Influence of size, composition and supramolecular organization of Photosystem I on trapping efficiency
Insights from the algae *Chlamydomonas reinhardtii* and *Nannochloropsis gaditana*
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CHAPTER 1

Introduction
Photosynthesis

Across evolution, living organisms have developed different strategies to cope with their energy needs, among which is photosynthesis. Photosynthesis is the ensemble of processes producing chemical energy using sunlight. Different types of photosynthesis exist in Nature, with the most widespread being the “chlorophyll-based form” (1), in which sunlight is harvested by chlorophylls (Chl) and carotenoids (Cars) (and bilins in some organisms). Oxygen is a by-product of photosynthesis in plants, algae and cyanobacteria, but is not produced by most of the prokaryotes, which are therefore called anoxygenic bacteria.

Oxygenic photosynthesis consists of four successive phases: (i) Light harvesting and excitation energy transfer by the antenna systems (ii) Charge separation in the reaction center (photochemistry) (iii) Secondary electron transfers, resulting in the synthesis of NADPH (reductive agent) and ATP (energy) (iv) Carbon fixation, for which NADPH and ATP are used in the Calvin-Benson cycle to synthesize stable organic products from CO₂. The three first phases are called ‘light reactions’ and the last phase ‘dark reactions’. However, only the first phase depends directly on the light (photon absorption) whereas the others could be considered light-driven reactions.

Light harvesting in oxygenic organisms relies on pigments coordinated to multiprotein complexes. These complexes are embedded in (or associated with) membranes called thylakoids. In photosynthetic eukaryotes, thylakoid membranes form the inner network of an organelle specialized in photosynthesis called the chloroplast. Four major trans-membrane proteins are involved during the first three phases: Photosystems (PS) I and II, cytochrome b₆f and ATP synthase. Electrons travel linearly through these complexes (Figure 1), from water, the first electron donor, to NADP⁺, the final electron acceptor of the electron transport chain (ETC).

The electron transfer from water to NADP⁺ is not spontaneous as shown by the redox potential of their related couples (E°’ (O₂/H₂O) = +0.82V > E°’ (NADP⁺/NADPH) = -0.32V). After excitation by sunlight, Chls become highly reductive. In the reaction centers (RCs) of the PSs, these highly reductive excited Chls can react with nearby oxidative species. Photochemistry consists in the formation of successive radical pairs by charge separation (CS). Primary electron transfer steps end with final radical pairs involving P₇₀₀⁺ in PSI and P₆₈₀⁺ in PSII (each named after its absorption wavelength, (2) and details for PSI below). P₇₀₀⁺ and P₆₈₀⁺ have strong oxidative power and extract electrons from plastocyanine (PC) and water. These RC Chls, back in their ground states, are then ready for new light excitation. Secondary chemical reactions involve the successive acceptors of the ETC starting from ferredoxin (Fd) reduction on the donor side of PSI and from plastoquinone (PQ) reduction on the donor side of PSII (Figure 1). In conclusion, sun light provides the energy necessary to fill
the free energy gap between water and NADP$^+$ and to initiate a cascade of spontaneous oxido-reduction reactions (also called the “Z-scheme”).

Figure 1: Thylakoid membrane of higher plants with the four main photosynthetic multiprotein complexes: the two photosystems (PS) and their Light Harvesting Complexes (LHC), PSI-LHCI and PSII-LHCII, the cytochrome b6f (cytb6f) and the ATP synthase. Under light, electrons travel linearly from H$_2$O to NADPH (solid arrows) and at the same time protons translocate from the stroma to the lumen (long dashed arrow). The proton gradient is used by the ATP synthase for the production of ATP. Cyclic electron transfer (short dashed arrow) only produces ATP which adjusts the NADPH:ATP stoichiometry. Fd: ferredoxin; FNR: ferredoxin-NADP-reductase; PQH$_2$: plastoquinol; PC: plastocyanin. Picture adapted from (3).

After CS in the RC, a high electric field of $10^7$ V.m$^{-1}$ is created across the membrane (considering a voltage of 100 mV across a membrane of 10 nm (4)). Due to water oxidation and the PQ cycle, protons (H$^+$) accumulate in the lumen (the inner space of the thylakoid) resulting in a proton gradient across the membrane. This proton gradient is used by the ATP synthase to produce ATP.

**Antenna principle**

As just mentioned, the photosynthetic ETC is activated thanks to the energy harvested from the sun. In principle, the more light is harvested, the more electrons are transported from water to NADP$^+$. Considering that a Chl has a cross-sectional area of about 15 Å$^2$ and that the photon flux is 100 µmoles photons/m$^2$/s on average, the average probability for a photon to be absorbed on this Chl is equivalent to one photon per second. In the case of a Chl in an RC, this would lead to the generation of one electron per second (considering a quantum yield of 1). Within the PSs, pigments are densely packed around the RC Chls and can transfer their excitation energy to it. In this way, the RC benefits from energy harvested by the entire network, which increases the probability of generating an
electron in the RC. For instance, 155 Chls (a and b) and 35 carotenoids (26 β-carotene, five lutein and four violaxanthin molecules) compose the PSI pigment network in higher plants ((5), Figure 2).

