

## Functional analysis of Photosystem I light-harvesting complexes (Lhca) gene products of *Chlamydomonas reinhardtii*

Milena Mozzo<sup>a,b</sup>, Manuela Mantelli<sup>b,c</sup>, Francesca Passarini<sup>b,c</sup>, Stefano Caffarri<sup>b</sup>, Roberta Croce<sup>a,\*</sup>, Roberto Bassi<sup>c</sup>

<sup>a</sup> Department of Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

<sup>b</sup> Univ Aix Marseille, Fac Sci Luminy, Lab Genet & Biophys Plantes, CEA, iBEB, SBVME, CNRS UMR Biologie Végétale et Microbiologie Environnementales, F-13009 Marseille, France

<sup>c</sup> Dipartimento Scientifico e Tecnologico, Facoltà di Scienze MM.FF.NN. Università di Verona, Strada Le Grazie 15, 37134 Verona, Italy

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### ABSTRACT

The outer antenna system of *Chlamydomonas reinhardtii* Photosystem I is composed of nine gene products, but due to difficulty in purification their individual properties are not known. In this work, the functional properties of the nine Lhca antennas of *Chlamydomonas*, have been investigated upon expression of the apoproteins in bacteria and refolding *in vitro* of the pigment–protein complexes. It is shown that all Lhca complexes have a red-shifted fluorescence emission as compared to the antenna complexes of Photosystem II, similar to Lhca from higher plants, but less red-shifted. Three complexes, namely Lhca2, Lhca4 and Lhca9, exhibit emission maxima above 707 nm and all carry an asparagine as ligand for Chl 603. The comparison of the protein sequences and the biochemical/spectroscopic properties of the refolded *Chlamydomonas* complexes with those of the well-characterized *Arabidopsis thaliana* Lhcas shows that all the *Chlamydomonas* complexes have a chromophore organization similar to that of *A. thaliana* antennas, particularly to Lhca2, despite low sequence identity. All the major biochemical and spectroscopic properties of the Lhca complexes have been conserved through the evolution, including those involved in “red forms” absorption. It has been proposed that in *Chlamydomonas* PSI antenna size and polypeptide composition can be modulated *in vivo* depending on growth conditions, at variance as compared to higher plants. Thus, the different properties of the individual Lhca complexes can be functional to adapt the architecture of the PSI–LHCI supercomplex to different environmental conditions.

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*Chlamydomonas reinhardtii* is a unicellular green alga, with two flagella and a single chloroplast used as model organism for the study of photosynthesis and biofuel production [1]. The thylakoid membrane complexes, which catalyse the light phase of photosynthesis, share many properties with those of higher plants. The recent publication of the *C. reinhardtii* genome [2] has shown that algal and higher plants light-harvesting antenna proteins underwent an higher level of differentiation with respect to the components of Photosystems I and II core, cytochrome *b<sub>6</sub>f* and ATPase, probably as a result of adaptation to specific environmental conditions [3]. This makes possible to study the relation between the structure/function of the antenna system in water vs. terrestrial environment. Both PSI and PSII antenna systems present clear differences in *C. reinhardtii* with respect to land plants: *C. reinhardtii* PSII antenna lacks CP24 (Lhcb6)

and Lhcb3, while the PSI antenna is larger [4–9] and contains 9 polypeptides [4] rather than the four found in higher plants [10].

In this work, we focus on Photosystem I, the plastocyanin-ferredoxin oxido-reductase. This supramolecular complex is composed of two moieties: the core complex, responsible for charge separation and electron transport, and the outer antenna system (LHCI), which extends the light harvesting capacity and ensures photoprotection [11,12]. Both PSI and PSII antenna proteins bind Chl *a*, Chl *b* and xanthophylls. Nevertheless, the PSI antenna complexes (Lhca gene products) differ with respect to PSII-associated Lhcb proteins for the presence of stable chlorophyll–chlorophyll interactions yielding absorption bands at wavelength > 700 nm, implying uphill energy transfer to the P700 reaction centre [13]. These “red shifted spectral forms” are typical of PSI and can be associated to either Lhc and/or core complex subunits depending of the taxa, while the energy levels of these transitions can be shifted up to 742 nm [14]. It has been suggested that the function of the “red forms” is to increase the absorption cross section of PSI, which is particularly useful under a dense canopy, where the light available is limited by the overwhelming absorption of PSII complexes up to 690 nm [15]; the “red forms” were also shown to be active in photoprotection, allowing to focus excitation

**Abbreviations:** At, *Arabidopsis thaliana*;  $\beta$ -DM, n-dodecyl- $\beta$ -D-maltoside; Car, carotenoid; Chl, chlorophyll; Cr, *Chlamydomonas reinhardtii*; Lhca and LHCI, light harvesting complex of PSI; LHCII, light-harvesting complex II; PSI and PSII, Photosystems I and II

\* Corresponding author. Tel.: +31 503634214; fax: +31 503634800.

E-mail address: [R.Croce@rug.nl](mailto:R.Croce@rug.nl) (R. Croce).

energy into antenna protein domains containing specific carotenoids with high efficiency in Chl triplet quenching [16–18] and in dissipation of singlet energy [19].

Most of the available information on the red forms associated to antenna proteins derives from higher plants, where they are responsible for the fluorescence emission at 735 nm, typical of PSI [20]. Among the four Lhca subunits, Lhca3 and Lhca4 have the highest content of red forms. Nevertheless, all Lhcas exhibit red-shifted emission as compared to Lhcb3 [21,22]. The low temperature fluorescence emission spectrum of *C. reinhardtii* PSI–LHCI supercomplex shows two peaks at 698 nm and 708–715 nm, the relative intensity of which changes depending on the purification procedure [4,5,8,23–26]. This result is confirmed by analysis of *C. reinhardtii* cells, the fluorescence of which peaks at 715 nm at low temperature [27,28]. This clearly shows that the blue shifted emission of PSI–LHCI of *C. reinhardtii* as compared to PSI–LHCI of higher plants is a natural property of the system and it is not due to the effect of detergent during the purification. Transient absorption measurements confirmed the presence of two pools of emitting chlorophylls [29,30]. Attribution of spectral features to individual Lhca subunits has been impossible up to now due to difficulty in purifying individual antenna subunits. Biochemical studies indicate that the antenna system of *Chlamydomonas* is composed of 6–14 Lhca subunits [4,6,9,24], making it significantly larger than the system of plants [5,7]. Moreover, it has been shown that the composition and size of *C. reinhardtii* LHCI is modulated in response to culture conditions [31–33] with a 6 subunits subset being assembled even in the absence of PSI core complex [24]. The above reports strongly suggest that *C. reinhardtii* LHCI is substantially different in composition, biogenesis and spectral properties with respect to land plant LHCI. In order to gain information on the properties of algal LHCI and to interpret responses to different growth conditions in terms of functional adaptation, we have undertaken the analysis of the individual pigment–proteins. This was accomplished by over-expressing the individual *lhca1*–9 genes in bacteria and refolding the apoproteins *in vitro* with purified pigments. This approach was shown to yield pigment–protein complexes with biochemical and spectral properties identical to the native proteins. This was verified each time the purification of the corresponding native protein from thylakoids was possible [34–36]. Even the kinetics of energy transfer could be closely reproduced down to femtosecond resolution [37]. Here, the biochemical and spectroscopic properties of the algal Lhcas are compared to those of Lhcas of *Arabidopsis thaliana* [22,34,36,38,39] and with those of the partially purified native Lhcas of *Chlamydomonas*, previously described.

