali *et al.* (2016). Illumination of the detergent suspension of the complexes LHCII with light resulted in an increase in the pool of LHCII monomers at the expense of the pool of trimers. To verify whether the light-induced monomerization of LHCII complexes also took place *in vivo*, the fluorescence lifetimes were imaged with FLIM in a single intact chloroplast of the abaxial epidermis of the spinach leaf. The measurement was performed at three different laser frequencies corresponding to the white light intensities of 9, 45; and 1,200 µmol(photon) m^{-2} s^{-1}. The analysis of Chl *a* fluorescence lifetimes in the chloroplast revealed that the mechanism of monomerization of LHCII trimers is triggered in the thylakoid membrane at light-limiting conditions. At higher light intensities, energy traps are formed from monomers characterized by efficient energy quenching and involved in the protection of the photosynthetic apparatus from excess excitations. Therefore, the presented analysis strongly supports the hypothesis, that light-induced monomerization is coupled with the regulation function of LHCII complexes in changing light conditions. It is a very important aspect of photosynthesis because provides an energetically favorable cluster of LHCII in the thylakoid membrane. The recent study on PSII adaptation to

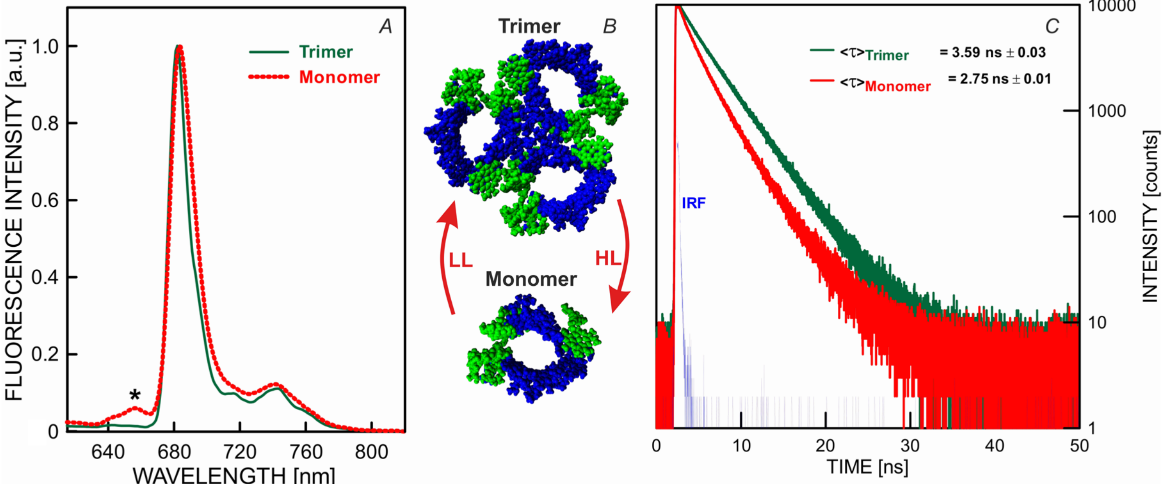

Fig. 3. Light-induced monomerization of the light-harvesting complex of PSII (LHCII) trimer. (*A*) 77 K fluorescence emission spectra of the LHCII trimers and monomers. The excitation wavelength was 470 nm. The spectra were normalized at the maximum. The individual fluorescence emission of the Chl *b* pool which is not energetically coupled to Chl *a* is marked with an *asterisk*. Measurements were done on LHCII trimers and monomers located in polyacrylamide gel (after electrophoretic separation). *See* more details in Janik *et al.* (2015). (*B*) Schematic representation of the LHCII monomer and trimer structure (top view). For clarity, only Chl *a* (*blue*) and *b* (*green*) molecules are presented. LHCII monomerization is presented as high light (HL)-induced process (*C*). LHCII trimers are typical for low light intensities (LL). Fluorescence lifetime analysis of Chl *a* in LHCII monomers and trimers. Intensity weighted average lifetime $<\tau>$ values ± SD for 10 independent replicates are included additionally. The fluorescence decay kinetics were fitted with components characterized by the following lifetimes: $\tau_1 = 3.69$ ns, $\tau_2 = 1.88$ ns, $\tau_3 = 0.36$ ns in the case of LHCII monomer ($\chi^2 = 0.99$); $\tau_1 = 3.73$ ns, $\tau_2 = 1.89$ ns, $\tau_3 = 0.32$ ns in the case of LHCII trimer ($\chi^2 = 0.96$), excitation and detection were set at 470 and 680 nm, respectively. Data was obtained from Janik *et al*. (2015).