Chls are substituted porphyrins whose nitrogen atoms coordinate a central magnesium atom. The conjugated doubled bonds of the porphyrin ring permit π-electrons delocalization and the absorption of visible light. The various substituents to the ring change its molecule symmetry tuning the absorption properties. A long phytol chain makes the molecule hydrophobic. Cars are linear polyene chains potentially terminated by rings at one or both ends. Xanthophylls contain oxygen in their molecular structure while carotenes are unsaturated hydrocarbons. Depending on the length of the conjugated system different wavelengths can be absorbed.

According to the Franck-Condon principle, the most probable transition from the ground state will occur with the vibrational level of the excited electronic state whose wavefunction overlaps the most with the one of the ground state. In the excited state, the electrons reorganize which modifies the energy landscape compare to the ground state. In Figure 3, electronic transitions are represented for Chl b: the most probable transitions are \((S_0, v_0)\) to \((S_n, v_2)\) in the blue region and \((S_0, v_0)\) to \((S_1, v_2)\) in the red region \((v_2\) is an arbitrary
choice to visualize the principle). These most probable transitions are named the Soret bands in the blue and the Q bands in the red. If there is no overlap of the wavefunctions, the transition is not possible (also said “forbidden”). An $S_0$-$S_1$ transition is forbidden for Cars leading to the absence of red absorption.

Figure 3: (A) Absorption (dark green) and fluorescence (brown) spectra of Chls $b$ (in acetone) schematized by (B) a simplified Jablonski diagram. Different excited levels are populated after absorption (solid red or blue arrows) and depopulated after fluorescence (solid brown arrow) or internal conversion (IC, blue and red dashed arrow). Other de-excitation pathways are possible but not represented (see main text).

The absorption intensity is also determined by the polarization properties of the transition. For the Chls, $Q_y$ (first excited state, $S_1$ in the Figure 3) and $Q_x$ (second excited state, not represented in Figure 3) transitions correspond to transition dipole moments $\vec{\mu}$ of the molecule that are oriented (more or less) along the two axes of the porphyrin ring (Figure 2). The transition is enhanced when the electric field is polarized parallel to the
transition dipole moments. By comparing the transition strength upon different electric field polarizations, we can estimate the molecule orientation.

The intensity of a transition to one excited state (all vibrational levels together) is quantified by the oscillator strength \( f \). It can be seen as a proportion of electrons in the molecule able to oscillate with the frequencies \( \nu \) of the absorption band of the excited state. The oscillator strength \( f \) is proportional to the area under the absorption spectrum, \( A = \int_{\Delta \nu} \varepsilon(\nu) \, d\nu \) with \( \varepsilon(\nu) \) the extinction coefficient (in \( M^{-1} \cdot cm^{-1} \)). \( \varepsilon(\nu) \) will vary depending on the refractive index \( n \) (6).

The oscillator strength of the \( Q_y \) transition of Chl \( b \) is 0.7 times the one of Chl \( a \) (7).

The dipole strength \( d = \left( \mu \right)^2 \) is another quantification of the transition strength and is related to the extinction coefficient as follow \( d = 9.186 \times 10^{-3} = n \int_{\Delta \nu} \frac{\varepsilon(\nu)}{\nu} \, d\nu \) (in Debye\(^2\), (6, 8, 9)). The dipole strength enables to obtain the radiative rate \( k_{\text{rad}} \) (10). The intrinsic fluorescence lifetimes are consistent with experiment \( (\tau_i = \frac{1}{k_{\text{rad}}} = \frac{\tau_{\text{obs}}}{\Phi_F} \) with \( \tau_{\text{obs}} \) the measured value \textit{in situ} and \( \Phi_F \) the fluorescence yield) when considering carefully the influence of the refractive index (8).

To summarize, energy levels are populated in different proportion depending on the excitation wavelengths, on the selection rules of the Franck-Condon principle and on the orientation of the transition dipole moments of the molecules with respect to the electric field polarization.