## 1. Experimental procedures

### 1.1. Construction of Lhcas expression plasmids

Plasmids were constructed amplifying by PCR the Lhca genes from the cDNA phage library Stress II of *C. reinhardtii* (strain CC-1690) supplied by the Chlamydomonas Center-Duke University ([www.chlamy.org](http://www.chlamy.org)). cDNA of *C. reinhardtii* are rich in G-C content; thus different DNA polymerases and amplification conditions (reaction mix, annealing temperature (Ta)) have been used, in order to optimise each amplification reaction. Taq Platinum High-Fidelity (Invitrogen) was used for *lhca1*, 2, 3, 4, 6, 8 and Platinum Pfx (Invitrogen) for *lhca9*. The polymerase GoTaq (Promega) was used for *lhca5* and 7.

The reaction mixes were:

(1) 1 µg DNA template, buffer 1X, 2 mM MgSO<sub>4</sub>, 0.2 mM dNTPs, 0.5 µM each primer, 2U Taq Platinum High-Fidelity or Platinum Pfx (Invitrogen).

(2) 1 µg DNA template, GoTaq buffer 1×, 2 mM MgSO<sub>4</sub>, 0.2 mM dNTPs, 0.5 µM each primer, 1.25 U GoTaq, 5% DMSO, 1 M betaine.

Cycling conditions for amplification with mix 1:

94 °C×3 min, (94 °C×30 s, Ta °C×30 s) repeated 5 times, 68 °C×50 s, (94 °C×30 s, 57 °C×30 s, 68 °C×1 min) repeated 25 times, 68 °C×10 min. The annealing temperature (Ta) was 48 °C for *lhca1*, 2 and 3, 44 °C for *lhca9* and 46 °C for *lhca4*, 6, 8.

Cycling conditions for amplification with mix 2:

94 °C×3 min, (94 °C×30 s, Ta °C×30 s) repeated 5 times, 72 °C×50 s, (94 °C×30 s, 57 °C×30 s, 72 °C×1 min) repeated 23 times, 72 °C×10 min. The annealing temperature (Ta) was 54 °C for *lhca5* and 52 °C for *lhca7*. Primers were designed to generate restrictions sites for *Bam*HI at 5'-end and *Hind*III at 3'-end, except for *lhca4* (restriction sites for *Sac*I at 5' and *Eco*RI at 3').

*lhca1*:

F-atggtatccGCCCTGTCGATGCGCACC, R-cgaagcttGAAGAAGGGGA-CGGAGATGC;

*lhca2*:

F-atggtatccGCCATGCTGCTGAAGTCCC, R-gcaagcttCACCTGTTCACGGGCTCG;

*lhca3*:

F-atggtatccATGCTCACCAAGTCCGCGC, R-gtaagcttCGCGACCAGC-TTGCCGAAG;

*lhca4*:

F-gagagctcGCGTTCGTTCTTGCTAAGTCC, R-ccgaattcCAGGCGGG-CCAGGTCGTC;

*lhca5*:

F-gaggtatccGCCGCCCTTATGCAGAAGTCC, R-ccaagcttCTGGTGCC-GGGCCACAG;

*lhca6*:

F-gaggtatccATGCTGGTTGCCAAGAACG, R-ccaagcttAGGCCACAGG-CCCTGCAG;

*lhca7*:

F-gaggtatccGCCCTGTCGATGATCGC, R-ccaagcttGGCGATGGGGTTC-GACAC;

*lhca8*:

F-gaggtatccGCCCTTACCATGAAGCGC, R-ccaagcttCAGGAAGGGCA-GGGAGAC;

*lhca9*:

F-gaggtatccATCGCTGCTAAGAGCCAGG, R-ccaagcttAGCGGAGCCCA-GGGTCTC.

The amplified regions were cloned in the pETMHis vector, a pET-28a(+) (Novagen) home modified expression vector which add a minor number of bases to the cloned sequence and 6 histidines at the C-terminus. Correct DNA amplification and insertion into the expression plasmid were verified by DNA sequencing.

### 1.2. Isolation of overexpressed Lhca apoproteins from bacteria

Lhca apoproteins were expressed in *E. coli*, using the Rosetta2 (DE3) pLysS competent cells (Novagen). Two different bacteria growth media were used, in order to improve the protein expression yield: Superbroth [40] for the expression of *lhca1*, 2, 3, and Terrific Broth [41] for the expression of *lhca4*, 5, 6, 7, 8, 9. Protein induction was carried out with IPTG 1 mM for 5 hours at a temperature of 37 °C, under agitation (200 rpm). Lhca apoproteins were obtained as inclusion bodies and purified as previously described in [42]. Accumulation of the expressed proteins was analyzed and quantified by denaturing SDS-PAGE followed by Coomassie staining [43].

### 1.3. Pigments purification

*C. reinhardtii* (WT strain cw15-) was grown following standard procedure [44] and cells were pelleted by centrifugation at 4200 g for 10 min. Pellets were used for the pigment extraction. Total pigments

purification (chlorophylls *a*, *b* and carotenoids) was performed as in [45,46] but with the following modifications: initial pigment extraction was obtained in absolute ethanol and NaCl was not present in the aqueous solution. The carotenoid extraction was performed as in Ref. [46].

#### 1.4. *In vitro* reconstitution of Lhca–pigment complexes

Recombinant Lhcas were refolded *in vitro* in presence of purified pigments from *C. reinhardtii*, as described previously [34]. The reconstitution mix contain 420 µg of apoprotein, 240 µg of chlorophylls (Chl *a/b* ratio of 3.0) and 80 µg of carotenoids (loroxanthin, neoxanthin, violaxanthin, lutein and β-carotene). The reconstituted complexes were purified from the excess of pigments and apoprotein as described in [47]. In short, the reconstitution mix was loaded on a sucrose gradient (0.1–1 M, 0.06% β-DM, 10 mM HEPES pH 7.6, rotor SW41, 41,000 rpm, 20 h at 4 °C). The reconstituted complexes migrated in a well defined band and were largely separated from free pigments and unfolded protein. The band containing the reconstituted complex was subjected to anion-exchange chromatography (EMD-DEAE 650 (S) Fractogel) and the complex eluted from the column with a linear gradient of 500 mM NaCl, 50 mM Tris pH 7.6, 0.025 % β-DM. This step allowed the completed separation of the folded (holo-) and unfolded (apo-) proteins, which are eluted from the column at different salt concentrations. In the last step, the reconstituted purified complexes were loaded on a second sucrose gradient, which allowed to separate the last free pigments and to check the aggregation state of the complexes. All reconstituted products were present in the sucrose gradient in a sharp, well defined unique band, corresponding to the monomeric aggregation state. The yield of the reconstitution procedure was between 1.5% and 6.5%, for all complexes with the exception of Lhca3, which scored only 0.4% (Table 1). The individual complexes were reconstituted at least 3 times and the products of the different reconstitutions showed identical biochemical and spectroscopic properties.