low- and high-light conditions confirmed new thinking about the role of LHCII monomers in photoprotection (Kim *et al.* 2020).

Distinctive roles for the xanthophyll cycle pigments: violaxanthin – 'a molecular spacer' and zeaxanthin – 'a molecular rescuer'

According to the commonly accepted view, the xanthophyll cycle is of particular importance in the regulation of the antenna function of the photosynthetic apparatus. So, the investigations of the molecular organization of the LHCII complex induced by xanthophyll cycle pigments, violaxanthin and zeaxanthin, were conducted in model systems (Janik *et al.* 2016, Welc *et al.* 2016). The obtained results indicated that violaxanthin and zeaxanthin exerted different effects on the LHCII complex organization in the artificial lipid membrane. Native electrophoresis showed that violaxanthin induced mainly the trimeric form of the LHCII complex, whereas zeaxanthin promoted the formation of monomeric and dimeric forms (Fig. 4) (supported in an experiment where violaxanthin and zeaxanthin were incorporated into LHCII complexes in a detergent environment).

Chromatographic analysis proved that both xanthophylls were weakly bound to the LHCII monomer while violaxanthin was strongly bound to the trimeric form. Molecular modeling was employed to determine the molecular mechanism underlying the formation of trimeric LHCII complexes in the presence of violaxanthin and their degradation to monomers in the presence of zeaxanthin. It was indicated that the binding of the xanthophyll cycle pigments to the LHCII monomer was competitive and that the binding to the complex had varied strength. This may result from the different orientation of the rings in the pigment molecules and the presence of two additional polar groups in violaxanthin, which ensures its potential to form additional hydrogen bonds, in comparison to zeaxanthin. Violaxanthin can form four and zeaxanthin two LHCII complex-binding sites. Violaxanthin and zeaxanthin were found to compete for the binding site with PG, which is located on the border of two monomers in the LHCII trimer. Additionally, zeaxanthin exhibits stronger binding to the Chl *a* 610 molecule [according to the nomenclature in Standfuss *et al.* (2005)], which sterically hinders aggregation of LHCII monomers into trimers in the presence of zeaxanthin. This interpretation is consistent with the observation of Welc *et al.* (2016) and Xu *et al.* (2015) that zeaxanthin is located at the periphery of the pigment–protein complex rather than in the internal binding sites of LHCII.

Another important finding from the analyses of the lipid–protein–pigment systems is the formation of supramolecular LHCII structures (n-LHCII) in the lipid membrane in the presence of the xanthophyll cycle pigments. The intensity-weighted average Chl *a* fluorescence lifetime measured for the n-LHCII structures, obtained by electrophoretic separation of the lipid–protein samples (in the detergent environment), is

