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Review

Evolution and functional properties of Photosystem II light harvesting complexes in eukaryotes ☆

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ABSTRACT

Photoautotrophic organisms, the major agent of inorganic carbon fixation into biomass, convert light energy into chemical energy. The first step of photosynthesis consists of the absorption of solar energy by pigments binding protein complexes named photosystems. Within photosystems, a family of proteins called Light Harvesting Complexes (LHC), responsible for light harvesting and energy transfer to reaction centers, has evolved along with eukaryotic organisms. Besides light absorption, these proteins catalyze photoprotective reactions which allowed functioning of oxygenic photosynthetic machinery in the increasingly oxidant environment. In this work we review current knowledge of LHC proteins serving Photosystem II. Balance between light harvesting and photoprotection is critical in Photosystem II, due to the lower quantum efficiency as compared to Photosystem I. In particular, we focus on the role of each antenna complex in light harvesting, energy transfer, scavenging of reactive oxygen species, chlorophyll triplet quenching and thermal dissipation of excess energy. This article is part of a Special Issue entitled: Photosystem II.

1. Introduction

Solar energy is the most abundant renewable energy source available in our planet. Evolution generated a wide range of organisms capable of using solar energy in order to produce biomass through the photosynthetic process. Light harvesting is one of the most intensively investigated processes in plant biology, photosynthesis-driven carbon fixation being crucial to both feedstock and renewable bioenergy production. Oxygenic photosynthetic organisms, from cyanobacteria to vascular plants, are adapted to different environmental conditions, even extreme. The central structure for light energy conversion are the photosystems, i.e. multisubunit transmembrane pigment–protein complexes that catalyze electron transport from electron donors located on the inner thylakoid surface to acceptors located on the outer face [1,2]. Each photosystem is composed by a moiety named *core complex*, containing the *reaction center*, where light dependent charge separation and the first steps of electron transport occur. A second moiety, called *antenna* is located peripherally with respect to reaction centers and is specialized in harvesting light and in transferring excitation energy to the reaction center, thus increasing the amount of photons absorbed per photosystem. Core complex subunits are highly conserved through evolution, while peripheral antenna proteins are diversified [3]. The first peripheral antenna system, operating in an oxygenic environment, appeared 3–3.5 billion years ago with the phycobilisomes in cyanobacteria. These consist into arrays of soluble proteins hanging on the surface of photosynthetic membranes [4]. During eukaryotic evolution phycobilisomes were lost, in favor of light-harvesting complex (LHC), antenna proteins integral into thylakoid membranes. LHC proteins have three membrane-spanning regions and coordinate chlorophyll (Chl) and carotenoid (Car) ligands, with different composition depending on taxa. Besides acting in light harvesting, LHC proteins also have roles in photoprotection: in excess light conditions, reaction centers cannot efficiently quench Chl excited states, yielding into an increased lifetime and a higher probability of intersystem crossing to triplet states [5]. Chl triplets readily react with molecular oxygen, yielding into the harmful singlet oxygen ($^1O_2$) [6]. Lhc proteins catalyze thermal dissipation of Chl singlet excited states thus limiting the formation of triplet states [7]. Among different redox states of oxygen, the fully reduced form H_2O and the fully oxidized form O_2, differing by 4 e^-, are relatively stable. Intermediate states, produced by univalent electron transport within photosystems, are toxic and need to be scavenged, a function performed by LHC-bound xanthophylls with far enhanced efficiency with respect to lipid free carotenoids [8–11]. This

Abbreviations: NPQ, non-photochemical quenching; Chl(s), chlorophyll(s); Car(s), carotenoid(s); LHC, light harvesting complexes; Lhca, light harvesting complexes of Photosystem I; Lhcb, light harvesting complexes of Photosystem II; VCP, violaxanthin-cycle pigment; PG, phosphatidylglycerol; DGDG, digalactosyldiacylglycerol; A. t., *Arabidopsis thaliana*; C. r., *Chlamydomonas reinhardtii*; P. p., *Physcomitrella patens*; ELIPs, early light-induced proteins; SEPs, stress-enhanced proteins; OHPs, one-helix proteins; CBP, chlorophyll binding proteins; ROS, reactive oxidative species; FCP, fucoxanthin binding complexes

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activity is essential for photoprotection even at low light intensity [12,13].

During evolution and transition from aquatic to sub-aerial environment, photosynthetic organisms have experienced a progressive increase in the concentration of O_2 in their environment, leading to an increasing risk of reactive oxygen species (ROS) formation. This condition favored the selection of antenna proteins more efficient in quenching chlorophyll excited states in excess light conditions and in ROS scavenging at all light intensities. These considerations suggest that LHC proteins were critical for evolution of photosynthesis in aerobic environment, characterized by a high level of oxidative stress. Following endosymbiotic event(s) with formation of a plastid compartment within eukaryotic cells, the appearance of multiple LHC isoforms, each tuned into a specific combination of light harvesting and photoprotection function within a supramolecular photosynthetic assembly, likely increased the ability to resist ever-changing environmental conditions and thus to colonize new environments. The selection of mechanisms that allows an efficient balance between light absorption and electron transport to acceptor substrates, typically CO_2, through the dissipation of energy absorbed in excess and/or the efficient scavenging of ROS, was likely a key event in the evolution of oxygenic photosynthesis.

Mechanisms for heat dissipation of excitation energy are present in phycobilisomes already, although their efficiency is low [14,15]. In case of high light stress, LHC components of the PSII antenna system efficiently dissipate the energy absorbed in excess as heat, through a photoprotective mechanism known as *N*on-*P*hotochemical *Q*uenching (NPQ). This process limits over-reduction of electron transport chain and energy transfer to oxygen, with the formation of toxic ROS [16,17]. Additional regulative functions associated to Lhc proteins are the direct quenching of chlorophyll triplet states by xanthophylls, the scavenging of ROS species when formed and the so-called state1–state 2 transitions, achieved by a mobile pool of LHCII that can switch back and forth between PSII and PSI [18–20].

In this article we review the structural and functional properties of LHC antenna proteins of Photosystem (PS) II, the Lhcb proteins. Interactions between Lhcb and PSII core form PSII–LHCII supercomplexes, which further extend into a three-dimensional macrostructure across the paired membranes of the grana stacks [21,22]. PSII is likely the site in which antenna proteins were more exposed to evolutionary pressure, due to the lower photosynthetic efficiency of PSII (~0.8) compared to PSI (~1). Lhcb proteins are thought to have appeared once during the evolution of the antenna system, implying that the antenna proteins from plants, green algae, red algae and Stramenopiles share a common ancestor [23]. Encoded in the nucleus and following translation in the cytoplasm, LHCs are targeted to the chloroplast via an N-terminal extension called transit-peptide, that is recognized by the import apparatus [24]. Two translocons traversing the outer (Toc) and inner (Tic) envelope membrane catalyze protein import into the chloroplast stroma [25,26]. In the stroma, the transit-peptide is removed and the apoprotein co-insertionally assembles in the thylakoid membrane with chlorophylls and carotenoids [27].

In the following we will discuss the functional properties of the different Lhcb proteins and attempt defining the relations between their structure and role in light harvesting and photoprotection.

2. The evolution of Lhcb proteins

The evolution of green organisms and their photosynthetic machineries is intimately linked to the extended light-harvesting complex protein super family (Fig. 1). In this gene family, several subclasses can be identified: the Lhcb proteins, the FCP proteins and the LHC-like proteins. The main differences in PSII antenna protein content in the different organisms are reported in Table 1.

2.1. Lhcb proteins

Major LHCII trimers are composed of three subunits called Lhcb1, Lhcb2 and Lhcb3, which form homotrimers (Lhcb1, Lhcb2) or combine into heterotrimers (Lhcb1, Lhcb2, Lhcb3) [28]. There are over 77% sequence similarity among Lhcb1, Lhcb2 and Lhcb3, suggesting that their three-dimensional structures are virtually identical. The trimerization of Lhcb1, 2 and 3 gene products to form LHCIIs requires the sequence motif WYGPDR at the N terminal region. Such a motif is present in LHCII and Lhcb5, but not in Lhcb4 and Lhcb6 [29], and is required for trimer formation. In *Chlamydomonas reinhardtii* (*C. r.*), the components of the trimeric LHCII complexes are encoded by nine *Lhcbm* genes, called *Lhcbm1–Lhcbm6*, *Lhcbm8*, *Lhcbm9* and *Lhcbm11*[30]. None of the *C. r.* Lhcb mature polypeptides could be specifically associated with a single subtype of *A. t.*, implying that diversification between the *Lhcbm* isoforms occurred after the divergence of *Chlamydomonas* from the green lineage. In another green alga species, *Ostreococcus tauri*, Lhcb1–3 and Lhcbm proteins are not present. However 4 genes encoding Lhcp complexes, specific Lhc subunits for *Prasinophyceae*, have been identified [31], supporting the view that independent specialization of proteins encoding the

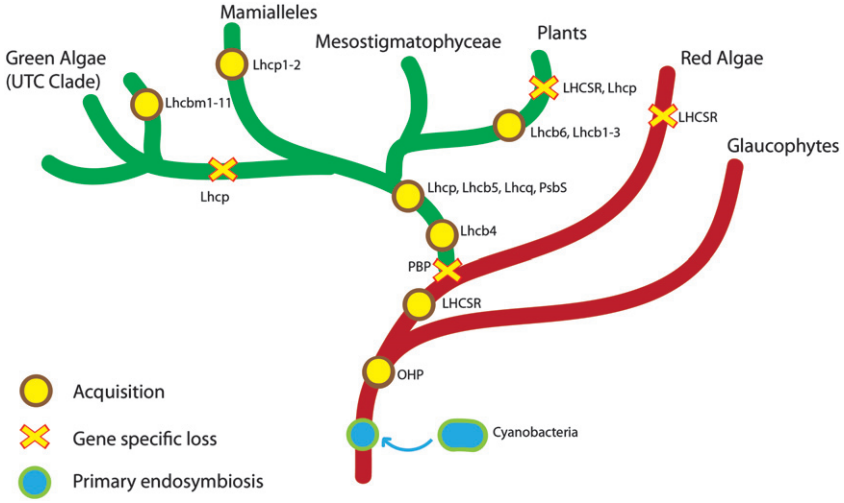

Fig. 1. Scheme for a phylogenetic tree of major LHCs in the principal photosynthetic eukaryotic lineages. The major eukaryotic groups are shown along with some key evolutionary events such as gene acquisition or gene specific loss. The primary endosymbiogenesis permitted the acquisition of photosynthesis from a cyanobacteria. OHP, one-helix protein; PBP, phycobilin protein.