#### 1.5. Pigment analysis

Pigments were extracted with acetone 80%, fractionated and quantified by HPLC and by fitting the spectra of the acetone extracts with the spectra of individual pigments [36].

#### 1.6. Spectroscopy

The absorption spectra were recorded at 77 °K (LT) in 20 mM Hepes pH 7.5, 70% w/v glycerol and 0.06% β-DM, using a Cary 4000 (Varian) spectrophotometer. Fluorescence emission spectra at 77 °K

were measured using a CaryEclipse (Varian) spectrofluorimeter and corrected for the instrument response. The samples were excited at 440 and 475 nm. The spectral bandwidth was 5 nm (excitation) and 3 nm (emission). The chlorophyll concentration was about 0.02 µg/ml in 20 mM HEPES, 70% w/v glycerol and 0.03% β-DM.

Circular dichroism spectra in the visible region were measured at 10 °C on a AVIV 62ADS spectropolarimeter. The samples were in 20 mM HEPES, 0.2 M sucrose and 0.06% β-DM.

## 2. Results

### 2.1. Sequence analysis

The sequences of *lhca1*–9 ORFs were obtained from GenBank, from literature [8] and from the EST deposited in the Chlamydomonas centre database ([www.chlamy.org](http://www.chlamy.org)); references are reported in Table 1. Sequence alignment of Lhca1–9 of *C. reinhardtii* (Cr-Lhca) with Lhca of *Arabidopsis thaliana* (At-Lhca) showed identity lower than 35% (ClustalV program, data not shown). Higher levels of conservation were observed within the three putative transmembrane helices, thus suggesting a conserved folding. Within Cr-Lhca1–9, the identity was of 39–52%. By integrating our data with those previously reported [3,8,48], the Lhca proteins were clustered in 3 sub-classes: (i) “higher plants-like” including Lhca1 and 3; (ii) “green algae specific” including Lhca2 and 9; (iii) “higher plants orthologous” antennae including Lhca4, 5, 6, 7 and 8.

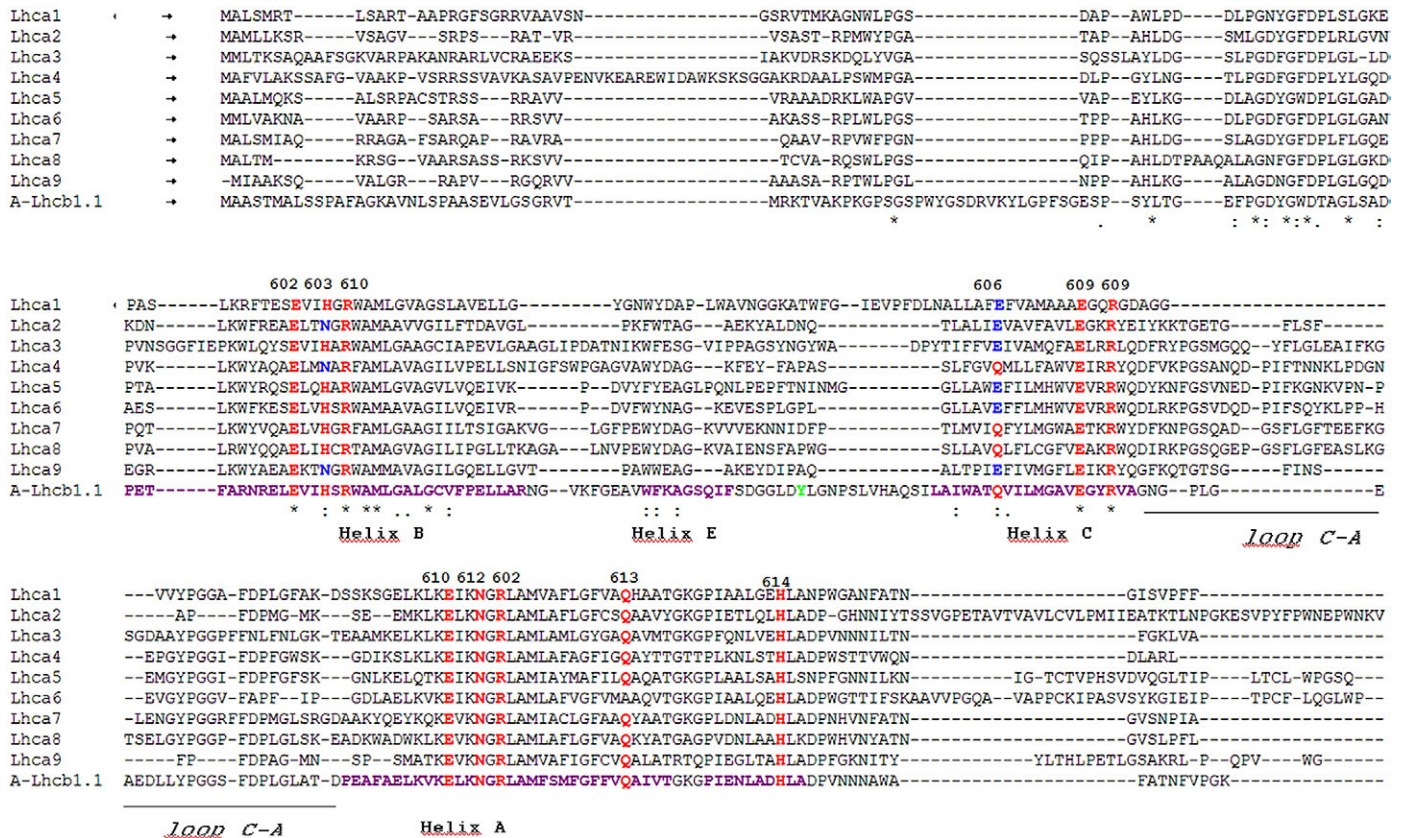
The spectral properties of Lhc proteins are determined by the number and organization of chromophores. In order to identify the amino acid residues (AA) acting as Chl ligands in Cr-Lhca proteins, the sequences were aligned to that of At-Lhcb1.1 (Fig. 1), whose chlorophyll ligands are known [49–51]. Most of the ligands are conserved in each Cr-Lhca sequence, with the exception of Chl *a* 613 (nomenclature according Liu et al. 2004 [50]) ligand in Lhca6. In few cases the nucleophilic residue responsible for the Chl binding in At-Lhcb1.1 was substituted in Cr-Lhca with a different residue, which can, nevertheless, still act as a ligand. The glutamine (Q) ligand of Chl 606 is conserved only in Lhca4, 7 and 8, while a glutamic acid (E) is found in the same position in Lhca1, 2, 3, 5, 6, 9. The same substitution was observed in several Lhc complexes of higher plants, where it has been shown to modulate the binding affinity for Chl *b* [52]. Substitution at the Chl 603 ligand was found in Lhca2, 4, 9, carrying asparagine (N), rather than the more common histidine (H). Asparagine as Chl 603 ligand in At-Lhca3 and At-Lhca4 was shown to be necessary for the formation of red forms [53]. The determinants for the carotenoid binding are less well known, but for the case of Tyrosine (Y) 111 in Lhcb1.1 which strengthens the binding of neoxanthin in the N1 site [50,54]. This residue is not conserved in any of the Cr-Lhca.