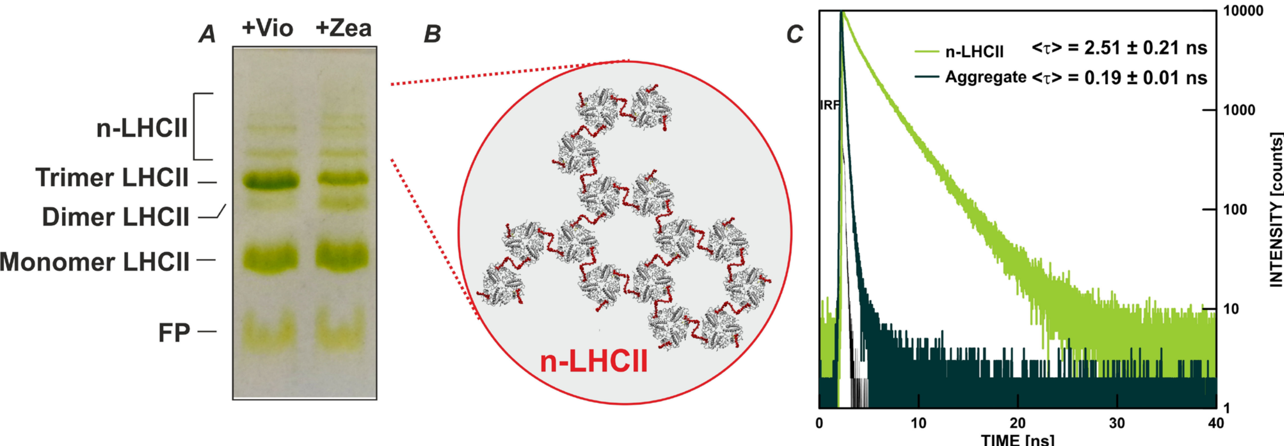

Fig. 4. Supramolecular structures of the light-harvesting complex of PSII (LHCII) promoted by exogenous violaxanthin and zeaxanthin. (*A*) Native electrophoresis of the LHCII sample modified with exogenous violaxanthin (+Vio) or zeaxanthin (+Zea). (*B*) Schematic representation of n-LHCII structure under the presence of exogenous violaxanthin (top view). The model is based on PDB data (ID: 2bhw). For clarity of presentation, only endogenous neoxanthin and exogenous violaxanthin are presented (*in red*). (*C*) Fluorescence decay kinetics of Chl *a* emission in the n-LHCII located directly in the polyacrylamide gel. Excitation and detection were at 470 and 680 nm, respectively. Intensity weighted average lifetime <τ> values ± SD for 5 independent replicates is presented in the figure. The fluorescence decay kinetics were fitted with four components characterized by the following lifetimes: τ_1 = 3.7 ns, τ_2 = 2.5 ns, τ_3 = 1.3 ns, and τ_4 = 0.2 ns. For comparison, fluorescence decay kinetic of aggregated LHCII [in buffer, 20 mM Tricine and 10 mM KCl (pH 7.6)] is added. FP – free pigments. Data from Janik *et al*. (2016).

ca. 2.5 ns (Fig. 4) [similar to average Chl *a* fluorescence lifetimes measured for a single chloroplast (2.23 ns) and a leaf in the presence of violaxanthin therein (2.5 ns)]. It is shorter than the average lifetime of the trimeric (*ca.* 3.5 ns in the detergent) and monomeric forms (*ca*. 2.7 ns in the detergent); at the same time, it is longer than that of the aggregated LHCII form (*ca*. 0.2 ns at a low detergent concentration). A comparable value of Chl *a* fluorescence lifetime (2.14 ns) was measured for model lipid–protein membranes with additional violaxanthin by Zhou *et al.* (2020). The 77 K fluorescence emission spectrum of Chl *a* in LHCII for the n-LHCII in the detergent environment is characterized by a fluorescence maximum at 680 nm, as in the case of the LHCII trimer emission spectrum in the detergent. These results indicate that the n-LHCII, although formed by more than one trimer, are not typical oligomeric structures, for which the fluorescence emission maximum is noted at 700 nm. Considering the possible physiological significance of the presence of n-LHCII structures in thylakoid membranes, a question should be put forward whether the lipid phase of the thylakoid membrane is characterized by the presence of a pool of violaxanthin that is not bound directly to antenna complexes, which could stabilize such structures. As shown by chromatographic analysis of thylakoids isolated from darkened spinach leaves, a considerable violaxanthin pool does not bind directly to the LHC complex proteins. Pigment molecules from this fraction can therefore contribute to the stabilization of the n-LHCII structures by interacting with the hydroxyl groups in neoxanthin molecules in adjacent LHCII trimers.