Table 1
Summary of main properties of Photosystem II antenna proteins in different organisms and model species.

Organism	Model species	Trimeric PSII antenna proteins	Monomeric PSII antenna proteins	LHC-like proteins	Chlorophyll content	Main carotenoids content bound by antenna proteins	NPQ mechanisms
Higher plants	*Arabidopsis thaliana*	Lhcb1–3, Lhcq	Lhcb4–6, Lhcb7–8 (substechiometric amount)	PSBS, ELIPS,OHPs, SEPs	Chl *a*, Chl *b*	Lutein, neoxanthin, violaxanthin (zeaxanthin)	PSBS-dependent
Mosses	*Physcomytrella patens*	Lhcbm proteins (13 genes) Lhcb3	Lhcb4–6, Lhcb9	PSBS, ELIPS, LhcSR1–2, OHP, SEPs	Chl *a*, Chl *b*	Lutein, neoxanthin, violaxanthin (zeaxanthin), loroxanthin	LhcSR proteins-dependent; PSBS-dependent
Green algae	*Chlamydomonas reinhardtii*	Lhcbm1–6, Lhcbm8, Lhcbm9, Lhcbm11 (trimeric complexes), Lhcq	Lhcb4–5	PSBS[a], ELIPS, LhcSR1–3, OHP, SEPs	Chl *a*, Chl *b*	Lutein, neoxanthin, violaxanthin (zeaxanthin), loroxanthin,	LhcSR3-dependent
	Ostreococcus tauri	Lhcbp, Lhcq	Lhcb4–5	ELIPS, LhcSR, OHP, SEPs	Chl *a*, Chl *b*	Dehydrolutein, violaxanthin, (zeaxanthin), prasinoxanthin, lutein,	N.A.
Diatoms	*Pheodactylum tricornutum*	FCP (A–F)		Lhcx1–4, OHP, SEPs	Chl *a*, Chl *c*	Fucoxanthin, diadinoxanthin (diatoxanthin)	Lhcx (LhcSR like)-dependent

N.A: information not yet available. Lhcq are reported in the table even if the functions, properties and distribution among different organisms are still unclear.
[a] *Psbs* gene is present in the genome, but the protein is not accumulated.

major light harvesting complex occurred in the different taxa belonging to *viridiplantae*, like Lhcbm proteins of *C. r.* and Lhcb1–3 proteins in plants.

In the case of the moss *Physcomitrella patens* (*P.p.*), *Lhcb1–2* genes cannot be identified with the corresponding genes in plants, the closest relatives being thirteen *Lhcbm* genes, encoding apoproteins for heterotrimeric LHCII complexes, while a *Lhcb3* gene is found [3,32].

The first Lhcb protein distinct from the major LHCII was reported in the '80s; due to its apparent molecular mass of 29 kDa, it was called CP29 (Chlorophyll-Protein of 29 kDa) [33] and later Lhcb4 [34]. In *Arabidopsis thaliana*, Lhcb4 is present in three different isoforms named Lhcb4.1, Lhcb4.2 and Lhcb4.3, the latter isoform having a different expression profile as compared to Lhcb4.1 and Lhcb4.2 [35]. Lhcb4.3 also shows a deletion of the N terminal domain. Due to this differences with respect to Lhcb4.1 and Lhcb4.2, Lhcb4.3 was recently suggested being a distinct Lhcb antenna protein and renamed as Lhcb8 [35]. Whether this corresponds to a difference in biochemical or spectroscopic properties among CP29 complexes, is still unknown. In 1986, the smallest LHC component of PSII was identified and called CP24 on the basis of the molecular mass of its apoprotein. It was later called Lhcb6 [36,37]. The last monomeric antenna isolated was CP26 [37,38], later called Lhcb5. By EST and genome data analysis, a new Lhcb protein, named Lhcb7, was recently identified and suggested having a place in PSII antenna system; Lhcb7 was shown to be a new PSII antenna protein, somehow similar to Lhcb5, but its contribution to the PSII antenna system is not clear yet, since this gene is rarely expressed and shows high relative expression levels in petals, sepals and in conditions of oxidative stress [35].

The monomeric antennae Lhcb4, Lhcb5 and their respective orthologs are found in all classes of green plants [39]. The wide distribution of Lhcb4 and Lhcb5 in the green algae suggests that these proteins are involved in functions established early during the evolution of the LHCII-type antenna systems [40]. Recently, Lhcb4 has been shown to migrate towards PSI during state transitions in *C. r.* thus contributing to the re-distribution of excitation energy between PSI and PSII. So far there is no evidence for migration of Lhcb4 to stroma membranes in plants [20,41].

In the case of the minor antenna Lhcb6, its orthologs were not detected in green algae as *C. r.*[30] or *Ostreococcus tauri*[31]. Lhcb6 indeed appears to be associated to land environment, since it is first found in the moss *P.p.* and then maintained in higher plants. Besides Lhcb6 and Lhcb3, one additional Lhcb protein is first detected in *P.p.*, named Lhcb9. However, this antenna subunit did not fix in later stages of evolution and is restricted to mosses [3].

Other proteins closely related to Lhcbs were identified in the different organisms, however bearing unknown functions, as in the case of the gene products called Lhcq [30], recently identified in green algae and in some land plants as *A. t.*[31,39]. Lhcq proteins form a supported subgroup at the base of the LHCII branch. These proteins have a trimerization motif like other major LHCII proteins, suggesting a possible role in substituting components of the peripheral LHCII antenna system in specific conditions [40].

2.2. FCP proteins

The photosynthetic apparatus of diatoms and brown algae presents differences as compared to that of green algae and land plants. Diatoms have thylakoid membranes lacking stacks (grana) and the two photosystems are not laterally segregated [42]. Nevertheless, evidencehas been reported for membrane domains formed by different lipid species, suggesting a macrodomain organization of thylakoid membranes [43]. Antenna system in these organisms is composed by proteins named FCP (fucoxanthin-binding complexes) or more recently Lhcf: these proteins bind Chl *a*, Chl *c* and carotenoids as fucoxanthin, diadinoxanthin and diatoxanthin [44,45]. Diadinoxanthin and diatoxanthin appear to be involved in a xanthophyll cycle similar to the violaxanthin/zeaxanthin cycle of plants, which is also present in diatoms [46]. Diatoxanthin is the de-epoxidized form being accumulated, together with zeaxanthin, upon high light stress. Despite differences in pigment binding, FCP proteins share high level of similarity with protein components of the plant major LHCII, with an average of ~30% of identity compared to *A.t.* Lhcb1. Still their functional properties are not known well enough to verify this suggestion. Other types of Lhc proteins named Lhcc and Lhcr are instead present in *Cryptomonads* and red algae, but their properties have not been described yet [47].

2.3. LHC-like proteins

LHC-like proteins are proteins that share homology with Lhcs, although similarity can be rather low. In most cases their function is unknown, with some interesting exceptions. The LHC-like protein family can be divided into four sub-classes: the four-helix protein PsbS [48], three-helix early light-induced proteins (ELIPs) [49], two-

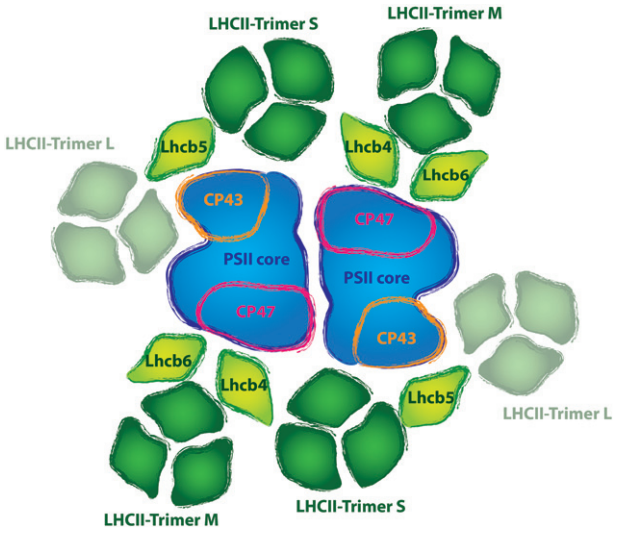

Fig. 2. Arrangement of the PSII $C_2S_2M_2(L_2)$ supercomplex. Lhcb1, Lhcb2 and Lhcb3 combine randomly to form the LHCII-trimer; S, LHCII trimer strongly bound; M, LHCII trimer moderately bound; L, LHCII-trimer loosely bound.

helix stress-enhanced proteins (SEPs) [50] and one-helix proteins (OHPs) [51]. These LHC-like proteins are present in different photosynthetic organisms: the OHPs are ubiquitously distributed among photosynthetic organisms [51], while SEPs are absent in cyanobacteria and ELIPs are found in viridiplantae but absent in red algae and diatoms [47]. Members of the OHPs, SEPS and ELIPS families are in all cases induced by high light stress, when the expression of most other Lhc proteins is down-regulated [50,52–54]. It was proposed that these LHC-like proteins might play a photoprotective role within the thylakoids, either by transient binding of free chlorophyll molecules and/or by acting as sinks for excitation energy [53,55,56]. Over-expression of ELIP 2 in *Arabidopsis thaliana* was shown to decrease the level of chlorophyll biosynthesis precursors, suggesting that ELIPS act in down-regulating chlorophyll accumulation in potentially stressing conditions [57]. These genes are thought to be representative of the ancestor gene for LHC proteins. This new hypothesis for the evolution of the extended LHC protein family suggests SEPs as the best candidate for the ancestor of LHC proteins. Two-helix sequence gene duplication would provide a simple and parsimonious explanation for the origin of the second, less-conserved CB-TM helix in LHC proteins [47].