**Table 1**  
Lhca from *C. reinhardtii* (the nomenclature used in this paper is according to Stauber et al., 2003 [6]).

Stauber et al. [6]	Elrad and Grossman [69]	Tokutsu et al. [8]	Bassi et al. 92 (protein)	Accession number	MW (kDa)	Reconstitution efficiency %	Chl <i>a</i> /b	Chl/Car	loro	neo	Viola	lut	β - car
Lhca1	Lhca4	Lhcl-6	p22.1	AB122119	23.9	6.4 (4)	2.7	3.6	2.6	3.5	11.7	20	–
Lhca2	Lhca9		p19	XM_001690979	28.4	3.1 (4)	2.5	4.5	1.8	–	10.7	17.6	–
Lhca3	Lhca6	Lhcl-2	p14.1	AB122115	30.3	0.4 (7)	3.8	3.2	4.2	–	9.4	21.6	7.8
Lhca4	Lhca5	Lhcl-1	p14	AB122114	28.7	1.5 (4)	1.5	3.3	2.8	–	16.6	18.4	3.9
Lhca5	Lhca1	Lhcl-3	p15.1	AB122116	28.2	2.1 (3)	2.4	5.7	0	–	9.4	12	2.6
Lhca6	Lhca8	Lhcl-5	p18.1	AB122118	27.8	3.1 (4)	1.8	4.5	2.7	–	6.4	18	3.1
Lhca7	Lhca2	Lhcl-7	p15	AB122120	26.2	1.7 (4)	1.9	3.7	3.2	–	8.3	20.5	4.8
Lhca8	Lhca3	Lhcl-4	p18	AB122117	25.9	6.5 (3)	1.6	3.4	3.1	–	5.2	25	7
Lhca9	Lhca7		p22.2	AF244524	22.8	3.3 (3)	3.1	4.8	0	–	12.9	15.3	0

Alternative nomenclatures present in literature and accession number are indicated [4,8,69]. Molecular weight (MW) of the expressed apoproteins, reconstitution efficiency percentage, with the number of sample preparation repetitions (into brackets), and pigment composition of the refolded antennae are reported. The pigments values are normalised to 100 Chl *a*; the standard error is less than 0.1, in the case of Lhca3 is 0.5. Loro, loroxanthin; neo, neoxanthin; viola, violaxanthin; lut, lutein; β-car, β-carotene.





**Fig. 1.** Sequence alignment of Lhca1–9 apoproteins from *C. reinhardtii*. Trans-membrane helices are in purple, chlorophyll binding sites (nomenclature of Liu et al., [50]) are in red (identical in the two species) or in blue (homologous in the two species). The neoxanthin binding site is in green. The sequence of Lhcb1.1 from *A. thaliana* is used as reference.

## 2.2. Reconstitution conditions and purification of the refolded complexes

The cDNA of the nine Cr–Lhca was amplified from a phage library, the apoproteins overexpressed in *E. coli* and refolded *in vitro* with *C. reinhardtii* pigments, which differ from those of higher plants for the additional presence of loroxanthin. All Cr–Lhca complexes were obtained in monomeric state as assessed by sucrose gradient ultracentrifugation (see [Experimental procedures](#) for details).

## 2.3. Pigment composition

All pigment–protein complexes showed a Chl *a/b* ratio between 1.50 and 3.1 ([Table 1](#)) with the exception of Lhca3 (Chl *a/b* 3.8). However, reconstituted Lhca3 is rather unstable and thus it might not being fully representative of the protein pigment composition *in vivo*. The main carotenoid species were lutein and violaxanthin. Some of the complexes, namely Lhca3, 7 and 8, bound small amount of  $\beta$ -carotene. Lhca1 was the only protein which could bind neoxanthin ([Table 1](#)), although in sub-stoichiometric amount. Loroxanthin was found in most complexes, but in very low amount ([Table 1](#)), suggesting the absence of a specific binding site for this xanthophyll specie.