After absorption, the excitation energy decays rapidly by successive heat dissipation steps: (i) dissipation to the lowest vibrational level \( (S_n, v_n) \) of the excited state; then (ii) dissipation from \( S_n \) state to one of the vibrational levels of \( S_1 \) (internal conversion, IC in Figure 3) and finally (iii) dissipation to the lowest vibrational state of \( S_1 \). Since internal conversion is very fast (rate constant \( \sim (200 \text{ fs})^{-1} \) (11-13)), very little fluorescence is observed from \( S_2 \) of Cars, and \( S_n \) in Chls exhibits none. Fluorescence is a radiative process during which the molecule returns from \( (S_n, v_0) \) to one of the vibrational levels of the ground state by emission of a photon (Figure 3). From \( S_1 \), both IC and fluorescence are possible with predominant IC in Cars (rate constant \( \sim (10 \text{ ps})^{-1} \) in Cars (14) against \( \sim (50 \text{ ns})^{-1} \) in Chls) and predominant fluorescence in Chls (rate constant \( \sim (2 \mu \text{s})^{-1} \) in Cars and \( \sim (13.3 \text{ ns})^{-1} \) in Chls (15)). Even though \( S_1 - S_0 \) transition is optically forbidden in Cars, this transition can receive some oscillator strength from the strongly allowed \( S_0 - S_2 \) transition after perturbation of the Car structure, explaining the observed fluorescence from \( S_1 \) (13, 15). In addition to fluorescence and IC, excitation energy can decay from \( S_1 \) via inter-system crossing (ISC). ISC consists in a spin-flip of \( S_1 \) leading to the formation of a triplet excited state \( T_1 \) from which phosphorescence will occur. For a Chl, when considering the IC, fluorescence and ISC (rate constant \( \sim (8 \text{ ns})^{-1} \) for Chl ISC (16)) competing pathways, the chance to create a Chl triplet is \( \sim 60\% \). This long living Chl triplet (phosphorescence with a lifetime of ms (17)), if not
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quenched, can react with molecular \(^3\)O\(_2\) (triplet in its ground state) and form reactive oxygen species (ROS). Singlet oxygen \((^1\text{O}_2)\) and hydroxyl radicals HO\(^-\) are the most reactive ROS leading to dramatic photodamage, such as oxidation of lipids, proteins and nucleic acids (18). Several photoprotective mechanisms exist (19-21) to avoid formation of ROS (Chl triplet interaction with Cars to form Car triplet which are lower in energy than singlet oxygen) or to quench them (direct scavenging of the ROS by Cars). The generalized coordinates (or the bond length in the case of a diatomic molecule) differ between \(S_1\) and \(S_0\) states and the energy landscapes of the two states do not overlap (Figure 3). During emission, different vibrational levels of the ground state will be populated depending on their wavefunction overlaps with \((S_1, v_0)\). The most probable transition energy is not expected to be with \((S_0, v_0)\) but rather with higher vibrational levels of \(S_0\) instead. This will result in the shift of the fluorescence maximum toward lower energy as compared with the absorption maximum in the \(Q_y\) (Figure 2). The difference in energy is called the reorganization energy (Figure 3) which is approximated as half of the Stokes Shift (22).

The proteic environment influences the electron distribution and therefore the energy levels of the pigments. The influence of the environment on a single pigment is described by the homogeneous and inhomogeneous broadenings of the absorption (or fluorescence) band (Figure 4). One pigment in a specific protein binding-site will experience (even slight) conformational changes of the protein. The \((S_0, v_0)\leftrightarrow(S_1, v_0)\) transition (also called the zero-phonon line) can take as many values as protein conformations whose distribution determines the inhomogeneous broadening. The inhomogeneous broadening is well described by a gaussian distribution (23) whose full width at half maximum (FWHM) \(\Gamma_{\text{inhom}}\) is represented in Figure 4.
Figure 4: Scheme representing contribution of the homogeneous and inhomogeneous broadenings in the absorption or fluorescence spectra of a Chl in a proteic environment. As an illustration in PSI (PDB 4XY8 (5)), Chl a1301 (nomenclature (24)) bound to PsaF (by a water molecule) experiences two conformations (either blue or orange) of the protein. With the decrease in temperature, the phonons of the protein are vanishing (from full color to dimmed color).

The homogeneous broadening corresponds to the broadening of the zero-phonon line by the phonon side-wing. Phonons are low-frequency vibrations of the protein (the bath) that can couple with the electronic transition. The difference in energy between the zero-phonon line and the phonon side-wing maximum corresponds to the reorganization energy which equals the product of $S$, the strength of the electron-phonon coupling (or Huang-Rhys factor), and $\nu$ the mean frequency of the protein matrix phonons (22, 25). The total broadening of the electronic transition is characterized by a FWHM $\Gamma_{\text{tot}}$ whose square $\Gamma_{\text{tot}}^2$ equals $\Gamma_{\text{inhom}}^2 + \Gamma_{\text{hom}}^2$.

We can consider now the absorption of a protein that binds more than one pigment. Each of them will have different maximum corresponding to different binding sites in the protein. Nevertheless, each absorbing form will have a contribution which can be decomposed in homogeneous and inhomogeneous broadenings as represented in Figure 5A for two different Chls $a$. Summing all the absorbing forms’ contributions will result in the pigment-protein complex absorption spectrum (black in Figure 5B).
Figure 5: (A) Absorption spectra of two different Chls $a$ decomposed in homogeneous (pink) and inhomogeneous (black) broadenings. (B) Absorption spectrum of a pigment-protein complex (black) containing several Chls $a$ (dark cyan) and Chls $b$ (dark orange).