An interesting group of LHC-like proteins is that of LHCSR proteins, for which clear evidence for their function has been recently obtained. These are found in green algae and mosses, but absent in lycopods and seed plants. LHCSRs are stress-related proteins that accumulate upon acclimation to high-light intensity conditions and are involved in photoprotection mechanisms (see below) [58–68]. Recent phylogenetic analysis with chlorophyll binding proteins (CBPs from a wide range of taxa) demonstrated that most of the stress-induced CBPs belong to the LHCSR clade, composed by LHCSR, Li818-like and LHCX [60].

A unique member of LHC-like protein family is the four-helix protein PsbS. It has been described as a PSII subunit, from its localization in the grana membranes and within PSII–LHCII supercomplexes [69]. Interestingly, *psbS* gene is also present in green algae where, however, the corresponding protein is not accumulated in the chloroplast even when expressed under a constitutive promoter [70]. Despite its homology to Lhc proteins, chlorophyll-binding residues are not conserved in PsbS [69] and the protein does not appear to bind pigments [71]. Reverse genetic analysis (Section 4.5), followed by complementation with point mutant variants of PsbS, evidenced the importance of lumen-exposed glutamate residues for NPQ activation. These PsbS lumen-exposed residues undergo protonation at low luminal pH, triggering thermal energy dissipation in neighbor Lhc proteins as described in Section 4.3. Since luminal pH acidification is a consequence of over-reduction of photosynthetic apparatus, PsbS appears to have an important role, although indirect, in photoprotection as pH signal transductor for luminal acidification in stress conditions [71–74].

3. Structure and organization

3.1. Three dimensional structures of LHCII and Lhcb4

After the bacterial reaction center[75], bacteriorhodopsin [76] and bacterial porins [77], LHCII was one of the first membrane proteins to have its structure determined [78]. Today the structure of the complex by X-ray crystallography is available at 2.5 Å of resolution [79,80]. LHCII subunits have three helix membrane-spanning regions named A–B–C, connected by both stroma and lumen-exposed loops, and two amphipatic helices exposed on the thylakoid lumen surface, named D–E (Fig. 3). Each monomer coordinates four xanthophylls and 14 porphyrin molecules, 8 Chl *a* and 6 Chl *b*. In addition, two different lipid molecules complete the LHCII structure, phosphatidyl glycerol (PG) and digalactosyl diacyl glycerol (DGDG). The most prominent feature of the

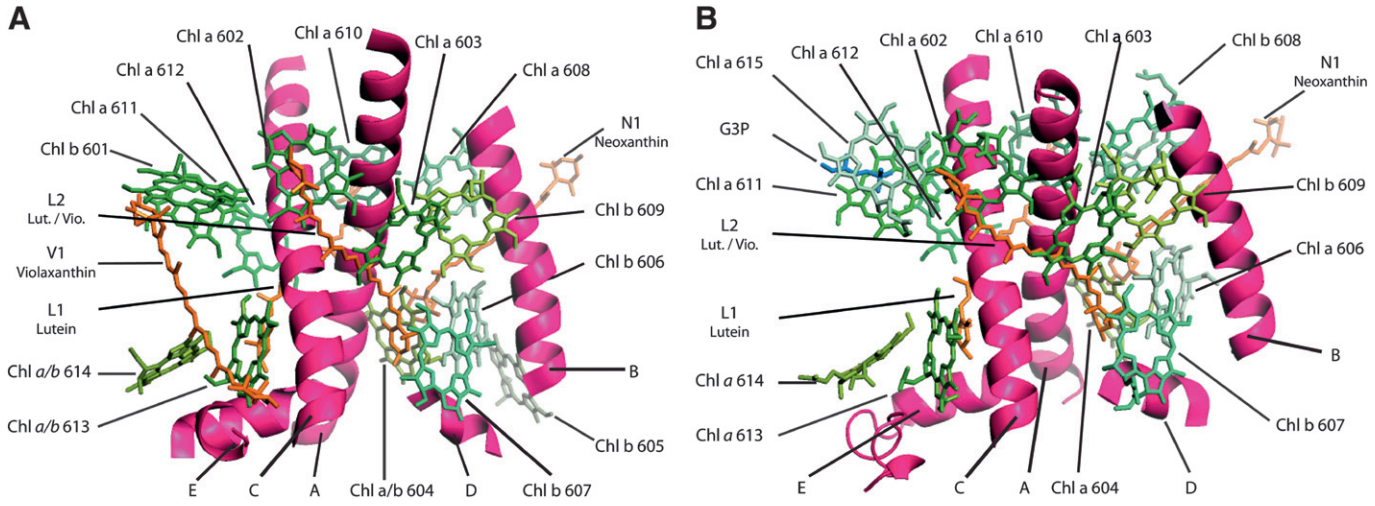

Fig. 3. Molecular model of LHCII monomer and Lhcb4 showing chlorophyll and xanthophyll chromophores bound to different binding sites. The model (A–B) has been drawn based on the crystal structures of LHCII trimer (A) [79] and Lhcb4 (B) [85]. Pink, polypeptide; green, chlorophylls; orange, carotenoids; blue, lipids.

LHCII protein backbone are the 32 amino acid-long trans-membrane helices A and C (named also helices 3 and 4) [78–80]. These regions possess the characteristic '*LHC motif*' (ExxxxRxAM), in which the Glu (E) from one LHC motif binds a Chl *a* molecule via a salt bridge to the Arg (R) of the other: this coordinated binding of chlorophyll stabilizes the central two helices [78]. The second membrane-spanning region is the 20 amino acid-long helix B (named also helix 1 [80]); which also participates in chlorophyll binding [79,81]. The two amphipathic helices at the luminal surface of the thylakoid membrane consist of 9 (D or helix 4) and 10 (E or helix 5) residues and are slightly bent with respect to the membrane plane [79,80].

Chlorophylls are coordinated to the polypeptide chain via their central Mg^{2+}, one facing the lumenal side and one the stromal side of the thylakoid membrane. Chls 601, 602, 603, 608, 609, 610, 611 and 612 comprise the layer facing the stroma, whereas Chls 604, 605, 606, 607, 613 and 614 are closer to the lumen. Interestingly, Chls *a* are arranged around helix A and B, while Chls *b* are in the vicinity of helix C. Seven chlorophylls are bound to amino acid side chains of histidine, asparagine, glutamic acid and glutamine (Chls 610, 612, 613, 603, 602, 609 and 614). Two are bound to the oxygen atoms of carbonyl groups (Chls 601 and 605), one to phosphatidyl glycerol (Chl 611) and four more via water molecules (Chls 604, 606, 607 and 608) (Table 2). Structural data, validated by site-specific mutagenesis and energy pathway modeling (see next section), evidenced that some Chls are strongly coupled to each other, in particular the trimer located at the stromal layer Chl 610–611–612, the trimer Chl 602–603–609 and the dimers Chl 613–614 and Chl 604–606: as discussed in the last paragraph of this review, the excitation coupling of this Chls and the interactions among the different Chl clusters determine the excitation energy pathway through the protein.

Xanthophylls play a role in both light absorption and photo-protection, thus are essential components of the antenna [79]. The major LHCII binds 2 luteins, 1 neoxanthin and 1 Violaxanthin Cycle Pigment (VCP; i.e. violaxanthin, anteraxanthin or zeaxanthin) [79]. The characteristic lutein binding motif is the DPLG sequence in the hook-like extension [81] (Fig. 4) at the stromal end of trans-membrane helix 4. Thanks to their cross-brace construction, the two lutein molecules bound to the L1 and L2 sites stabilize the LHCII complex. The lutein molecule in site L1 establishes a contact with amino acid residues in the range of Ser160 and Leu164 on the stromal side, and to Gln197 on the luminal side. Other residues involved in binding the xanthophylls in L2 are Asp47, Thr48 or Ala49 on the stromal side, and residues in the short sequence in between Trp97 and Ala100 on the luminal side. The β-cyclohexane rings of both lutein molecules are located towards the lumen; conversely, the ε-cyclohexane rings are positioned close to the stromal membrane surface. Also, binding sites exist in LHCII for both neoxanthin and a VCP, which are respectively labeled N1 and V1. Neoxanthin binding site stabilizing motif has been identify in the lumenal loop where the epoxycyclohexane ring OH group is bound through a hydrogen bond with Tyr112, while the cyclohexane ring at the stromal side protrudes out of the protein into the lipid bilayer [79,82,83]. The high specificity of this binding site for neoxanthin is clearly due to the fact that it can only accommodate the 9′-cis stereoisomer.

Besides chlorophylls and xanthophylls, non-proteic components of LHCII trimers include lipids [79]: PG is located parallel to the violaxanthin ligand in external site V1 and appears to be essential for trimerization [80], serving as hydrophobic glue between subunits [84]. In addition, DGDG is required for the formation of both 2D and 3D crystals of LHCII [84]. DGDG was localized in a central hydrophobic cavity on the lumenal side in the high-resolution structure of the pea complex [80]. An additional binding site was identified at the periphery of the complex, bridging the gap in between two adjacent trimers on the stromal side of the spinach complex in the highly curved proteolipid vesicles [79].

During the preparation of this review, the structure of a monomeric Lhcb protein from spinach was made available at 2.8 Å resolution [85]. This protein was identified as Lhcb4, although lacking its N-terminal region which was lost by proteolysis. Identification of this protein as Lhcb4 is likely to be a correct assignment since it contains binding sites for the three xanthophylls lutein (in L1), violaxanthin (in L2) and neoxanthin (in N1), which makes it different from both LHCII (having 4 xanthophyll binding sites) and Lhcb6 (lacking neoxanthin). Lhcb4 is very similar to a LHCII monomer as derived from the trimeric structure [79], due to the three membrane-spanning helical regions connected by both stroma and lumen-exposed loops and the two amphipatic helices exposed on the thylakoid lumen surface [85]. Differences consist into a somehow more packed structure for Lhcb4 as compared to LHCII. Indeed, the three transmembrane helices of Lhcb4—A, B and C—are all shorter than the corresponding helices in LHCII and also shorter are the loops connecting helix C to helices A and E. Moreover, the amphipathic helix D of Lhcb4 is more deeply included in the hydrophobic region protein core. Ground for the monomeric aggregation state can be found in the C terminal region, which contributes to trimerization with a specific motif [86] and is shorter in Lhcb4 as compared to LHCII [79,85]. The

Table 2
Different nomenclatures of chlorophyll-binding sites.