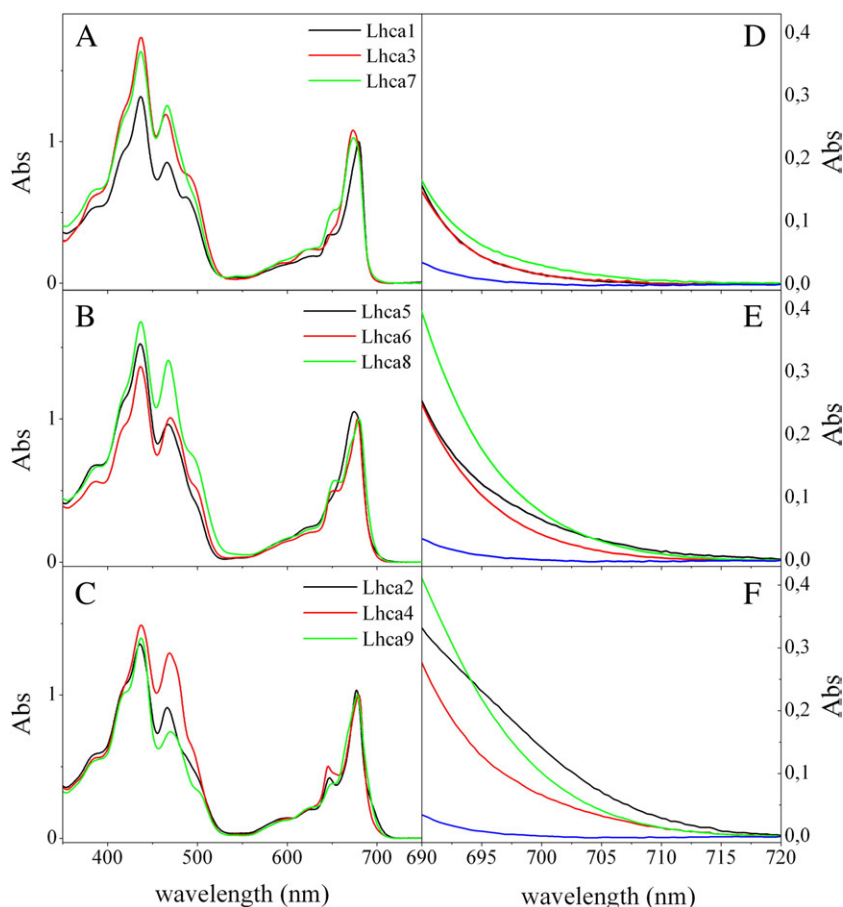
## 2.4. Absorption spectra

The Lhca1–9 absorption spectra were recorded at 77 K in the 350–750 nm range ([Fig. 2](#)). All complexes exhibit a main peak in the  $Q_y$  region in the 675–680 nm range ([Table 2](#)). The distribution of the Chls additional forms in each complex was analyzed using the second derivative of the absorption spectra ([Table 3](#)). In most complexes a single Chl *b* contribution was found, peaking at 644.4–653.2 nm; two

exceptions were Lhca4 and Lhca7, which exhibited 2 distinct Chl *b* contributions at 644.8/653.2 and 645.6/652.0 nm respectively ([Table 3](#)). The energy of Chls *a* was rather well conserved in all complexes, with two major contributions around 672.0 and 680.0 nm, similar to At–Lhcas [55] ([Table 3](#)). In the far-red absorption region (685–720 nm) only Lhca9 showed a clear contribution at 692.0 nm, while a contribution in the 693–697 nm range was detected in Lhca2. In all other complexes the far-red region was structureless ([Table 3](#)). However, comparison of the absorption spectra of Lhcas with that of LHCI, which does not contain red-shifted forms, showed that all Lhca exhibit more intense absorption in the red tail with respect to LHCI, an indication of the presence of “red Chls” ([Fig. 2](#)). In order to compare the amount of the red absorption in each complex, the percentage of absorption in the spectral windows between 690–750 and 700–750 nm was calculated. Lhca4 from *A. thaliana* was used as positive control and scored 11% and 5% above 690 and 700 nm, respectively ([Table 2](#)). The spectra of all Cr–Lhcas showed lower amplitude in the red as compared to At–Lhca4: Lhca2 and Lhca9 were the “red-most” antennae, with 8–10% of absorption above 690 nm and 2% above 700 nm. A second group of complexes was composed of Lhca4, 5, 6 and 8, which have 4–6% of the absorption above 690 nm. A third group, composed of Lhca1, 3 and 7, showed less than 3% of absorption above 690 nm. Only 1% of Chl absorption was above 700 nm in the second and third groups ([Table 2](#)).

## 2.5. Circular dichroism spectra

To get information about the pigment organization within Lhca subunits, circular dichroism spectra were measured at 10 °C ([Fig. 3](#)). A “+ − −” signature in the  $Q_y$  region with contributions at 647–652 nm (−), 661–669 nm (+) and 685–688 nm (−), was observed in all



**Fig. 2.** Absorption spectra at 77K of Lhca monomers. In panels (A–B–C) the absorption in the whole visible region is shown. In panels (D–E–F) the absorption in the 690–720 nm range of Lhca complexes is compared to that of LHCII from higher plants (solid thick line/blue line). The spectra are normalised to the maximum in the  $Q_y$  region.

complexes. The relative intensity of the two negative components was also very similar in most of the complexes, with the exception of Lhca4 which showed increased amplitude at 652 nm, in agreement with a higher Chl *b* content. In Lhca1 and Lhca3 an additional negative contribution was present at 673 and 675 nm, respectively (Fig. 3A). Furthermore, a negative signal at wavelengths longer than 700 nm was observed in Lhca2 and 9 (Fig. 3B), correlating with the high amplitude of the red absorption forms in these complexes. In the blue region, all spectra were characterized by three negative components peaking around 500, 480 and 470 nm, while the signal in the region below 450 nm was positive in all samples. Comparison of the spectra, showed that Lhca2 and Lhca9 were almost identical in the red (only a small difference was detected in the 683/695 nm range) and differ only for the presence of an additional Chl *a*/Chl *b* interaction visible at

470(–)/438(+) nm in the latter, suggesting a very similar pigment organization. Also Lhca5 showed a similar spectrum which was, however, narrower in the red region due to the absence of red forms in this complex. High similarity could also be observed between the spectra of Lhca7 and Lhca8 (Fig. 3C), again indicating similar pigment organization.

## 2.6. Fluorescence emission spectra

Fluorescence emission spectra recorded at 77K (Fig. 4) allow the direct detection of the red forms. Based on their emission maximum three subclasses of complexes could be distinguished: (i) the “blue Lhca” group, composed of Lhca1, 3 and 7, with emission maxima at 682.5–683.5 nm; (ii) the “intermediate Lhca” group, composed of

**Table 2**

Characteristics of the chlorophylls absorption and emission bands (expressed in nanometers) in the red visible region at 77 K.

	max Abs	max emiss	emiss-Abs	FWHM <sup>a</sup>	%abs > 700	%abs > 690
Lhcb1.1 At	676	680	4	19.5 ± 0.3	0.1	0.4
Lhca1 Cr	680	683	3	9.8 ± 0.3	0.3	2.3
Lhca2 Cr	677	717	40	45.5 ± 0.7	2.7	9.6
Lhca3 Cr	673.5	682.5	9	12.1 ± 0.3	0.2	1.9
Lhca4 Cr	679.5	689; 708.5	29	47.7 ± 0.7	1.2	5.3
Lhca5 Cr	675	684.5; 697.5	22.5	33.9 ± 0.3	1.1	4.6
Lhca6 Cr	678.5	687.5; 694.5	16	33.9 ± 0.5	0.6	4.1
Lhca7 Cr	673.5	683.5	10	14.0 ± 0.5	0.5	2.6
Lhca8 Cr	680	690; 694.5	14.5	26.2 ± 0.4	1.0	6.2
Lhca9 Cr	678	710	32	34.9 ± 0.7	1.5	8.1
Lhca4 At	674	732	58	43.8 ± 0.5	4.8	9.3

<sup>a</sup> FWHM indicates the half-band width of the Gaussian fitting of the emission spectra; the error is reported.