**PSs: Core & Lhcs. Differences between organisms**

The harvesting capacities of the PSs are achieved thanks to a very large number of pigments, either Chls or Cars, bound to different subunits of the PSs. Even though different, the subunits of PSI and PSII antenna systems can be grouped in two moieties: the core (or inner) and the peripheral (or outer) antennae. The core antenna only binds Chls $a$ while the peripheral antenna can bind other types of Chls, like Chls $a$ and $b$ in plants and algae.

**Well conserved core complexes**

In cyanobacteria, PSII core is made of 20 subunits binding 35 Chls $a$ and 11 β-carotenes (26) and PSI core is made of 12 subunits binding 98 Chls $a$ and 22 β-carotenes (24). The core of both PSs in eukaryotic organisms is almost identical to that of cyanobacteria (27). In particular, in higher plants (5, 28, 29), 12 subunits of the PSI core (PsaA-L) have been resolved among which 10 are homologous to cyanobacterial PSI (24, 30). PsaG, PsaH, PsaN and PsaO are four subunits unique to PSI core of higher plants (31, 32) but the two latter have not been resolved in the crystal structures yet. In higher plants, PsaG interacts with PsaB and the peripheral antenna (5, 29). This contributes to stabilize the whole supercomplex which was still observed in the absence of PsaG but to a smaller extent (33, 34). PsaH is located in higher plants where monomers interact in cyanobacterial PSI trimer (28, 30) and is important for the overall stability of higher plant PSI (5, 29, 35). Furthermore, PsaH was observed to interact during state transition with Lhcb4 (36, 37). PsaO was also reported to play a role during state transition in binding Lhcb4 (38). (Lhcb4 and state transition are described in the followings). No crystal structure is available for the PSI of the green alga *Chlamydomonas reinhardtii*. However, because of the well conserved core subunits compared with cyanobacteria and higher plant (39), we expect the same structural
features for PSI core of *C. reinhardtii*. The pigments composition is also very similar between cyanobacterial PSI and eukaryotic PSI cores (5, 24, 29).

**Peripheral antenna complexes**

On the contrary, the peripheral antennae of eukaryotic PSI vary a lot, not only as compared with cyanobacteria but also between different eukaryotic organisms (40). In cyanobacteria, the soluble proteins, which are called phycobilisomes, serve as peripheral antenna of both PSs, whereas, in plants and green algae, the peripheral antenna is made of trans-membrane proteins called Light Harvesting Complexes (LHC), either LHCIs (or Lhcas) for PSI or LHCIIIs (or Lhcbis) for PSII. Concerning PSI-LHCI of eukaryotic organisms, large differences have been observed: PSI outer antenna of *C. reinhardtii* is more than twice larger than in higher plants (Figure 6). More precisely, among the six Lhca genes reported in *Arabidopsis thaliana* (41), Lhca1-4 encode for PSI peripheral antenna (28, 42, 43) while the Lhca5 and Lhca6 proteins are present in sub-stoichiometric amount with the PSI core (44). In *C. reinhardtii*, nine genes were related to PSI outer antenna (45, 46) with all of them being expressed. The products of all the genes assembled in the PSI supercomplex (47, 48) in the form of two concentric half rings on one side of the core ((48), Figure 6). The pigments number of the PSI core is increased by 58% with the presence of LHCIs in higher plants and by 128% in *C. reinhardtii* (Table 1) if we consider that each LHCI binds 14.25 Chls (on average) and three Cars (5).

![Figure 6: EM picture of PSI-LHCI in *C. reinhardtii* (48) superimposed with the crystal structure of PSI-LHCI in higher plants ((49), PDB 2WSC, stromal view) with the core antenna (green) and the peripheral antenna (brown). The core subunits PsaH and PsaL are represented in yellow and pink respectively. The asterisks locate the additional LHCIs present in *C. reinhardtii*. Blue and yellow arrows correspond to either Lhca2 or Lhca9 (of interest in the followings). Scale bar 10 nm. Figure modified from (48).](image)

Concerning PSII peripheral antenna, many Lhcb genes produce six proteins in *A. thaliana* (41) and 11 in *C. reinhardtii* (50, 51). LHCII trimers, the major LHCIIIs, are constituted by three Lhcb proteins in *A. thaliana* (Lhcb1-3) and by nine in *C. reinhardtii* (LhcbM1-9). Two trimers per monomeric PSII core were found in *A. thaliana* PSII supercomplex (52-55) versus three in *C. reinhardtii* (56, 57). Monomeric antennae in PSII-LHCII, which also called minor
antennae, are located between the core and the trimers. Three per monomeric PSII core were found in *A. thaliana* PSII supercomplex, namely CP24 (Lhcb6), CP26 (Lhcb5) and CP29 (Lhcb4) while only two are present in *C. reinhardtii*. CP24 is missing in *C. reinhardtii* PSII-LHClI supercomplex and its position is occupied by one LHClI trimer instead (57). Considering the number of Chls and Cars in each LHC (14 Chls and four Cars per monomer in the LHClI trimer (58), 10 Chls and two Cars estimated in CP24 (59, 60), 14 Chls and four Cars in CP26 that we assume to have the same pigment composition as a monomer of the LHClI trimer, 13 Chls and three Cars in CP29 (61)), the pigments number of the PSII core antenna is increased by 333% with the presence of Lhcbs in higher plants and by 424% in *C. reinhardtii* (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Core antenna</th>
<th>Peripheral antenna</th>
<th>Total for each type of pigments (and increase of core antenna %)</th>
<th>Total of Chls+Cars (and increase of core antenna %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI-LHCI</td>
<td>Chls 98</td>
<td>57</td>
<td>127</td>
<td>155 (58%)</td>
</tr>
<tr>
<td></td>
<td>Cars 22</td>
<td>13</td>
<td>27</td>
<td>35 (59%)</td>
</tr>
<tr>
<td>PSII-LHClI (monomeric)</td>
<td>Chls 35</td>
<td>121</td>
<td>153</td>
<td>156 (346%)</td>
</tr>
<tr>
<td></td>
<td>Cars 11</td>
<td>32</td>
<td>42</td>
<td>43 (290%)</td>
</tr>
</tbody>
</table>