Pan et al. [85]	Standfuss et al. [28]	Liu et al. [79]	Kühlbrandt et al. [78]	*Spinacia olearea* LHCII model	*Pisum sativum* LHCII model
				Mg^{2+} coordinated by	
Chl *a* 610	Chl 1	Chl *a* 610	Chl *a*1	Glu 180	Glu 180
Chl *a* 612[a]	Chl 2	Chl *a* 612	Chl *a*2	Asn 183	Asn 183
Chl *a* 613	Chl 3	Chl *a* 613	Chl *a*3	Gln 197	Gln 197
Chl *a* 602	Chl 4	Chl *a* 602	Chl *a*4	Glu 65	Glu 65
Chl *a* 603	Chl 5	Chl *a* 603	Chl *a*5	His 68	His 68
Chl *a* 604	Chl 6	Chl *a* 604	Chl *a*6	Water 309	Gly 78
Chl *b* 607	Chl 10	Chl *b* 607	Chl *a*7	Water 308	Unidentified
Chl *b* 608	Chl 11	Chl *b* 608	Chl *b*1	Water 302	Unidentified
Chl *a* 611[a]	Chl 7	Chl *a* 611	Chl *b*2	PG[b]	Unidentified
Chl *a* 614	Chl 8	Chl *a* 614	Chl *b*3	His 212	His 212
Chl *b* 609	Chl 12	Chl *b* 609	Chl *b*5	Glu 139	Glu 139
Chl *b* 606	Chl 13	Chl *b* 606	Chl *b*6	Water 310	Gln 131
–	Chl 9	Chl *b* 601	–	Tyr 24[c]	None
–	Chl 14	Chl *b* 605	–	Val 119[c]	None
Chl *a* 615[a]					

[a] Coordinated by a central ligand (glyceraldehyde 3-phosphate) determined by X-ray crystallography of spinach Lhcb4.
[b] PG, phosphatidyl-glycerol.
[c] Coordination of the Mg^{2+} occurs via the oxygen of the backbone carbonyl group.

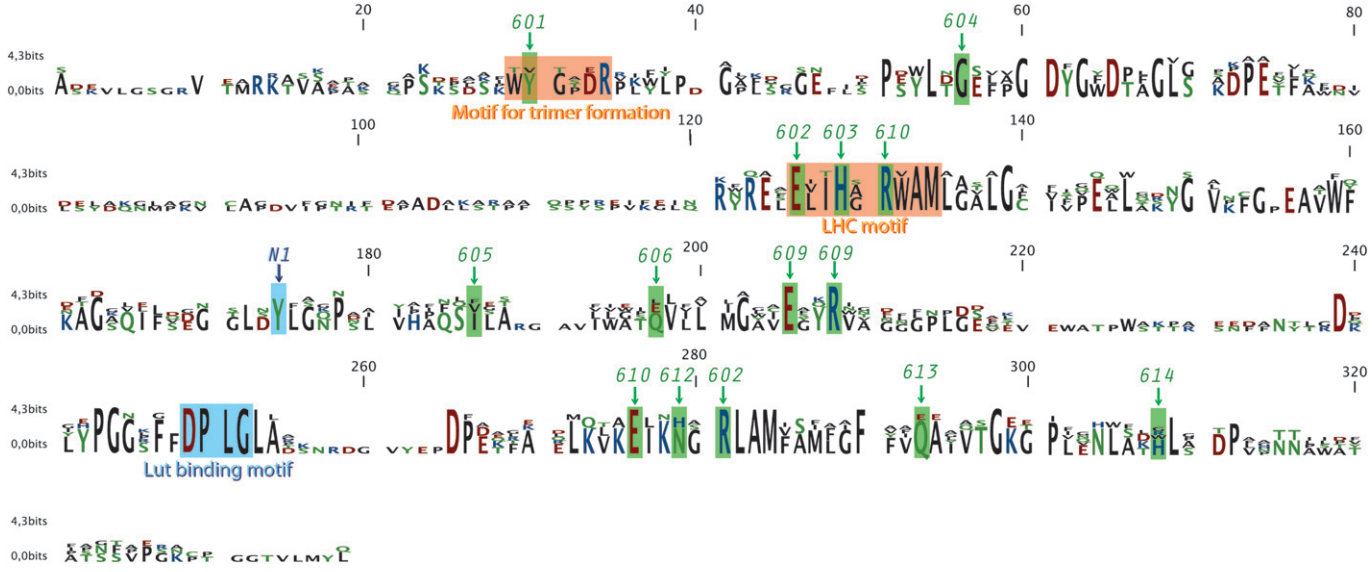

Fig. 4. Sequence logos of several major light-harvesting proteins. The sequences from *A. t.* (Lhcb1; Lhcb4; Lhcb5; Lhcb6), *C. r.* (Lhcbm1) and *P. p.* (Lhcbm1; LhcSR) were analyzed to obtain a consensus protein. Some conserved motifs are represented: that for trimer formation, LHC motif and Lutein-binding motif. A green arrow marks amino acid residues that bind chlorophylls. Only three chlorophylls are not represented here: Chl *b* 608, Chl *b* 607 and Chl *b* 611, respectively coordinated by water 302, water 308 and PG. The size of character are correlated with the conservation of the residue in the different Lhc subunit: the higher the character, the more the residue is conserved.

most surprising feature revealed by Lhcb4 crystal structure was the presence of 13 chlorophyll binding sites, which only in part coincide with those previously identified in LHCII subunits. In particular, Chl 605 and Chl 601 of LHCII are absent in Lhcb4, while Chl 615 has no counterpart in LHCII. The Chl 615 ligand was reported to be glyceraldehyde-3-phosphate (G3P), which bridges this chlorophyll to Chl 612 and Chl 611. It should be noted, however, that the binding of G3P to Lhcb4 has not yet been confirmed by chemical methods and thus this assignment should be considered as tentative. Further work is needed in order to verify that the atomic density attributed to G3P could be in fact assigned to phosphatidylglycerol (PG), a lipid molecule also found as a component of LHCII. Additional striking differences between LHCII and Lhcb4 were the absence of the peripheral V1 site in the latter and the localization of the xanthophyll cycle pigment violaxanthin in the inner site L2, which is instead occupied by lutein in LHCII. This finding confirms previous biochemical work [87–89] and supports the view that L2 is an allosteric site controlling the transition of this protein subunit between two conformational sites, as a basis for regulation of light harvesting efficiency vs energy dissipating conformations [90,91]. In the following sections we will integrate this structural information about pigment binding sites with biochemical data on native and recombinant Lhcb antenna proteins.

3.2. Organization of Lhcb proteins within PSII supercomplexes

The organization of pigment–protein subunits into PSII supercomplexes is summarized in Fig. 2. The antenna moiety of PSII is composed by two types of pigment-binding proteins, differing for their aggregation state. Trimeric LHCII proteins are built by Lhcb1–3 gene products, while monomeric Lhcb proteins consist of Lhcb4, Lhcb5 and Lhcb6 [34]. Three populations of LHCII trimeric particles were identified from their location within the PSII supercomplexes, by electron microscopy analysis upon mild solubilization of PSII membranes [92]. One of these trimeric isoforms, the S-trimer, is *strongly* bound to the complex and is associated with Lhcb5 which, in turn, is connected to the PSII core subunit CP43 [21,93]. Furthermore, many PSII particles were detected harboring an additional LHCII trimer which was named as the *moderately bound* LHCII (M-trimer), since it is more easily released from PSII than LHCII-S. LHCII-M is associated to Lhcb4 and Lhcb6 [38,93], while Lhcb4 is in turn connected to the core complex subunit CP47. Although with lower abundance, larger PSII complexes were observed and shown to bind a third, very *loosely* bound trimer (L-trimer) [94]. Recently, a reliable protocol for homogeneous preparations of the various types of PSII–LHCII supercomplexes was published [95].

4. Functions

4.1. Properties of isolated Lhcb proteins

In the last 30 years, several attempts were made to analyze *in vitro* the properties of the different Lhc proteins, in order to directly elucidate both structural and functional properties of each gene product, allowing for efficient light harvesting and/or thermal dissipation of excitation energy. Early analysis were performed on native LHCII trimers from higher plants, that can be readily isolated from thylakoid membranes [96]. These samples were indeed used for several experiments, such as determination of 3D structure [79,81], evaluation of excitation energy pathways [97–101] and investigation of photoprotection mechanisms [7,90,102–108]. In the case of monomeric complexes, as minor complexes Lhcb4–6, far less information is available about native complexes [88,108–114] since similar physical–chemical features and molecular mass of these proteins make their isolation problematic. Nevertheless, native Lhcb4 protein was isolated from spinach and used for crystallization, obtaining a three-dimensional structure with 2.8 Å resolution as described in the previous section [85]. Purification of native monomeric complexes in many cases requires an isoelectrofocusing (IEF) step [38], in order to separate the different Lhcb complexes on the basis of their isoelectric points. This procedure may yield into removal of pigments bound to peripheral sites of the complexes, such as site V1 of LHCII [115]. An alternative strategy for obtaining pigment–protein complexes in pure form, even when their abundance in plant tissues is very low, is *in vitro* reconstitution of recombinant apoproteins expressed in bacteria. Indeed, Lhc apoproteins have the property of spontaneously folding *in vitro* upon addition of chlorophylls and carotenoids [116–118]. As for Lhcb proteins, LHCII, Lhcb5, Lhcb4 and Lhcb6 were overexpressed and reconstituted *in vitro* in the presence of pigments, easily obtaining

holoproteins from Arabidopsis [83,109,119,120], tobacco [89], barley [121] and maize [118,122–124]. These proteins were reported to be indistinguishable from their native forms purified from plants [122]. The availability of recombinant proteins opened the possibility for detailed investigation of the function of these proteins by site-specific mutagenesis. In the following section, the results of the *in vitro* functional analysis on recombinant and native proteins are reported.