As mentioned earlier, the high density of LHCII complexes in the thylakoid membrane could result in an undesirable loss of absorbed light energy. Based on 77 K fluorescence emission spectra and Chl *a* fluorescence lifetimes of n-LHCII forms, it can be claimed that trimers in such a structure are separated from one another at a sufficient distance to prevent excitation energy self-quenching induced by aggregation of antenna complexes and ensure long-range distance energy transfer. The presence of n-LHCII structures in the thylakoid membrane in low or optimal light conditions at the predominance of violaxanthin in the pool of the xanthophyll cycle pigments may serve a regulatory role in the utilization of excitation energy in the photochemical processes of photosynthesis. The LHCII clusters stabilized by violaxanthin were also observed in the nonintact thylakoid membranes (Johnson *et al.* 2011). Nanoscale imaging of monolayers and multi-bilayers composed of exogenous xanthophylls confirmed the formation of LHCII ring-like clusters (Zhou *et al.* 2020).

Since the values of the fluorescence lifetimes for the n-LHCII structures (in the detergent environment) in the presence of violaxanthin and zeaxanthin are the same, it can be concluded that the molecular form of these structures is similar. However, the zeaxanthin-promoted structures, observed in chloroplast lipid environment (without detergent), are characterized by a relatively short Chl fluorescence lifetime (0.49 ns) (Janik *et al.* 2016). Moreover, fluorescence emission spectroscopy and chromatography analysis indicate that the zeaxanthin-stabilized supramolecular structure consists of a minimum of four pigment molecules and three LHCII monomers (four zeaxanthin molecules/LHCII trimer). The binding of another zeaxanthin molecule may lead to the destabilization of the trimer and disintegration of the supramolecular structure. It can be expected that the

LHCII molecular structures stabilized with zeaxanthin in the lipid membrane will exhibit a capability of efficient excitation energy quenching. On the other hand, the question of whether such energy quenching is based upon energy transfer directly from Chl to zeaxanthin molecule or is a result of specific LHCII organization induced by zeaxanthin is still open. The latest research pointed out that zeaxanthin, located peripheral to the LHCII complexes, rather promotes energy trap creation and then directly dissipates excess energy as heat. Since the presence of zeaxanthin in the LHCII complex promotes its monomerization and dimerization, it can be expected that oligomeric structures able to quench excess energy in light stress conditions will be formed from the monomers and dimers in the thylakoid membrane. Unfortunately, it has not been proved that 'trimer–monomer transition' is the main element of the 'molecular switching' mechanism during photoprotection.

It was proved that full photoprotective activity is achieved not only by xanthophyll cycle activity but also by its cooperation with the PsbS protein (Welc *et al.* 2021). The activity of xanthophyll cycle pigments is indirectly controlled by PsbS, which affects transmembrane localization and orientation of violaxanthin and zeaxanthin by influencing the molecular organization of membrane proteins. It seems that PsbS is not a 'seeding center' for LHCII aggregation but contrary, it allows irregular and less tight protein packing, which results in easy access and exchange of xanthophyll cycle pigments: violaxanthin and zeaxanthin.

LHCII dimer – a potential photoprotective structure

Quantitative and qualitative analysis of the oligomeric LHCII forms in leaves in high-intensity light conditions [1,200 μmol(photon) m^{-2} s^{-1}] demonstrated a large pool of monomers and a small fraction of dimers whereas LHCII in the darkened leaves had primarily the trimeric form (Fig. 5) (Janik *et al.* 2017a).

To determine whether the dimeric LHCII structure was induced directly by light, a detergent suspension of dark-adapted complexes was illuminated with varied-intensity light in the range of 11–1,240 μmol(photon) m^{-2} s^{-1}. It was observed that the number of LHCII dimers in the preparation strongly depended on light intensity. The greater the light intensity, the greater the fraction of dimeric structures was. Additionally, it was shown that the pool of LHCII trimers decreased with an increase in the pool of monomers and dimers in low-intensity light [11–95 μmol(photon) m^{-2} s^{-1}]. In turn, light from a high-intensity range [682–1,240 μmol(photon) m^{-2} s^{-1}] increased the pool of dimers at the expense of monomers. An increase in the number of LHCII dimers with increasing light intensity was also observed for a detergent suspension of thylakoid membranes isolated from spinach leaves. A light-induced dimeric LHCII structure was also observed using the FCS method. Two molecular forms of the LHCII complex, *i.e.*, trimeric (diffusion coefficient was 7 μm^2 s^{-1}) and monomeric (diffusion coefficient was 35 μm^2 s^{-1}), were found to be present in the darkened LHCII suspension in the detergent solution. Illumination of such a suspension with the light of 100 μmol(photon) m^{-2} s^{-1} resulted in the appearance of an additional LHCII form with a 22 μm^2 s^{-1} diffusion coefficient in the solution, which was identified as an LHCII dimer.