Table 1: Estimation of the Chls and Cars number in PSI and PSII of *C. reinhardtii* (C.r.) and *A. thaliana* (A.t.).

Not only the PS peripheral antenna size differs between organisms but also their affinity for Chl b: the Chl a/b ratio of LHCs in *C. reinhardtii* is lower than in higher plants; but in both organisms, the Chls a/b ratio of Lhcbs is lower than in Lhcas (5, 58, 62-64). Furthermore, *C. reinhardtii* has a particular Car composition with the presence of loroxanthin (48, 65), in addition to β-carotene, lutein, violaxanthin and neoxanthin present in higher plants.

**Light harvesting capacities versus PS efficiency?**

The question is whether ETC and PS performances always increase with the increase of the light harvesting capacities. This thesis focuses on light harvesting, excitation energy transfer (EET) and trapping capacities of PSI which determine the overall trapping efficiency of this photosystem.

The trapping efficiency $\Phi_{CS}$ is the quantum yield of CS and can be measured by comparing the excitation lifetime in the two following cases: when the excitation energy promotes CS or not. When CS is possible, this channel competes efficiently with ISC so that
the possibility to form triplets is strongly reduced (see above). Aside CS and ISC, fluorescence is another pathway for the energy to decay. As it can be measured, fluorescence gives indirectly access to CS yield, if all the other decay rates are unchanged. \( \Phi_{CS} \) can then be written as \( \Phi_{CS} = 1 - \frac{\tau_{CS}}{\tau_{no\ CS}} \) (15) where \( \tau_{CS} \) is the fluorescence lifetime (called average decay time, in the followings) when the RC is able of CS, and \( \tau_{no\ CS} \) the one when CS does not occur.

The average decay time \( \tau_{CS} \) can be interpreted in terms of migration and trapping times such as \( \tau_{CS} = \tau_{mig} + \tau_{trap} \) (15, 66, 67). The migration time \( \tau_{mig} \) is the time required to reach thermal equilibrium and is the time for the excitation to arrive at the RC for the first time. If this component dominates, the process is *diffusion-limited*. The migration time can contain a term representing the time of the last energy transfer step to the RC Chls therefore called delivery time \( \tau_{del} \). If this term dominates, the diffusion process is called *transfer-to-the-trap* limited (68). The trapping time \( \tau_{trap} \) is the ratio between the intrinsic time of the CS \( \tau_{iCS} \), and the probability that the excitation is located on the RC after thermal equilibration. This probability decreases with the increasing number of pigments, i.e. the antenna size. In a simple situation of the antenna composed of only isoenergetic pigments, the trapping time would be \( \tau_{trap} = N^{*}\tau_{iCS} \) with \( N \) the number of isoenergetic pigments in the antenna system. \( \tau_{trap} \) does not depend on the initial excitation location. If this term dominates, the process is *trap-limited*. If the antenna system is modelled as an ensemble of very well coupled (infinitely fast migration time) isoenergetic pigments, \( \tau_{CS} = \tau_{trap} = N^{*}\tau_{iCS} \). The reality is more complex and the three contributions (diffusion, transfer-to-the-trap and trap) in \( \tau_{CS} \) of PSI-LHCI are under debate (69).

**Excitation energy transfer**

The migration of the excitation energy in the antenna systems depends on the types of interaction between pigments. When bound to proteins, pigments are oriented and separated in a specific way that will influence their interaction (3, 15). To picture interaction of several pigments (isoenergetic if \( S_1 \) levels have the same energy \( \varepsilon_e \)), we usually represent them by superimposing their energy diagrams (Figure 7A). We can also think of them as an ensemble by describing the state space (Figure 7B): the ground state of the ensemble is when all the pigments are in their ground state (\(|g>\) ), \( n \) states correspond to the first exciton states when one of them is in \( S_1 \) (\(|e>\) ), \( n^{*}(n-1)/2 \) states correspond to the second exciton states when two of them are in \( S_1 \)...etc. If the interaction of each pigment with the related bath (independent bath for each pigment) dominates above the interaction between pigments, the first exciton states will all have the same energy \( \varepsilon_e \) (degenerated energy
levels) and the EET will be described in the frame of the Förster theory (red arrows in Figure 7B).