4.2. Pigment binding sites: affinity and occupancy in the different Lhcb proteins

Early analysis of native proteins, isolated by IEF from maize, reported that the composition and stoichiometry of pigments are different depending on antenna proteins. Based on Chl/protein ratios, Lhcb6, Lhcb5, Lhcb4 and LHCII were reported to bind 5, 9, 8 and 13 chlorophylls respectively, and 2 xanthophylls per polypeptide [110]. While the figure of Chl content per LHCII holocomplex proved to be very close to the final figure yielded by X-ray crystallography, this was not the case for monomeric Lhcbs, for which the development of milder isolation procedures yielded higher values for Chl/polypeptide ratios. It implies that while a fraction of pigments is strongly bound to specific aminoacid residues, others are more loosely bound and can be removed during purification procedures. The most extreme case is that of Lhcb6, in which the initial value of 5 Chls per polypeptide determined on the protein purified from corn [110] was corrected to 10 Chls when the recombinant protein was analyzed [122]. Similarly, in the case of Lhcb4, early analysis on the native protein suggested 8 Chls and 2 xanthophylls per polypeptide, with one site (L1) occupied by lutein and a second site having mixed occupancy by violaxanthin and neoxanthin [118]. Later, neoxanthin and violaxanthin were shown to have each its own binding site [83,125], bringing the pigment complement to an upper limit of 3 xanthophylls and 12 Chls, very similar to the figure revealed by X-ray crystallography [85]. It is likely that Lhcb5 also has a similar pigment complement as Lhcb4, since these two pigment–protein complexes are very similar to each other. In general, purified Lhcb pigment–protein complexes, when analyzed by biochemical methods, yield a lower Chl/Car figure with respect to that obtained by X-ray crystallography. This is likely due to the fact that electronic densities of chromophores in each of multiple sites can be detected even when a subset of them can be occupied only in a fraction of protein molecules within the complex. The most clear example is LHCII, in which 1–2 Chls and the violaxanthin bound in site V1, all located in peripheral sites, are lost during isoelectric-focusing purification steps [115]. A similar consideration can be made in the case of the occupancy of a Chl binding site by Chl *a*, Chl *b* or both. In Lhcb4 and LHCII, extra-chlorophylls lost in highly purified proteins are all located at the periphery of the complex and are not directly coordinated by protein residues but by lipids, water molecules, non-covalently bound glyceraldehyde-3-phosphate or other chlorophylls, suggesting that these peripheral chlorophylls may be more susceptible to detergent treatment.

Several chlorophyll binding sites, that were reported to be conserved by sequence analysis on Lhcb proteins, are summarized in Table 2. These sites are accessible to site-directed mutagenesis of the ligand residues to non-ligand variants, thus allowing to study the properties of these conserved chlorophyll and xanthophyll ligands by differential analysis [87,119,120,124,126]. A comparison of the pigment properties in the homologous binding sites of different Lhc complexes allowed identification of at least three different domains, that are conserved through the Lhc family members analyzed in detail so far (Table 3):

1) Site Chl 612 accommodates a Chl *a* with a red-shifted absorption at 680–682 nm. In Lhcb complexes, this Chl represents the lowest energy state of the complex [87,119,120,124,126].
2) Sites 602 and 603 also accommodate Chl *a* in all Lhcb complexes (although limited affinity for Chl *b* in the case of Lhcb5 cannot be excluded). Absorption peak of Chl 603 is conserved at 675 nm, while Chl 602 shows an absorption range between 674 (Lhcb1) and 678 (Lhcb5) [87,119,120,124].
3) Chl 606 has affinity for Chl *b*. In Lhcb1, Lhcb5 and Lhcb6 it binds Chl *b* only, while in Lhcb4 Chl *a* and Chl *b* binds with 40% and 60% affinity, respectively [87,119,120,124,126].

The properties of pigments in the other binding sites are less conserved. Most Chls in the C-helix domain of LHCII are involved in a H-bonds network [79]. The presence of Gln in Chl 606 binding site enhances affinity for Chl *b,* while Glu allows for binding both Chl *a* and Chl *b* as demonstrated by site-specific mutagenesis [124]. Indeed Gln forms H-bond with formyl groups of Chls *b* bound to sites Chl 609 and Chl 607, thus stabilizing the binding of Chls *b* in this region. Chl 609, involved in the H-bond network in Helix C, is a Chl *b* in Lhcb1 and Lhcb6 (Gln in Chl 606 binding site), while it is a mixed occupancy site in all other complexes. Chl *b* is also bound to Chl 613 and Chl 614

Table 3
Occupancy of chlorophyll binding sites and absorption of individual chlorophylls in Lhcb proteins.

Pigment binding site	Lhcb1		Lhcb4 (CP29)		Lhcb5 (CP26)		Lhcb6 (CP24)	
	Chl	Abs. maxima	Chl	Abs. maxima	Chl	Abs. maxima	Chl	Abs. maxima
Chl *a* 610	Chl *a*	679	Chl *a*	669 (*)	Chl *a* (*)	*	Chl *a*	680/670
Chl *a* 612	Chl *a*	681	Chl *a*	680	Chl *a*	682	Chl *a*	680/670
Chl *a* 613	Chl *a*/b (50/50)	662/650	Chl *a*/*b* (70/30)	668/638	Chl *a*/*b* (75/25)	679/655		
Chl *a* 602	Chl *a*	674	Chl *a*	676	Chl *a*	678	Chl *a*	670
Chl *a* 603	Chl *a*	675	Chl *a*	675	Chl *a*	675	Chl *a*	670
Chl *a* 604	Chl *a*/b (50/50)	678/652					Chl *b*	*
Chl *b* 607	Chl *b*	652					Chl *b*	*
Chl *b* 608	Chl *a*	679					Chl *b*	*
Chl *a* 611	Chl *b*	646/660			Chl *b* (*)	*	Chl *a*	680/670
Chl *a* 614	Chl *a*/b (50/50)	665/650	Chl *a*/*b* (30/70)	679/639	Chl *a*/*b* (85/15)	665/655		
Chl *b* 609	Chl *b*	652	Chl *a*/*b* (60/40)	678/650	Chl *a*/*b* (60/40)	679/653	Chl *b*	*
Chl *b* 606	Chl *b*	652	Chl *a*/*b* (40/60)	678/652	Chl *b*	638	Chl *b*	637,5
Chl *b* 601								
Chl *b* 605								
Car L1	L	489	L	494,5	L	491	L	496
Car L2	L/V	495/492	V/N	492/?	L/V/N	494/497/*	V	499
Car N1	N	486	N	486	N	487		
Car V1	V	484,8						

The nature of the chlorophylls in the different chlorophyll binding sites and their absorption (Abs.) maxima in Q_y are shown in Lhcb1 (Croce et al. [125]; Caffari et al. [115]; Remelli et al. [124]), Lhcb4 (Bassi et al. [87]; Gastaldelli et al. [131]), Lhcb5 (Ballottari et al. [119]) and Lhcb6 (Passarini et al. [120]; Marin et al. [137]). *Absence or uncertain information about chlorophyll nature and absorption maxima.

located in the Helices A and D, respectively: these two sites have mixed occupancy in Lhcb1, Lhcb4 and Lhcb5 and are not conserved in Lhcb6 [120].

Chl 610 is coordinated by an ionic pair (Glu on Helix A and Arg on Helix B), which is crucial for protein stability, as experimentally proven in Lhcb1, Lhcb4 and Lhcb5. This is not the case for Lhcb6, where a complex can be obtained, although with lower efficiency, upon mutation at the Glu/Arg residues forming the ionic pair [120]. This complex lost an absorption form at 670 nm, being an indication supported by analysis of the corresponding mutant in Lhcb. Chl 601 and Chl 605 are coordinated by Tyr and Leu residues on the loop between Helix E and Helix C, and are close to the N terminus of Lhcb1: these residues are not conserved in other Lhcb complexes, and thus are thought not to be present in monomeric Lhcb complexes. The situation of Chl 604, Chl 607, Chl 608, and Chl 611, that are not directly coordinated by aminoacid residues in LHCII and Lhcb4, is not clear in Lhcb5 and Lhcb6. Excitons that characterize the excitation energy landscape in Lhcb proteins were solved in the case of LHCII by extensive modeling and combination of different spectroscopic data [99]. More recently, the 14 lowest electronic energy levels were directly measured by 2D spectroscopy analysis [97]. The two methods yielded quite similar results as reported in Table 4.

Xanthophyll chromophores are more difficult to investigate. Four types of xanthophyll-binding sites have been reported in plant Lhcb proteins, called respectively L1, L2, N1 and V1, that differs with respect to their capacity of inducing a different extent of red-shift on their ligands. Site L2 induces the strongest absorption red shift, followed by L1, N1 and V1 [7,115,119,127,128].

Xanthophyll binding to Lhc proteins appears to be stabilized through multiple weak interactions, with no single mutation being efficient in causing depletion of a ligand from a specific site. The only partial exception is the mutation in a tyrosine residue in the luminal loop (discussed in section 3.1), which causes a small but specific loss of neoxanthin [83]. This lack of specific ligands is in agreement with the major factors for binding carotenoids into pigment–proteins, being the pairing of aromatic residues with end-rings, a feature also found in carotenoid biosynthesis enzymes [129]. Consistent with the conservation pattern of these residues, Lhcb4 and Lhcb5 bind neoxanthin while Lhcb6 does not [122]. Both occupancy and organization of xanthophyll binding sites in Lhcb proteins have been defined essentially by analysis of recombinant proteins with modified pigment composition, as guided by the early low resolution model of LHCII [78] that showed only two carotenoid molecules cross-bracing transmembrane helices A and B and indicated as sites L1 and L2. While site L1 was later confirmed to host lutein in all Lhc proteins, site L2 showed a different nature in several aspects: first, its occupancy is different depending on protein, i.e. lutein in Lhcb1–3 and violaxanthin in Lhcb4–6; second, violaxanthin can be substituted by zeaxanthin upon its accumulation in high light conditions in Lhcb4–6 proteins [88,89,128]. This L2 site has allosteric nature, and binding of zeaxanthin induces a conformational change to a shorter fluorescence lifetime state [90]. Peripheral sites other than L1 and L2 have been identified in LHCII, binding respectively neoxanthin (site N1) and violaxanthin (site V1) [79,115,125]. Early work suggested that neoxanthin in Lhcb4 and Lhcb5 shared with violaxanthin the site L2; however, the finding that neoxanthin has its own binding site [83,85] rather suggests distinct binding sites in all members of the family. Site V1 is only present in LHCII [79,85], its occupancy does not induce conformational changes in the protein [115].

Table 4
Exciton energies determined from the 2D electronic spectroscopy experiment compared with those calculated from previous models.