To find out whether LHCII dimers induced by strong light had a different function than that of the dimers formed at low-light intensities, we measured the average Chl *a* fluorescence lifetimes in the LHCII complex organized in the dimeric structure under the impact of low (*ca.* 1 ns) and high light (0.6–0.9 ns) and incorporation of a zeaxanthin molecule into the complex (1–2 ns). These results indicate that LHCII dimers formed under the impact of high-intensity light quench the excitation

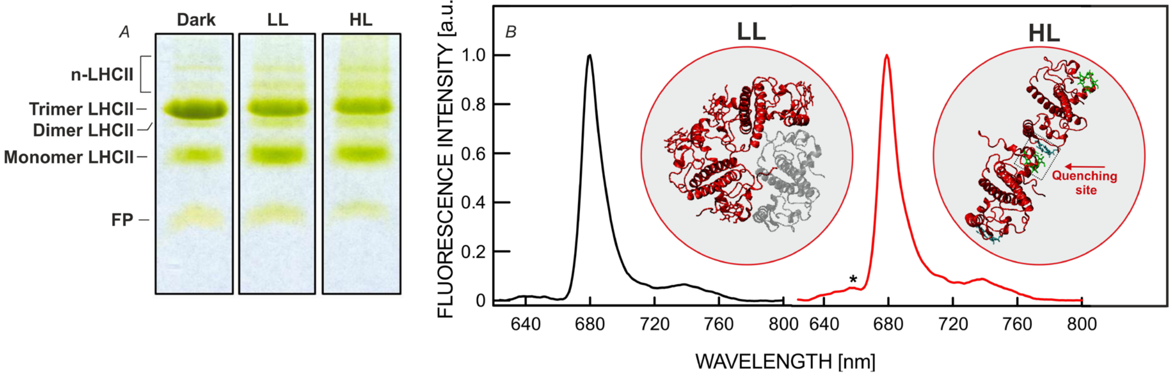

Fig. 5. Different light-harvesting complexes of PSII (LHCII) dimeric forms induced by low or high-light intensity. (*A*) Native electrophoresis of the LHCII samples adapted to the dark or illuminated with low (LL) or high (HL) intensity of light. (*B*) Fluorescence emission spectra of the LL-induced (*black line*) or HL-induced LHCII dimers (*red line*). The spectra were normalized to 1 at the maximum. Excitation was at 470 nm. Additionally, the schematic structure of both dimeric forms is included [top view, based on PDB data (ID: 2bhw)]. The quenching structure is proposed for two peripheral Chl molecules: Chl *a* (Chl 14, *cyan* color) and Chl *b* [Chl 8, according to nomenclature in Standfuss *et al.* (2005), *green* color] located in neighboring LHCII monomers. FP – free pigments. Data obtained from Janik *et al*. (2017a).

energy more efficiently than dimers induced by low light. It can therefore be concluded that the dimers observed in low-light conditions are merely elements of the disintegration of LHCII trimers in the monomerization process, whereas the dimers induced by strong light arise *de novo* from existing monomers (Fig. 5). Therefore, they are a specific molecular form of LHCII appearing in response to exposure of plants to strong light. This conclusion was strongly supported by the results of LHCII dimer measurements performed with the molecular spectroscopy techniques. The greater similarity of the high-intensity light-induced dimeric forms to LHCII monomers than trimers was observed. The specific molecular organization of energy-quenching LHCII dimers was also deduced from the CD spectra measured for the different LHCII molecular forms. The CD spectrum of dimers induced by strong light exhibited a band at 690 nm, which was not present in the spectra of LHCII trimers and monomers illuminated with strong light.