**Table 3**

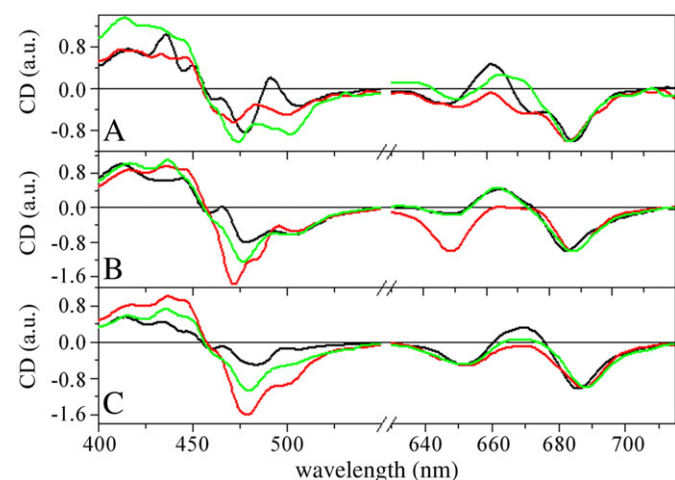
Chlorophyll absorption maxima as obtained from the second derivative analysis of the absorption spectra.

	Chl b	Chl a	Chl a	Chl a	Chl a
Lhca 1	644.4	662.4	672	681.2	
Lhca 2	646	660.8	678.8		693–697
Lhca 3	649.2		671.2	680.8	
Lhca 4	644.8; 653.2			681.2	
Lhca 5	645.2		672	681.2	
Lhca 6	646.8		667.2	679.2	
Lhca 7	645.6; 652		671.6	680.8	
Lhca 8	651.2		671.2	682.8	
Lhca 9	646.8		667.2	680.4	692

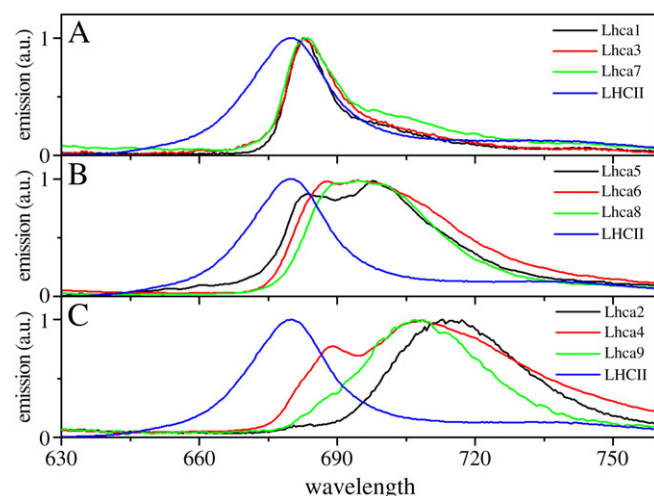
Lhca 5, 6 and 8, with maxima between 694.5 and 697.5 (iii) the “red Lhca” group, composed of Lhca2, 4 and 9, with showed maxima between 707 and 715 nm (Figs. 4–5; Table 2). The spectra were very broad as it was the case for Lhca of *A. thaliana* [37]. The full-width-half-maximum (FWHM) of the bands, calculated upon Gaussian fit, correlated with the energy of the red forms: the largest values of FWHM were associated with the red-most bands (Table 2). A second emission form, peaking between 684.5 and 690 nm (Table 2), thus blue-shifted with respect to the main peak, was present in Lhca4, 5, 6 and 8, suggesting that these proteins can assume two distinct conformations, characterized by a different distribution of the sites energy especially for the low energy forms. It is possible that *in vivo* when assembled in the PSI–LHCI supercomplex, the protein–protein interactions stabilize one of these conformations, most probably the red one as it is also the case for the Lhca complexes of higher plants [55].

### 3. Discussion

The antenna system of *C. reinhardtii* PSI is composed of nine pigment–protein complexes, which have very similar physico-chemical properties. This complexity has so far hampered the possibility to purify each of them to homogeneity. To study these complexes we have thus used a different approach, by cloning the nine genes from *C. reinhardtii*, overexpressing the apoproteins in *E. coli* and reconstituting the holoproteins *in vitro* with the pigments purified from *C. reinhardtii*. This approach has been used previously for the antenna complexes of higher plants (for an overview [56]) and it was

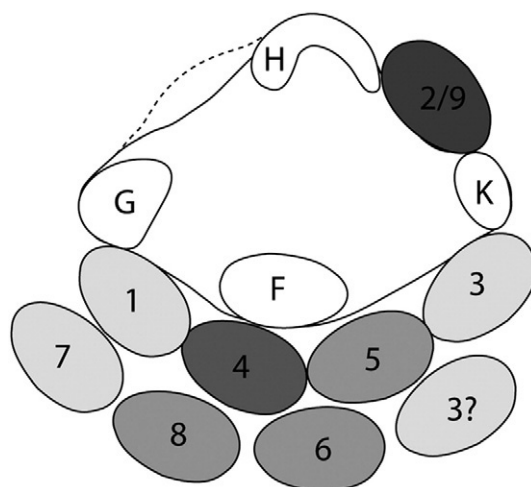


**Fig. 3.** Circular dichroism spectra at 10 °C of Lhca monomers: (A) Lhca1 (black), Lhca3 (red), Lhca5 (green); (B) Lhca2 (black), Lhca4 (red), Lhca9 (green); (C) Lhca6 (black), Lhca7 (red), Lhca8 (green). The spectra are normalized to the minimum I the  $Q_y$  region.



**Fig. 4.** Emission fluorescence spectra at 77 K of Lhca monomers. The spectrum of LHCI from higher plants is reported in all panels as a reference (thin solid line/blue line). The excitation wavelength was 500 nm.

shown to yield to pigment–protein complexes which were indistinguishable from the native ones, purified from plants, in both their biochemical and spectroscopic properties. This was demonstrated every time it was possible to purify to homogeneity monomeric and dimeric antennas [34,36,38,39]. Although no data are available about individual *C. reinhardtii* Lhcas, their average properties are known based on the analysis of few preparations containing several complexes [4,5,8,23–26]. From these data it is clear that the Lhca antenna complexes of *C. reinhardtii* have specific properties which differentiate them from the Lhcb antenna and from the Lhca complexes of higher plants. In particular *C. reinhardtii* LHCI antenna has fluorescence emission with energy intermediate between Lhcb complexes and plant LHCI. This property and several others (see below) are well reproduced by the recombinant complexes, clearly indicating that they closely reproduce their native counterparts also in the case of *C. reinhardtii* Lhcas.



**Fig. 5.** Model for the Cr–LHCI organisation, showing the absorption properties of the single Lhca complexes. Model of *Chlamydomonas* PSI with indicated the energy of Lhca monomers. Position of antennae are given according to Stauber et al., [9]. The three Lhca groups identified in this work are indicated by grey scale tonalities: (a) light grey, “blue Lhca” comprising Lhca1, 3, 7; (b) grey, “intermediate Lhca” comprising Lhca5, 6, 8 and eventually Lhca3 and (c) dark grey, “red Lhca” comprising Lhca2, 4, 9. Position of Lhca1, 3, 4 and 2/9 are given according to Stauber et al., [9]. The dashed line indicates an extra density in the PSI–LHCI of *Chlamydomonas* shown by electron microscopy, but that apparently is too small to accommodate an Lhc complex.