Exciton	Experimental energy (cm^{-1})[a]	Theoretical energy (cm^{-1})[b]
1	14,700	14,699
2	14,770	14,751
3	14,810	14,804
4	14,880	14,858
5	14,910	14,918
6	14,990	14,952
7	15,030	14,992
8	15,130	15,022
9	15,210	15,210
10	15,290	15,306
11	15,360	15,363
12	15,430	15,416
13	15,480	15,456
14	15,510	15,512

[a] From Calhoun et al. [97].
[b] From Novoderezhkin et al. [99].

4.3. Light-harvesting and energy transfer properties of Lhc proteins

Chlorophylls bound to Lhcb proteins absorb light energy and transfer it to the PSII reaction center. Several experiments were performed in order to establish excitation energy pathways between the different chlorophylls bound to Lhcb proteins and between the different antenna protein subunits to the core complex. Excitation energy transfer efficiencies have been estimated by comparing 1-T absorption and fluorescence excitation spectra [127] in the Soret region: these measurements allowed analysis of the fraction of blue light excitation that effectively reaches the lowest energy levels of Chl *a*, yielding values of energy transfer efficiencies of almost 90% for Chl *b* to Chl *a* and 60%–90% for Car to Chl *a* in the different Lhcb [119,128,130,131]. The wide range of Car to Chl transfer efficiency, in particular, is due to the modulating effect of xanthophyll composition, with the lowest efficiency observed in the presence of zeaxanthin. These results demonstrated that Lhcb pigment–protein configuration allows efficient transfer of absorbed energy to the lowest excited state energy level; nevertheless, this efficiency can be modulated by xanthophyll composition through conformational change, likely favoring energy dissipation in stress conditions [132].

Time-resolved transient absorption analysis also enabled to determine kinetics of Car to Chl energy transfer, revealing that carotenoids mainly transfer energy to Chl via S2 carotenoid excited states to Bx and Qx states of Chl *b* and Chl *a* respectively, in less than 100 fs [133]. A small part of the excitation energy may also be transferred from Car to Chl directly from the Car S1 excited state, even if with slower lifetimes (~1 ps) [133,134].

Intramolecular excitation energy pathways were modeled for LHCII, Lhcb4, Lhcb5 and Lhcb6 on the basis of pump–probe spectroscopy in the fs time range [99,135–138] and two-dimensional electronic spectroscopy [100]. In each case, the overall excitation energy transfer was reported to occur first among chlorophylls clustered in different layers within Lhcb proteins, and then from clusters located at the lumenal side to clusters on the stromal side of the membrane. The lowest excited energy states were indeed reported in chromophores located at the stromal side of Lhcb proteins, namely the Chl 610–611–612 cluster: here, excitation energy is focussed and transferred to other antenna proteins or to the reaction center complexes. In LHCII trimers, excitation energy transfer within the Chl 610–611–612 chlorophyll cluster was reported to be characterized by a Chl *a*–Chl *a* energy transfer step, occurring in less than 100 fs [99,100]. A similar timescale was also associated to energy transfer among dimers of strongly coupled chlorophylls, such as Chl 602 and 603, and among Chl 613 and Chl 614, even if in these case slower kinetic components (hundreds of fs) were also reported [99]. Energy transfer from Chl *b* to Chl *b* is completed in less than 1 ps. Chl *b* to Chl *a* energy transfer occurs in less than 100 fs due to several components, but mainly following two parallel pathways: i) at the stromal side, Chl *b* 609 and Chl *a* 603 are strongly coupled, allowing for energy transfer in less than 100 fs; ii) at the luminal side, the best coupling is observed in Chl *b* 605–Chl *a* 604 dimer, resulting in Chl *b* to Chl *a* energy transfer in less than 100 fs, while Chl *a* 604 can also

accept energy from other Chl *b* ligands at the luminal side within a few ps. Chl *a* 604, however, produces a bottleneck for excitation energy from Chl *b*, since energy transfer from this chlorophyll to the lowest excitons occurs in 12–20 ps [99,100]. Excitation energy transfer analysis on Lhcb5 and Lhcb6 yielded similar results as compared to LHCII; however, a single sub-picosecond component was associated to Chl *b* to Chl *a* energy transfer, due to the putative absence of Chl 604, thus altering the excitation energy pathway from Chl *b* to Chl *a* reported in LHCII [137]. In the case of Lhcb6, a peculiar role of Chl 603–Chl 602 dimer is proposed in connecting the Chl *b* cluster in the C helix and the low energy trimer Chl 610–611–612, located in the stromal chromophore layer [137].

Recently, information became available on the excitation energy transfer from different complexes composing the antenna system of PSII to the core complex: LHCII trimers, in particular, were reported to be oriented in the PSII supercomplex for optimal energy transfer to CP43, with Chl 612 in LHCII being only 17 Å apart from Chl 11 in CP43. Depending on its placement within the PSII supercomplex, LHCII transfers to other monomeric antenna complexes [139,180,181]. Energy transfer to Chls located at the stromal side of Lhcbs enable these proteins to easily transfer their excitation energy to other neighbor subunits [99,140].

4.4. Lhcb antenna proteins and photoprotection

Lhcb functional properties are strongly modulated by bound xanthophylls. In particular, carotenoid composition was associated to photoprotection properties of thylakoid membranes, the most effective sites providing photoprotection was reported to be Lhc proteins [11]. Saturation of electron transport chain indeed should be avoided by photosynthetic organisms in order to prevent ROS formation in the antenna system. Xanthophyll biosynthesis mutants lacking beta-xanthophylls have been shown to be extremely photosensitive [13] and produce singlet oxygen even at low light intensity. Xanthophylls bound to Lhcb proteins are indeed crucial for preventing ROS formation by triplet chlorophyll excited state quenching [7,10,128] and finally by scavenging of ROS produced. The systematic analysis of photoprotection in xanthophyll biosynthesis mutants has shown that photoprotection activity of all xanthophylls, including zeaxanthin, is enhanced by binding to Lhc proteins [11,141]. The observation that lutein-only plants are highly sensitive to light and accumulate $^1O_2$, despite lutein being the best $^3Chl^*$ quencher among plant xanthophylls [142], implies that ROS production is an active process even in low light conditions, and scavenging within Lhc proteins is indispensable to normal operation of photosynthesis. Neoxanthin appears to be selective for scavenging superoxide [143], while violaxanthin and zeaxanthin appears to be specific for $^1O_2$, although with the latter exhibiting enhanced activity.

4.4.1. Chlorophyll singlet excited states quenching

Xanthophylls prevent over-reduction of the photosynthetic apparatus by quenching singlet chlorophyll excited states, in order to reduce the possibility of energy transfer to oxygen. Singlet chlorophyll excited states are quenched when bound to protein, and the intensity of quenching depends on protein conformation and the composition of bound xanthophylls complement. Beside this intrinsic quenching property, Lhc proteins may be induced to dissipate as heat a variable portion of excitation energy absorbed. Indeed, the fluorescence decay of Lhcb proteins is characterized by multiple components, with lifetimes distributed in a wide range from 4 ns, which is the only component found in trimeric LHCII, to less than 100 ps found in LhcSR proteins [59,90]. Binding of zeaxanthin generally increases the amplitude of fast lifetime components.

The molecular mechanisms by which Lhc proteins quench part of light energy absorbed is still under debate, with different theories based on several experimental evidence. Quenching of energy in excess *in vivo* (NPQ mechanism) has two major components: qE, activated within seconds from excess light exposure, its triggering needs membrane energization by a trans-membrane pH gradient; qI, that has a slow onset and needs pH gradient for its build up, but is then maintained for 1–2 h even in the presence of uncouplers. The qI component depends on the activity of violaxanthin de-epoxidase, activated by lumen acidification in excess light, which leads to accumulation of zeaxanthin. Once zeaxanthin accumulates, qI becomes insensitive to uncouplers, implying that zeaxanthin itself is sufficient for establishing quenching [109,144–146]. The fast quenching component qE is dependent on pH transmembrane gradient for its induction, and relaxes very rapidly upon treatment with uncouplers or in the dark.

At present, it is not clear whether or not the qI and qE forms of quenching rely on the same fundamental mechanism(s) or not. In the following we will thus discuss each of them separately. Early studies associated qI to PSII photodamage. However, the slowly relaxing quenching component was observed to occur *in vivo* in the absence of photoinhibition and relaxes together with zeaxanthin epoxidation in the presence of D1 synthesis inhibitors; therefore, it appears to depend on zeaxanthin accumulation into Lhcb proteins, that was shown to be effective in decreasing their fluorescence lifetime both in leaves and membranes [109]. The long-lived component of NPQ, dependent from zeaxanthin accumulation, was distinguished from photoinhibitory quenching and renamed as qZ [144,145]. These results are consistent with *in vitro* studies showing decrease of Chl singlet excited states upon binding of zeaxanthin to Lhcb proteins [109,147]. Early evidence claimed that zeaxanthin had a S1 excited state energy level lower than violaxanthin, and could thus preferentially accept energy from singlet Chl excited state S1 [148]; this model was challenged by the measurement of similar S1 excited state energies for lutein, violaxanthin and zeaxanthin bound to Lhcb proteins [149]. Recent results have shown that zeaxanthin binding to LHC proteins is not sufficient for quenching: binding of zeaxanthin to site V1 of LHCII trimers does not change Chl fluorescence yield of the complexes [115], as well as its binding to the LHCSR protein from *Chlamydomonas reinhardtii*[59]. Quenching is instead observed upon zeaxanthin binding to site L2, where it can induce a conformational change to a dissipative form of the protein [109,119,128,146,150,151]. Lutein is also active in quenching when bound to site L2, although to a lower extent [119,128,152]. The case of site V1 in LHCII trimers can be explained based on the observation that this site is located at the periphery of the monomers, far away from Chl *a* binding sites, in which excitation is concentrated due to their lower excited state energies. In the case of LHCSR3, however, the complex binds mostly Chl *a*, making it unlike zeaxanthin being in contact with Chl *b* only. It thus appears that zeaxanthin-dependent quenching requires a specific geometry of interaction with Chl *a*, that can be obtained through a conformational change, specifically on site L2. Mutational analysis on chlorophyll binding sites suggests that Chl 603, the closest to site L2, is involved: its removal causes increase in fluorescence lifetime [119,153], while lutein and zeaxanthin binding to this site shifts protein to a dissipative conformation.