It is known that the ability to quench excitation energy by the LHCII molecular forms is a result of their specific organization of Chl molecules. It was proved previously that the photoprotective energy dissipation was associated with the influence of Chl molecules located in close contact with each other and an appropriate spatial conformation (Liu *et al.* 2004, Standfuss *et al.* 2005, Duffy *et al.* 2008) Therefore, a structure of the LHCII dimer capable of efficient excitation energy quenching was proposed, in which Chl 14 and Chl 8 [by the nomenclature proposed by Standfuss *et al.* (2005) Chl *b* and Chl *a*], away from each other in the structure of a single monomer, are located at a distance lower than 1 nm. This newly formed Chl pair could be a potential candidate for a specific molecular structure providing dimers the capability of photoprotective energy quenching under strong light.

The light-induced monomerization of LHCII trimers facilitates the formation of dimeric structures capable of effective excitation energy quenching in varying light conditions. On the other hand, it cannot be excluded that LHCII dimers can aggregate with each other into larger supramolecular structures (macro-domains, lamellar aggregates (Jennings *et al.* 1991, Barzda *et al.* 1996) characterized by the even greater capability of efficient energy quenching in excess excitation conditions.

Phosphorylation of LHCII complexes is the system regulating light utilization by chloroplasts

As discussed earlier, phosphorylation of LHCII complexes changes their molecular organization and leads to the reorganization of the structure of thylakoid grana. At illumination of spinach leaves with high-intensity light [*ca*. 1,200 µmol(photon) m^{-2} s^{-1}], only 30% of the pool of LHCII complexes are phosphorylated (Tikkanen and Aro 2012). To determine which molecular LHCII forms are present in the thylakoid membrane during illumination of plants with high-intensity light and their influence on the organization of lipid membranes, investigations were conducted on lipid–protein model systems (Janik *et al.* 2013). Immunodetection analyses revealed that 30-min illumination of spinach leaves with high-intensity light resulted in the phosphorylation of 35% of the pool of LHCII complexes. Furthermore, it was found that complexes isolated from leaves illuminated with strong light contained zeaxanthin (0.16 ± 0.01 molecule per LHCII monomer). Application of the AFM-IR techniques for measurement of the lipid–protein systems showed that lipid multi-bilayers containing nonphosphorylated complexes exhibited the presence of areas with a high content of LHCII complexes separated by areas comprising primarily lipids. The distribution of the lipids and LHCII complexes was homogeneous for systems containing phosphorylated complexes. These observations indicated the formation of transmembrane LHCII structures with the involvement of nonphosphorylated complexes. The measurement of infrared absorption spectra demonstrated that these structures were stabilized by hydrogen bonds, which facilitated the formation of antiparallel β-sheet protein structures. Due to the interaction in the lipid membrane plane, the nonphosphorylated LHCII complexes formed aggregates what was associated with an increase in the intensity of a fluorescence emission band with a maximum at 700 nm (Fig. 6) and shortening of the Chl *a* fluorescence lifetimes in the analyzed system.

A look within liposome vesicles with different ratios of lipids to LHCII protein resulted in a conclusion that LHCII in native thylakoid membranes is aggregated (Natali *et al.* 2016). Mediation of LHCII trimers (paired with PSII) in thylakoid membrane stacking by Albanese *et al.* (2020) was also confirmed.

The absence of lamellar LHCII aggregates in samples comprising nonphosphorylated complexes can be explained by the formation of transmembrane structures, which naturally limit the free movement of LHCII complexes and thus their interactions in the membrane. As shown earlier, van der Waals forces and Coulomb interactions caused by the different charge distributions on the surface of adjacent thylakoid membranes are involved in the stabilization of the structure of thylakoid grana in chloroplasts. In the presented studies, we showed that the thylakoid grana could be stabilized by transmembrane LHCII structures reinforced by hydrogen bonds between the polar portions of the protein part of LHCII located in adjacent lipid membranes.