### 3.1. The monomeric antenna: common properties

Reconstituted Cr-Lhca pigment–proteins exhibited a Chl *a/b* ratio between 1.50 and 3.1. Pigments used in the reconstitution procedure were extracted from *C. reinhardtii* and were used at the same Chl *a/b* ratio found in the thylakoid membrane from this organism. It should be underlined that when the same procedure was used for plant Lhc proteins that could also be purified from the chloroplast in their native form, the reconstituted complexes showed the same chromophore composition and stoichiometry as their native counterparts [47,54,57,58].

As compared to At-Lhca [22,24–26,38], the Cr-Lhca complexes show a general enrichment in Chl *b*. These findings are in agreement with data reported on purified PSI-LHCI from higher plants (Chl *a/b* 8.5) and from *C. reinhardtii* (Chl *a/b* 4.5), which indicate higher affinity for Chl *b* binding in the antenna complex of *Chlamydomonas* vs. those of land plants [4,5,7,59]. Interestingly, the lowest Chl *a/b* ratio was found for Lhca4, 7 and 8, which have the specificity of carrying a Q residue as a ligand for Chl 606 (B6) in helix C. In higher plant LHCI, this residue is involved in hydrogen bonding with the formyl group of two Chl *b* [50]. In different Lhc proteins the presence of a glutamine residue at this position correlates with the selectivity for Chl *b* in site 609 and 606 (as in Lhcb1), while the presence of a glutamate produces mixed Chl *a*/Chl *b* occupancy (as in Lhcb4). Accordingly, the selectivity for Chl *b* can be reversed by the reciprocal mutation [52]. On this basis we suggest that the organization in the helix C domain of Lhca4, 7 and 8 complexes is similar to that of At-Lhcb1 and that an extensive network of H bonds is present in these complexes, which is responsible for their higher affinity for Chls *b*.

In all Cr-Lhca complexes, lutein and violaxanthin are the major xanthophyll species. However,  $\beta$ -carotene is associated to several complexes, although in low amount. Loroanthin, a natural pigment of *C. reinhardtii*, was found to be associated to most complexes, but its amount was always lower than 10% of the total carotenoids, indicating that this xanthophyll shares binding sites with other carotenoid species. Tyrosine (Y) 111 residue, forming an H-bond stabilizing the neoxanthin binding in Lhcb5 [54], is not conserved in Cr-Lhcas; consistently, neoxanthin is not a ligand for Lhcas, with the exception of Lhca1, where, however, it only accounts for a very small fraction of the xanthophyll complement. It can be concluded that the two features which characterize the pigment content of Lhcas in comparison to Lhcb5 in higher plants, e.g. the absence of neoxanthin and the presence of  $\beta$ -carotene, are conserved in Lhcas of *C. reinhardtii*, indicating that the determinants for the affinity of the carotenoid binding sites are maintained between algae and higher plants.

The site energy of the pigments is very sensitive to the environment and thus it represents a tool to compare the protein organization between the members of the Lhc family [60]. Most of the Cr-Lhca have a CD spectrum almost identical to that of At-Lhca2 [22]. An exception is Lhca4, which exhibits a larger amplitude of the 652 nm Chl *b* signal which is comparable to that at 673 nm from Chl *a*. This is similar to the case of Lhca4 from *A. thaliana* [38]. We conclude that, despite the low sequence identity, the pigment organization is highly conserved and most of the complexes seem to be “*A. thaliana* Lhca2-like” when compared to the homologous proteins of higher plants.

We also found that Lhca3 and Lhca5 complexes are rather unstable as monomers. We speculate that interactions with other antenna subunits could stabilize them similarly to what observed for Lhca1 and Lhca4 of higher plants, where the monomers exhibited a denaturation temperature of 45 °C, which increased up to 57 °C [38] upon dimerization. These protein–protein interactions can also lead to a slight change of the spectroscopic properties of the individual complexes, similar to what observed in higher plants [22,38].

### 3.2. The red forms

Preparations of LHCI from *C. reinhardtii* containing several complexes, showed two fluorescence emission components at 680–685 nm and 705–708 nm [4,24]. However, due to difficulty in purification, it was not possible to attribute the red emissions to specific subunits. The analysis of the reconstituted monomeric complexes indicates that all nine Lhca have emission forms red-shifted as compared to LHCI as it is the case in higher plants [22]. Three complexes (Lhca1, 3 and 7) showed emission peaks in the 680–690 nm range, thus accounting for the blue contribution observed in the native preparations. Three other complexes (Lhca5, 6 and 8) have an additional emission in the 695–700 nm range. This emission component is not clearly resolved in the fluorescence spectra of the native LHCI preparations, possibly due to energy transfer to the red forms of a neighbouring complex or to a change in the absorption properties of these complexes, due to protein–protein interactions, when embedded in the PSI-LHCI supercomplex, as previously proposed [4]. Three more complexes, namely Lhca2, 4 and 9, showed emission above 707 nm with the red-most form (715 nm) associated to Lhca2. Interestingly, the LHCI oligomeric preparation from Takahashi et al. [24], which has an emission maximum at 708 nm, is depleted in Lhca2 and Lhca9, thus containing only the red forms associated to Lhca4, which indeed emits at 708 nm. This indicates that most of the energy is transferred to this subunit. The absence of Lhca2 from this preparation explains the fluorescence blue shift in the oligomeric *C. reinhardtii* LHCI as compared to that of PSI-LHCI (peaking at 715 nm), and indicates that Lhca2 is responsible for the red-most emission *in vivo*. Up-regulation of Lhca4 and Lhca9 was observed under iron deficiency conditions and [32] it correlates with a shift to the blue of the emission spectrum (from 710 to 704 nm) and with a relative increase of intensity of the emission above 700 nm as compared to the emission below 700 nm [32]. This indicates an increased content of red forms emitting around 705 nm in the antenna, in agreement with the fluorescence characteristics of Lhca4 and Lhca9. In the same conditions down-regulation of Lhca5 was also detected, leading to the suggestions that Lhca4 can substitute for Lhca5, possibly providing a better sink for energy dissipation. The finding that these antennas contain the lowest energy forms supports the hypothesis. A similar effect was recently observed in higher plants, where Lhca5 could substitute for Lhca4 [59].