As for the qE quenching, its triggering in plants depends on the Lhc-like protein PsbS, activated by lumen acidification through protonation of two lumen-exposed glutamate residues [72,73,154,155]. Since PsbS does not bind pigments [71], interaction of PsbS with Lhcb proteins was proposed to be needed for activation of quenching sites in PSII antennas through conformational change. Aggregation in low detergent/low pH, in the absence of PsbS, was to mimic such a conformational change *in vitro*[102], while a number of spectroscopic features similar to those typical of aggregation was taken as evidence that aggregation occurs *in vivo* as well [103,106]. The actual quenching mechanism responsible for thermal dissipation upon aggregation was suggested to consist of energy transfer to the S1 excited state of lutein bound to site L1 [103] or formation of a charge

transfer state between two neighbor chlorophylls [156]. The 'aggregation-dependent' quenching was observed in all Lhcb proteins, although only monomeric complex exhibited a zeaxanthin-dependent enhancement effect [113,147]. A distinct mechanism for qE induction was proposed to rely on the formation of a 'Charge Transfer quenching', implying a charge separation with electron transfer from a xanthophyll, zeaxanthin or lutein, to a pair of interacting chlorophyll *a* molecules. The charge recombination at the ground state would result into heat dissipation [157]. The carotenoid radical cation has absorption in the near IR (800–100 nm, depending on species) and a transient NIR signal with ps lifetime was detected in quenched thylakoids and in monomeric antennae Lhcb4–6 upon binding of zeaxanthin, but not in LHCII trimers [91,104,152,157,158]. Site-specific mutagenesis on Lhcb4 showed that charge separation occurs between zeaxanthin bound to the L2 site and the Chl 603–Chl 609 (A5–B5) dimer [91,104]. Also, genetic evidence that zeaxanthin is not essential for NPQ activation, although greatly enhancing its amplitude, was provided both *in vitro* in Lhcb5 and *in vivo* by showing that over-accumulation of lutein partially complemented the *npq1* mutation. [152,158]. Further evidence for the role of lutein radical cation in quenching was provided by the analysis of LhcSR3 from *C. r.*, a gene product essential for qE in algae where PsbS is not accumulated [59,61]. This protein shows high levels of lutein radical cation and is constitutively quenched, irrespective of whether it binds zeaxanthin or not. Instead, its lifetime is further decreased by acidification. In order to account for variable level of quenching, disconnection of LhcSR3 from the PSII pigment bed was hypothesized [59]. The finding described above thus suggests that, in spite of differences on protein quality, the molecular mechanism for qE in higher plants and green algae might be the same. Upon triggering of quenching processes, an increased level of the population of S1 excited states from singlet chlorophylls (^{1}Chl*) was observed by selective two-photon excitation of carotenoid dark states [105], suggesting an increased level of interaction between these chromophores. Yet, carotenoid ^{1}S* state can be addressed to both thermal relaxation to ground state or radical cation formation. Moreover, the possibility that the complementary quenching mechanism might develop synergistically in different components of the PSII antenna system cannot be excluded, in light of the multiple quenching events detected *in vivo*[144] and on the formation of multiple protein domains in the grana partitions [74].

4.4.2. Triplet excited states quenching

Accumulation of chlorophyll triplet states (^{3}Chl*) by intersystem crossing from ^{1}Chl* may occur in case of insufficient photochemical quenching. Chlorophyll triplet excited states are quenched in few μs by energy transfer to carotenoid, yielding carotenoid triplet excited states which have short lifetimes and cannot transfer energy to oxygen. Carotenoids are very efficient in chlorophyll triplet quenching, reaching 95% efficiency in LHCII trimers [7]. Efficiency of triplet quenching is different depending on xanthophyll species bound and protein sequence, with a minimum level of 60% in Lhcb6 when lutein is bound to both L1 and L2 sites [128]. It appears that xanthophylls bound to both L1 and L2 sites are active in ^{3}Chl* quenching, but this is not the case for the peripheral N1 or V1 sites. Clearly, the efficiency of the reaction is higher in LHCII trimers with respect to Lhcb monomers. Chlorophyll triplet quenching by xanthophylls occurs upon electron exchange Dexter-type mechanism [159], requiring tight van der Waals interaction between chromophores. Since trimers have a much lower dissociation constant for xanthophylls bound to the L2 site with respect to monomers, it is likely that the formers have a more tight Chl–xanthophyll interaction within these sites, allowing for ^{3}Chl* quenching. Monomers undergo exchange of violaxanthin in L2 with zeaxanthin [88,89] suggesting less tight interaction between chromophores. Within site L2, violaxanthin is less efficient than lutein in triplet quenching within Lhcb6 [128,142], while zeaxanthin is more efficient [128]. Although the dataset is incomplete and more Lhc proteins should be analyzed in detail, it is tempting to suggest that the capacity for xanthophylls exchange, although useful in singlet state quenching, implies a trade-off between the capacity of regulating ^{1}Chl* *vs* the efficiency of ^{3}Chl* quenching.

4.5. Reverse genetic of Lhcb proteins for in-vivo function

Despite overall similarity, Lhc proteins bear specific functional properties, as revealed by sequence alignments and mutagenesis analysis [34]. Reverse genetic has been used in order to investigate the properties of specific gene products. In particular, several mutations on *lhcb* genes are available in *A. t.* and *C. r.* In *A. t.*, insertional mutants are available on genes encoding the monomeric complexes Lhcb4 [74], Lhcb5 [109] and Lhcb6 [160], while among genes encoding components of the major LHCII, a knock-out (KO) mutation has been reported for the Lhcb3 gene [161]. Lhcb1 and 2 proteins are encoded by multiple genes, that have not been individually studied but have been collectively down-regulated [162]. Finally, KO mutants for the Lhc-like genes *psbS* and *ELIPS* have been reported [48,163]. In *C. r.*, insertional mutants are available on the *lhcbm1* gene, encoding a highly expressed subunit of the major LHCII complex, and on the *lhcSR* gene, coding for a LHC-like protein essential for qE [61,164]. Genotypes down-regulated by RNA antisense techniques are available for *lhcb1 + 2*, *lhcb4* and *lhcb5* in Arabidopsis [162,165,166] and *lhcb4* and *lhcb5* in *Chlamydomonas reinhardtii*[167]. The most striking observation from the functional analysis of these genotypes is that light harvesting function is not severely impaired by the depletion of any of the components of the PSII antenna proteins: the system reveals robustness, indeed it compensates the absence of a gene product by the over-accumulation of others homologous members without substantial decrease in antenna size [166]. Similar consideration applies for photoprotection in high light conditions, since in most cases Lhcb mutants do not exhibit strong sensitivity to excess light. Overall, these results suggest that photoprotection, light harvesting and excitation energy transfer between Lhc components and to PSII RC are conserved properties of the LHC subfamily. Verification is obtained from the analysis of the *ch1* mutant of Arabidopsis, strongly depleted in all Lhc proteins, which grows very slowly due to small antenna size and is over-sensitive to excess light due to ROS accumulation [11,168,169]. The above general statement needs some qualification: depletion in several Lhcb components, such as Lhcb4 and Lhcb6, causes an increase of F_0 and a decrease in PSII quantum yield. TEM analysis of grana domains showed that, upon depletion in Lhcb6 and Lhcb4, PSII core complexes are less homogeneously distributed in the membrane, implying longer pathways for excitation energy migration to the reaction center [170]. Although koLhcb have a similar photosynthetic rate as compared to WT, far more specific results can be detected by analyzing specific regulatory mechanisms in which Lhcbs are involved. State 1–state 2 transitions consist into the migration of PSII antenna proteins to PSI upon over-reduction of plastoquinone (PQ) pool, in order to balance excitation pressure between the two photosystems [171]. Specific kinases, STN7 in *A. t.*[172] and STT7 in *C. r.*[173], act in phosphorylating LHC proteins, a sub-set of which migrates toward PSI in stroma membranes. *C. r.* genotypes depleted in Lhcb4 or Lhcb5 are impaired in state transitions, while this is not the case for the corresponding *A. t.* mutants, suggesting these subunits are essential for this regulative process in green algae but not in higher plants [165,167]. In *A. t.*, the impairment of state transitions in *lhcb1–2* antisense plants confirms the major role of LHCII trimers into this process (Fig. 5). Mutations on either monomeric Lhcbs and Lhcb3, instead, make state transition faster [160,170]. Several koLhcb mutants exhibit effects on the kinetic and amplitude of NPQ, the most dramatic effect being observed in the case of koLhcb6 mutant, which was impaired in growth rate and photoprotection. The absence of Lhcb6 caused disconnection of the outer antenna moiety, made by