![Figure 7: Excitation energy transfer processes](image)

If the interaction between pigments is larger than the one with the baths, the first exciton states will have energy around $\varepsilon_e$ within an energy band ($\Delta \varepsilon_e$). In this case, interaction between pigments can be described in the frame of the Redfield theory (red arrow in Figure 7C) and the excitation delocalized over the ensemble. Since the exciton states of the first band are not eigenstates of the individual pigments, the excitation of the individual pigments will oscillate (Figure 7C). The population of each state will also depend on the orientation of the transition dipole moments of the related pigments as well as on their spatial arrangement (15). Energy exchange with the bath, even though weak, triggers transition to other energy states and finally result in a spatial migration of the excitation. A lot of debate exists in the scientific community whether different exciton states lead to coherent oscillations. Whether this excitonic coherence exists long enough to be of relevancy for biological processes is beyond this thesis scope but a review is available in (70).

To come back to the Förster theory, the interaction energy $V$ between weakly coupled pigments D and A can be approximated by a dipole-dipole interaction:

$$V = \frac{1}{4\pi\varepsilon} \frac{\mu_D \cdot \mu_A^*}{R_{DA}} 3(\mu_D \cdot \mathbf{r}_{DA})(\mu_A^* \cdot \mathbf{r}_{DA})$$

where $\mu_D$, $\mu_A$ are the transition dipole moments of D and A respectively (in Debye), $\mathbf{r}_{DA}$ is the normalized vector between the centers of D and A distant of $R_{DA}$, and $\varepsilon$ is the permittivity of the medium. The relative orientation of the transition dipole moments as well as their arrangement in the pair, together with the distance between the two pigments will influence the strength of the interaction. The EET is described between a donor (D) that transfers its energy to an acceptor (A) with a rate
described by the Förster equation (71-73): $k_{DA} = \frac{1}{\tau_R^6} \frac{R_0^6}{R_{DA}^6}$ where $\tau_R$ is the intrinsic radiative lifetime of the donor D and $R_0$ the Förster radius such as $R_0^6 = \frac{9 \ln 10}{128 \pi^6 c^4 N n^4} \kappa^2 J(v)$ where $c$ is the speed of light in vacuum, $N$ the Avogadro number, $n$ the refractive index of the surrounding, $\kappa$ the dimensionless orientation factor $\kappa = \mathbf{\mu}_D \cdot \mathbf{\mu}_A - 3 (\mathbf{\mu}_D \cdot \mathbf{r}_{DA}) (\mathbf{\mu}_A \cdot \mathbf{r}_{DA})$ which uses the normalized transition dipole moments $\mathbf{\mu}_D$, $\mathbf{\mu}_A$ of D and A respectively) and $J(v)$ the spectral overlap integral $J(v) = \int_0^\infty \frac{\varepsilon_A(v) F_D(v)}{v^4} \, dv$ with $\varepsilon_A(v)$ the molar extinction coefficient of the acceptor and $F_D(v)$ the fluorescence emission of the donor (normalized to 1 on the frequency scale $v$). If the spectral overlap integral $J(v)$ increases, the rate of energy transfer from D to A increases. When pigments are isoenergetic, they have similar absorption and emission spectra (represented in Figure 8 with the Stokes shift) which overlap over some frequency (case 1). For non isoenergetic pigments, energy transfer to a pigment lower in energy will be faster (larger integral in case 2 than in case 1, Figure 8) while the transfer from this pigment lower in energy will be slower (case 3).

Figure 8: Overlap integral (green hatch) between the fluorescence emission (dash) of a donor (D) and the molar extinction coefficient (solid) of an acceptor (A). The two pigments 1 and 2 are either isoenergetic (Case 1) or non isoenergetic, with the more energetic pigment 1 (black) being the donor (Case 2) or the acceptor (Case 3) of energy to (or from, respectively) a less energetic pigment (red).

The forward and backward rate constants ratio between pigment 1 and pigment 2 is expressed at equilibrium using the (Gibbs) free energy difference $\Delta G$ function of the equilibrium constant $K$: $\Delta G_{12} = G_2 - G_1 = -k_B T \ln(K_{1\to2})$ with $K_{1\to2} = k_{1\to2}/k_{2\to1}$, where $k_{1\to2}$ is the rate constant of EET from pigment 1 to pigment 2 (and reverse for $k_{2\to1}$), $k_B$ the Boltzmann constant and $T$ the absolute temperature. Under constant pressure and temperature, a negative free energy difference corresponds to a spontaneous process. The free energy difference is defined as $\Delta G_{12} = \Delta H_{12} - T \Delta S_{12}$ with $H$ the enthalpy and $S$ the entropy. When we consider only two pigments, the entropy difference is zero and the rate constants are
directly related to the energy levels of the pigments (when $S_1$ energy levels are taken to represent the enthalpy). A transfer from high energy pigment to low energy pigment will be “spontaneous” ($K_{1\rightarrow 2} > 1$, down-hill) while the reverse transfer not ($K_{2\rightarrow 1} < 1$, up-hill). The population of the highest level occurs thanks to thermal disorder.