It was shown that in higher plants, the red forms represent the low energy band of an excitonic, interaction involving Chls 603 (A5) and 609 (B5) [53,60–63]. The strong broadening of the band was shown to be due to the mixing of an excitonic state with a charge transfer state [19,55,64]. It has also been shown that the presence of an asparagine (N) residue as ligand for Chl 603 is needed in order to induce the large red-shift of the absorption band [53]. All these features are conserved in Lhca2, Lhca4 and Lhca9 of *C. reinhardtii*, which show the red-most emissions: (i) only these three complexes have a N residue in the putative binding site of Chl 603; (ii) their CD spectra have a component around 695 nm, thus indicating that the red-most form originates from an excitonic interaction; (iii) their emission bands are very broad with values comparable to that of Lhca4 of *A. thaliana*, suggesting the presence of a charge transfer state. It has also been shown that the red forms of higher plants are characterized by a very high value of optical reorganization energy [65] with Stokes shift in the order of 25 nm for the red-most-shifted forms associated to Lhca3 and Lhca4 [55,66]. Unfortunately, the red absorption tail of the spectra of most Lhcas is structureless thus preventing the determination of the absorption maximum of the lowest energy state. An exception is Lhca9, which shows a contribution at 692 nm, likely representing the lowest energy state of the complex. Using this value for the absorption and 707 nm for the emission, a Stokes shift of 15 nm (300 cm<sup>−1</sup>) and optical reorganization energy (S<sub>v</sub>) of 150 cm<sup>−1</sup> were calculated. The S<sub>v</sub> values of the red forms of higher plants Lhca was 120 cm<sup>−1</sup> for Lhca2,

200 cm<sup>-1</sup> for Lhca3 and 240 cm<sup>-1</sup> for Lhca4 [55,63], increasing linearly with the emission peak wavelength of the complexes. Remarkably, the data from Lhca9 fit extremely well in this relationship.

Lhca4, 5, 6 and 8 show two distinct emission peaks. This characteristic is somehow unexpected in isolated monomeric Lhc proteins since the equilibration time within a network of tightly interconnected chlorophylls is very fast (e.g., [37]). A likely explanation is that Lhca proteins in solution exist as an equilibrium mixture of two conformations with different energy of the lowest states. In the case of red forms this could well correspond to differences in the interaction between Chls 603 and 609, which originated the long wavelength emission band.

It can be concluded that, despite the low level of sequence conservation between the Lhca complexes of *A. thaliana* and *C. reinhardtii*, the red forms maintain specific features and are likely originated from the same pool of chromophores, indicating high conservation of the domain hosting Chl 603 through evolution.

### 3.3. Composition of the LHCI antenna

LHCI antenna complex from *C. reinhardtii* is easily modulated in size. Data in literature demonstrate that in normal growth conditions the antenna complex is composed of 7 to 14 subunits [4,5,7]. The analysis of the PSI–LHCI supercomplexes by electron microscopy, shows the presence of particles with 9–10 antenna subunits, although smaller particles were also detected [5]. It was also shown that the relative amount of individual antenna is regulated depending of nutrients supply concentration [31,32], suggesting a higher flexibility in the assembly of the system as compared to higher plants, where the composition of the LHCI antenna is fixed [59,67]. Recently, a stoichiometry of the Lhca complexes in the PSI–LHCI supercomplex has revealed that not all nine gene products are present in 1:1 ratio with the core proteins, again suggesting flexibility [9].

In Fig. 5 a working model for the organization of the antenna complexes in the PSI supercomplex, in which the energy distribution in the system, as obtained in this work, is also visualized, is presented. This model has been obtained by integrating the available structural data [5,7] with information about the stoichiometry of the antenna complexes [9] and the composition of purified LHCI-containing fractions [4,24,31] and complemented with the energy levels associated with each subunit, as obtained in this work. Although this model represents a summary of the available data on the structural and spectroscopic properties of Lhca antennas, it should still be considered as preliminar due especially to the lack of precise information about the localization of each complex. An oligomeric preparation containing all Lhca complexes but Lhca2, Lhca3 and Lhca9 could be obtained from a *C. reinhardtii* mutant strain which does not have a PSI core. This suggests that large part of the LHCI antenna assembles independently from the core [24], forming a stable supercomplex and that these antennas are close together also in the PSI–LHCI structure. The position of Lhca7 is assigned based on its enrichment in the LHCI supercomplex [24], and its absence in the LHCI-705 fraction [4], suggesting that it is located at the periphery of the supercomplex. The depletion of both Lhca3 and PsaK during iron starvation [31,32], which induces the dissociation of the LHCI from the PSI core [32], indicates that Lhca3 is located in between the core and the antenna and on the side of PsaK. Two Lhca3 are shown, based on the recent proposed stoichiometry and they are located as proposed by Stauber and coauthors [9]. The position of Lhca5 is assigned based on its behaviour, similar to that of Lhca3 under Fe starvation conditions, in which a decreasing of both proteins is observed due to a possible destabilization of this part of the antenna system. Following the suggestion of Stauber et al., [9], the complexes which we found to be the red-most Lhcas (2 and 9) are located in between the H and K subunits. The present model locates a red-form carrying complex in the same position as higher plant Lhca4 (the red-most

subunit) with respect to the core complex [10,68]. However, differently from plants, no red-form enriched Lhca subunit would be present in the position of the plant Lhca3. Lhcas subunits with intermediate content in red-forms would be located in the inner layer of the double crescent structure of the *Chlamydomonas* antenna, allowing a preferential energy transfer from the antenna system to the core through the Lhca4 complex. This organization of the antenna is in agreement with the time resolved measurements by Ihalaenen et al., [65] which showed that the overall lifetime is shorter for *Chlamydomonas* PSI than for *Arabidopsis* PSI, despite the higher number of Chls coordinated by the former. It was suggested that this difference is due to the presence of less-red shifted Chls associated to the antenna of *Chlamydomonas*, which is clearly the case of Cr–Lhca4.

### 3.4. Conclusions

In this work the nine Lhca antenna complexes of *C. reinhardtii* were individually characterized for the first time. The results indicate that, despite the low sequence identity between the apoproteins, the pigment binding and the spectroscopic properties of the subunits are very similar. The major differences are related to the presence of red forms, primarily associated to Lhca2, Lhca4 and Lhca9. Most Lhca proteins seem to have an “At-Lhca2-like” pigment organization, as indicated by the CD spectra.

Moreover, all spectroscopic fingerprints which characterize Lhca as compared to Lhcb complexes in higher plants, are conserved in the complexes from *C. reinhardtii*, namely: the absence of neoxanthin and the presence of  $\beta$ -carotene; presence of red-shifted absorption and emission spectral forms; presence of the N as ligand for Chl 603 in the three complexes, responsible for the lowest energy forms; large bandwidth, both in absorption and emission, for the red forms; large values of Stokes shifts for the red forms; presence of a excitonic interaction involving the red Chls.

It can be concluded that all the determinants of the main biochemical and spectroscopic properties of Lhca complexes have been conserved through evolution from green algae to higher plants.

However, the individual Lhca complexes differ in their red form content and thus in their capability of using the far-red light. Changes in the protein composition, depending on the growth conditions, will thus results in an *ad hoc* antenna system produced to perform better under different environmental conditions.

These results represent a base for engineering the PSI antenna for maximal performance in high-density cultures typical of photo-bioreactors.

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