Additionally, it was demonstrated that phosphorylation of LHCII complexes might not only have a regulatory role in photosynthesis at the illumination of plants with low-intensity light but also can provide protection to the photosynthetic apparatus from excess light. The low level of phosphorylation and de-epoxidation of violaxanthin induce the formation of LHCII aggregates characterized by high efficiency of excess excitation energy quenching in the lipid membrane in light stress conditions (Fig. 6). Similarly, LHCII protein–protein interaction in nanodisc platforms (Son *et al.* 2021) or in a buffer with a low concentration of DM followed by a dense array of proteins have been postulated to collapse into a highly dissipative structure (Crepin *et al.* 2021).

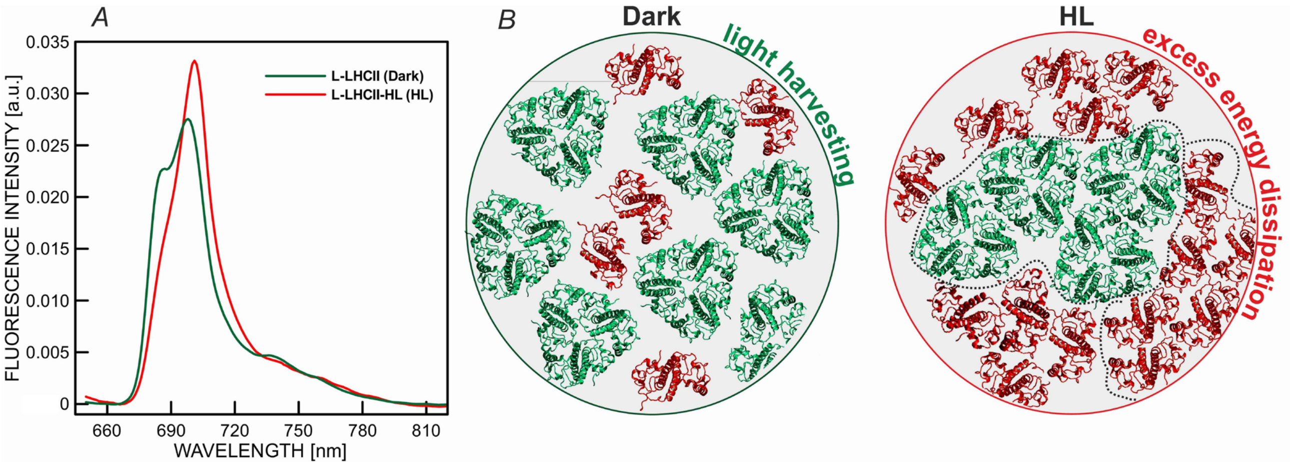

Fig. 6. Model of the molecular organization of light-harvesting complex of PSII (LHCII) under dark or high light (HL) conditions. (*A*) Fluorescence emission spectra of LHCII embedded into artificial chloroplast lipid membranes. LHCII was isolated from dark (L-LHCII) or high light (L-LHCII-HL) intensity-adapted spinach leaves. The excitation wavelength was 470 nm. The spectra were normalized to get the same area beneath each spectrum. (*B*) A proposed representation of the putative LHCII organization in thylakoid membranes. Under dark conditions, the LHCII complexes are tightly and loosely connected. In this state, LHCII preferentially absorbs light quanta. In dissipative conformation (HL), the LHCII complexes are organized into energy-excess quenching architecture. Possible photoprotective domains are outlined by *dashed black lines*. The schematic structure of LHCII is based on PDB data (ID: 2bhw). Data from Janik *et al.* (2013).

Summary

LHCII is the most abundant membrane protein in the biosphere and plays numerous biological functions in the photosynthetic apparatus. Among such functions are: harvesting of energy of photons, internal conversion, transfer of the electronic excitation energy towards the photosynthetic reaction centers, and stabilization of the functional forms of individual thylakoid membranes and entire grana structures. Interestingly, LHCII complexes have elaborated over the millions of years of biological evolution the capability of act as light intensity sensors that can convert this information on a series of molecular mechanisms acting in symphony to regulate a balance between the light-harvesting and quenching excessive excitations to protect the photosynthetic apparatus against photodegradation. Several of such mechanisms rely on the reorganization of supramolecular structures of LHCII, between single monomers, dimers, trimers up to large molecular aggregates.

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