The populations of the two levels are related by the Boltzmann distribution $P_2/P_1 = \exp\left(\frac{(E_2 - E_1)}{k_B T}\right)$ with $E$ the $S_1$ energy levels. When the temperature decreases, the pigment with the lowest $S_1$ will be populated the most. It follows that the 77K fluorescence is dominated by the “red” forms emission. When there is more than one pigment of each type, the pool size of pigments 1 ($N_1$) and pigments 2 ($N_2$) will lead to an entropy difference $\Delta S_{12} = -k_B \ln\left(\frac{N_1}{N_2}\right)$. The entropy difference will possibly contribute to the free energy difference so that the latter become negative and the transfer spontaneous even though up-hill at first. The detailed balance $\frac{k_{1\rightarrow 2}}{k_{2\rightarrow 1}} = \frac{N_2}{N_1} \exp\left(\frac{(H_2 - H_1)}{k_B T}\right)$ characterizes equilibrium between pools of pigments.

To summarize, the type of interactions will define the EET between pigments described either in the frame of Redfield theory or Förster theory. Both types of interaction take place in the antenna systems. The Förster theory applies for transfer times above 1 ps. The transfer between two isoenergetic Chls $\alpha$ takes $\sim$1.3 ps on average when they are distant by 1.5 nm and randomly oriented (15). We will use the Förster theory to study the EET between weakly coupled non isoenergetic Chls $\alpha$ in the antenna systems in PSI-LHCI. In particular, we will focus our interest on special Chls which absorb at lower energy than the RC or, more generally, than the bulk Chls $\alpha$, the so called “red forms” (63, 74-77).

This thesis aims at characterizing the EET and trapping kinetics of PSI-LHCI in order to determine PSI efficiency relative to the size and spectral properties of the antenna. We will thus focus on describing PSI-LHCI in the followings.

**PSI-LHCI enriched in red forms**

The “red forms” are excitonically coupled Chls $\alpha$ (43, 78-81) whose lowest exciton state mix with a charge transfer (CT) state (80, 82-85). Since the red form excited state has a CT character, its electron distribution will be very different from the ground state leading to significantly different dipole moments between ground and excited states. This explains the large Stokes shift and the large homogeneous broadening observed for the red forms (63, 76, 80, 82, 86). Furthermore, from the CT character, the dipole moment of the red form excited state will easily feel polar changes in the proteic environment cause by (even small) conformational changes of the protein. Both homogeneous and inhomogeneous broadenings of red-form containing antennae range between 170-360 cm$^{-1}$, each depending
on species, (23, 80, 82, 87) and are larger than broadenings reported for red-forms devoid antennae, like LHCII (88, 89).

The red forms can be located in both core and/or peripheral antennae (Figure 9). In the core antenna, candidates were proposed based on potentially strong excitonic interactions calculated from the crystal structure of cyanobacterial PSI ((24), purple in Figure 9).

Figure 9: Stromal and transmembrane views of PSI-LHCI of higher plants (PDB 4XK8 (5)) showing the red forms candidates in the core antenna (purple, PDB 1JBO) and the red forms characterized in the LHCIs of higher plants (red, PDB 4XK8).

Despite very similar PSI core subunits’ structure and pigment organization (see above), the red forms composition varies tremendously between organisms (77, 90, 91) possibility because the CT character gives to the red forms the capacity to sense even small
conformational changes of the protein. Gaussian deconvolutions of 6K absorption spectra permitted to identify several pools of red forms in cyanobacteria absorbing at 708, 719 or 740 nm (75, 90). One additional pool absorbing at 714 nm was identified by hole-burning studies (85, 92-94). Trimeric PSI is enriched in red forms compared to the monomeric PSI (75) with the red-most forms found at 740 nm in Arthospira platensis PSI trimers (90, 95-97). Only one pool at 705 nm was reported in higher plant PSI core (76). Time-resolved measurements do not confirm the presence of this pool in the core of higher plants (98). Similarly in C. reinhardtii, identification of red forms in the core is still debated (87, 99-105). At 77K, PSI-LHCI of C. reinhardtii emits at 712-717 nm (48, 106) then at higher energy than A. thaliana PSI-LHCI (maximum at 735 nm, (107)).

Even though well conserved between species, a noticeable difference in the PSI core structure is the size of the PsaB loop on the luminal side, whose extension was proposed to stabilize a Chl trimer B31-B32-B33 possibly the most red forms in Synechococcus elongatus (24). This PsaB extension is missing in other cyanobacteria and eukaryotes, which could destabilize the Chl trimer stacking and explain the differences in the energy levels of the red forms between these species (30, 49). A Chl bound to PsaG and Lhca1 has been recently found in higher plants (5, 29) and results in the formation of another stacked Chl trimer at the same place as in S. elongatus, even in the absence of the extended PsaB loop. This luminal trimer was therefore suggested to be responsible for the reddest forms also in higher plant PSI core, which somehow are not as low in energy as in S. elongatus.