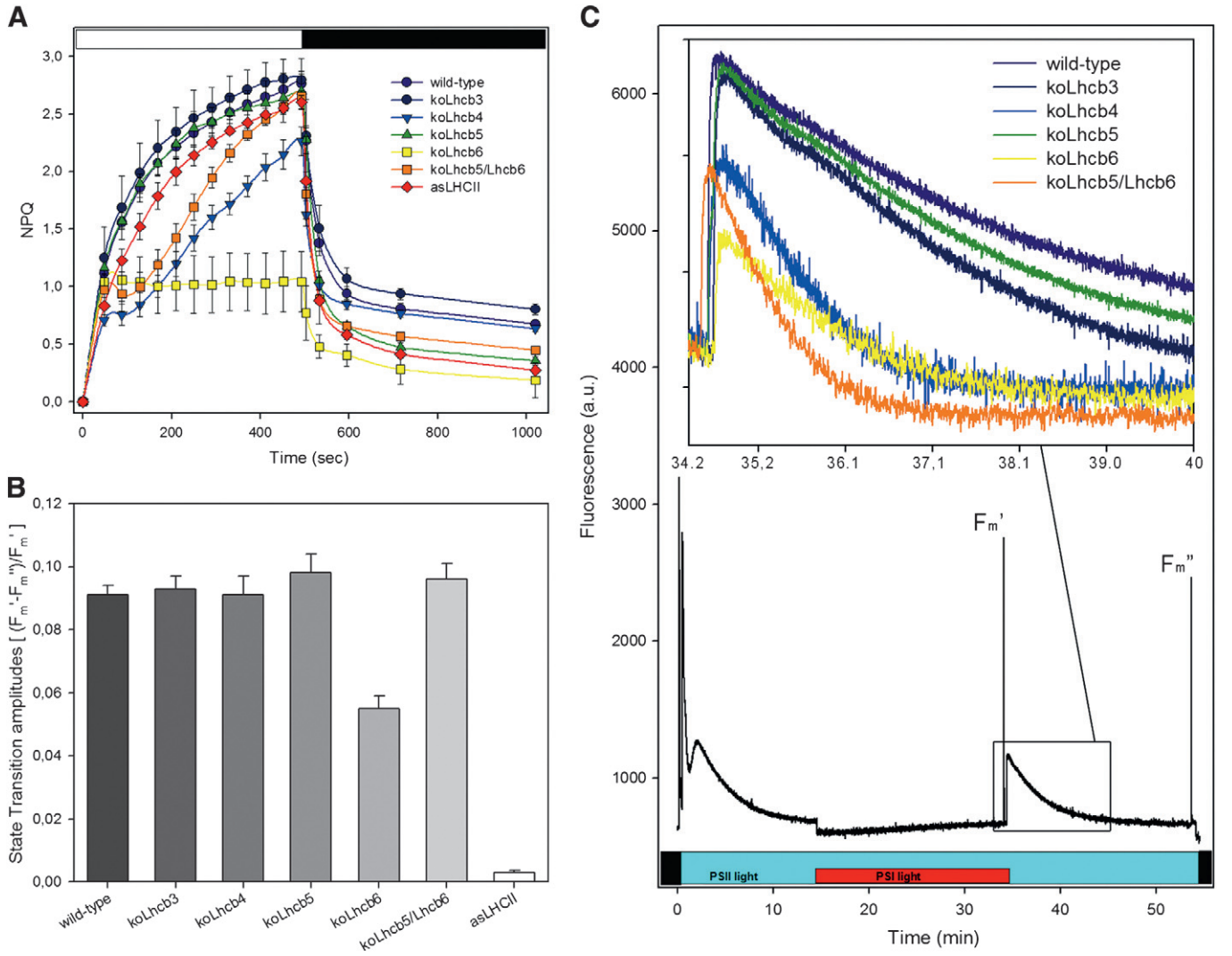

Fig. 5. NPQ and state transition analysis of wild-type and mutant genotypes. A) Kinetics of NPQ induction and relaxation were recorded with a pulse-amplitude modulated fluorometer. Chlorophyll fluorescence was measured in intact, dark-adapted leaves, during 8 min of illumination at 1260 μmol m^{-2} s^{-1} followed by 9 min of dark relaxation. All NPQ values of mutant plants after 530 s (dark recovery), with the exception of koLhcb3, are significantly lower than the corresponding wild-type values (means ± SD, $n = 4$, Student's t test, $P < 0.05$). B) State transitions induction on different insertional and antisense mutants on *lhcb* genes measured as the difference between maximum fluorescence emission of PSII in State I and State II (see point C). C) Fluorescence kinetics measured upon state transition induction in *lhcb* mutants. When illumination of leaves with far-red light is continued, the Chl fluorescence slowly rises until State I is reached: at this point, all LHCII has re-associated with PSII (F_m'). Turning off the far-red light induces a rapid increase in fluorescence, because background PSII-light now excites PSII more than PSI, thus causing Q_A reduction. The fluorescence rise phase is rapidly followed by a decay phase, as the transition to State II is induced and completed (F_m''). Such decay phase is highly informative, its slope reflecting the transition rate from State I to State II. Indeed, when the far-red light is turned off in State I, several mutants (e.g. koLhcb4 and koLhcb5/Lhcb6) showed a faster fluorescence decay, meaning their transition from State I to State II occurs much faster than the wild-type. D) Inset, kinetics of the fluorescence rise and decay phases upon induction of State II on *lhcb* mutants.

LHCII-M and LHCII-L, with the formation of regular arrays of C_2S_2 PSII supercomplexes within grana membranes, while domains enriched in disconnected LHCII trimers are formed at the periphery of grana disks [160]. Arrays slow down PQ diffusion rate and electron transport to cytochrome b_6f, resulting in lower transthylakoid ΔpH formation and consequent reduction of photosynthetic efficiency. Since transthylakoid ΔpH formation upon light exposure is moreover the feedback signal for activation of NPQ, koLhcb6 mutant presents a reduced rate and amplitude of quenching [160,170]. The absence of Lhcb6 protein in this mutant results in reduction of the qE component (Fig. 5) suggesting that, besides controlling PQ diffusion, Lhcb6 has a direct role in quenching as supported by the longer fluorescence lifetime of koLhcb6 mutant [174]. Similar effect, although less pronounced, was also reported in the case of Lhcb4 insertional mutant [74,174]. These phenotypes are consistent with the capacity of monomeric Lhcs of forming carotenoid radical cations *in vitro*[104] and of being a better quencher than LHCII trimers upon aggregation *in vitro*[113,147]. Antisense [165] or insertional [109] mutants on *lhcb5* gene have a distinct phenotype, compared to WT, only in the slow qI component of NPQ. The missing effect on qE can be understood in the framework of a dynamic model for the re-organization of the grana membranes during triggering of NPQ: in the dark, Lhcb6 is part of a pentameric complex, called B4C, together with Lhcb4 and the LHCII-M trimer [74,175]. Under excess light conditions, PsbS dissociates B4C [74]: the Lhcb4 moiety of B4C stays with PSII core, Lhcb5 and LHCII-S form C_2S_2 rich domains, while Lhcb6 forms a second domain with LHCII-M and LHCII-L. Therefore, two complexes active in charge-transfer quenching [91,104] and carrying pH sensitive residues [108,176,177] remain located within the C_2S_2 complex, thus allowing quenching to occur even in the presence of one active quenching center only. In this context, it is worth noting that multiple mutants such as koLhcb4/Lhcb6 and koLhcb5/koLhcb6, although strongly depleted in complexes active in charge-transfer quenching, still develop a substantial level of NPQ; this suggests that LHCII, which is maintained in these mutants, might have a role in quenching although through a different mechanism, in agreement with previous reports

suggesting cooperative protein–protein interactions of LHCII trimers in the membrane leading to conformational changes usually referred as 'aggregation' [102,103,147]. It's interesting to note that the reorganization of protein domains in grana partitions during NPQ started by dissociation of the B4C complex leads to formation of a LHCII-rich domain in the grana membrane, in which a different interaction between LHCII trimers and/or Lhcb6 could lead to a quenching event similar to the 'aggregation' of LHC proteins reported *in vitro* in condition of low detergent content, enhanced by co-aggregation of Lhcb6 and LHCII trimers: in any case the dissociation of B4C is a prerequisite for NPQ induction [74,102,147]. The study of the overall NPQ mechanism has been greatly facilitated by the isolation of the *npq4* mutant in *A. t.*[48], lacking PsbS protein and impaired in NPQ induction. This mutant, and the genotypes obtained by complementation with *psbS* gene carrying point mutations [72,73], were indeed essential for demonstrating the role of protonable (DCCD-binding) lumen-exposed residues. A mutant similar to *npq4*, blocked in NPQ induction, was isolated from *C. r.* It was demonstrated to lack the *lhcSR3* gene [61,178]. LhcSR3 is accumulated upon high light stress [65] and the mutant suffers photo-oxidative stress only upon the shift from low to high light, a situation in which thermal dissipation of light energy in excess is crucial in order to prevent photoinhibition of photosynthetic apparatus. Interestingly, mosses have both PsbS and LhcSR proteins expressed, and *P. p.* mutants on either *psbs* or *lhcSR.1*/*lhcSR.2* are both affected in NPQ and their action is additive [62]. Further information on the operation of NPQ in algae and mosses was provided by *npq5*, a mutant lacking Lhcbm1 [164], which presents a strong reduction in NPQ amplitude. Since *npq5* has normal levels of LhcSR3, it appears that Lhcbm1 is a partner for LhcSR during activation of NPQ. As in the case of down-regulation on *lhcb1–2* in Arabidopsis, a similar growth rate was reported in WT and *lhcbm1* mutant. Interestingly, *npq5* is not impaired in state transitions [164], suggesting differential roles of LHCII trimers in plants and algae. Reverse genetics thus revealed the possibility of Lhcb proteins to compensate each other, even if some functional role seems to be specific for some members of this gene family.

5. Conclusions

Light harvesting complexes are unique proteins evolved in eukaryotic organisms, most likely in response to increasing aerobic conditions. Increased oxidative stress forced autotrophic organisms to face new challenges originated by their early choice of porphyrins, which are efficient sensitizers, as light harvesting compounds. Energy transfer from chlorophyll triplet excited states to molecular oxygen progressively increased risk for ROS formation. This condition was further exacerbated in the event of land colonization, which forced photosynthetic organisms to deal with stronger and rapidly fluctuating level of irradiance. The new antenna proteins evolved in these conditions, besides collecting excitation energy with high efficiency like LHI and LHII systems of purple bacteria and phycobilisomes of cyanobacteria, also carry built-in mechanism for excess energy degradation into heat, for triplet Chl quenching and for ROS scavenging. The Lhcb proteins are crucial for plant growth and photoprotection, as demonstrated by the light sensitive phenotype of the mutants *ch1* in Arabidopsis and *chlorina* in barley, lacking antenna proteins due to the absence of Chl *b*, needed for their folding. Unfortunately, our knowledge of PSII antenna proteins relies mainly on the higher plant members of the family, while little information is available from other organisms. The recent elucidation of the biochemical properties of LhcSR3 of *Chlamydomans reinhardtii* was instrumental in showing that the properties of Lhcs from other organisms span over a larger range of activity with respect to the case of higher plants. Indeed, while LhcSR3 presents conserved pigment-binding sites compared to other Lhc proteins, the protein structure is tuned for quenching rather than for transfer to the reaction center. This finding supports early suggestions of Lhc proteins' conformational shift from a 'light harvesting' state to a 'dissipative' state [90,179]. This transition between conformations is likely to be regulated by different factors in different species. Besides those that have been found so far (zeaxanthin/violaxanthin, ΔpH, phosphorylation), others might be found in the future. The improvement of structural data available and increased resolution in transient spectroscopic measurement will help better understanding how these proteins may be used as harvester and quencher of solar energy, possibly in order to repeat such highly sophisticated performances in man-made solar energy harvesting devices.

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