More is known about the red forms associated with the peripheral antennae. Lhca3 and Lhca4 in higher plants have red forms that absorb at 704 nm and 708 nm respectively (80, 108). Lhca2, Lhca4 and Lhca9 in C. reinhardtii present characteristic features of red forms containing antenna, but given that their red absorption spectra at 77K do not show obvious structures, the lowest energy state could be determine only for Lhca9 and Lhca2 for which a contribution in the second derivative is detected at 692 nm and 693-697 nm, respectively (63). The 77K emission spectrum reveals that red forms in C reinhardtii Lhcas are higher in energy than A. thaliana Lhcas: the red emission with a maximum at 717 nm, was observed for Lhca2 in C reinhardtii (63), while Lhca3 and Lhca4 of higher plants emit at 725 nm and 733 nm respectively (80). The less red-shifted 77K emission peak correlates linearly with a decrease of Stokes shift in C reinhardtii (63).

Different roles were attributed to the red forms: (i) they increase the absorption cross section of PSI-LHCI in the red/far-red region (109) and (ii) in a context of non-equilibrium, they could help in concentrating excitation close to the RC (102, 110). Because of a CT character, the red forms were proposed to act as quenchers (82, 111) although no
correlation between the presence of the red forms and the lifetime of the complexes was observed (62, 112, 113).

Last but not least, the red forms’ influence on the EET and trapping kinetics of PSI has been observed in different organisms. The average decay time is ~22 ps for the PSI core of higher plants (114) and ranges from ~20 ps to ~40 ps in different cyanobacteria species depending on the red form content (90): the more red forms, the slower the trapping kinetics of the PSI core. Cyanobacterial PSI devoid of red forms has a lifetime of ~14ps (115). The red forms present in the peripheral antenna of eukaryotic organisms were also shown to significantly slow down the EET and trapping kinetics of PSI-LHCI (76, 114, 116, 117). In A. thaliana, the “blue” antenna complexes (low content of red forms, Lhca1 and Lhca2) transfer excitation energy to the core four time faster than the “red” antenna (high content of red forms, Lhca3 and Lhca4)(114). The overall lifetime of PSI-LHCI is ~50 ps in higher plants (114).

**Trapping on the RC of PSI**

The cofactors of the ETC of PSI form two symmetrical branches (one branch bound to PsaA and the other one to PsaB) arranged in three pairs of Chls a (P700, A, A0) and one pair of phylloquinone (A1) (Figure 10). Both branches transfer electrons to the sulfur-iron clusters (FX, FA, FB) on the stromal side. In *C. reinhardtii*, the PSI RC was reported to absorb at ~697 nm (100, 103, 118, 119). Since *C. reinhardtii* PSI core particles have very little absorption above 700 nm (100, 101), electron transfer steps of the RC could be well characterized in this alga: the accessory Chl A was revealed to be the primary electron donor, whereas P700 is oxidized in a second electron transfer step (2, 103, 105, 120)(Figure 10).
Figure 10: The cofactors of the PSI electron transport chain are in the middle of the PSI Chl network (from PDB 4XK8): view from the stromal side (spheres) or from two transmembrane sides (as indicated). The radical pairs are defined as proposed in (105) and (120).

The first radical pair $A^+A_0^-$ was shown to form after $\sim$6 ps. Within $\sim$2 ps, the antenna excited-state equilibrium seems to be completed and the red emission observed in PSI core was attributed to repopulation of the exciton state of the six RC Chls after recombination of the first radical pair (98, 104). In this model, which is called the “charge recombination model”, PSI is described as a shallow trap, limiting the decay kinetics of the supercomplex. Nevertheless, cyanobacterial PSI devoid of red forms has its fluorescence quantum yield drastically decreased at 77K (115) suggesting that the RC Chls are very good quenchers. As shown in higher plants (121), we have also observed that P700 and P700$^+$ in *C. reinhardtii* have similar quenching efficiency (data not shown). Other works proposed to model PSI kinetics differently: the first radical pair was only formed after $\sim$20 ps because of a limiting migration time (76, 122), or because of a shallow-trap not able to recombine (100, 118, 123). Because PSI kinetics can be modeled in multiple ways, conclusions on purely trap-limited or purely diffusion-limited kinetics are not possible (124).

**Acclimation**

As mentioned above, the quantum yield of the PSs depends on the absorption spectra of the PSs, their antenna size, the loss of excitation in the antennae before CS
(excitation energy transfer efficiency and photoprotection) and the trapping. These factors are adjusted to enable the highest performances of the ETC and the least photodamage (125) by remodeling the photosynthetic membrane either on a short time scale after sudden change of light intensity/quality (short term acclimation) or on a long time scale when change of light is maintained (long term acclimation). Both types of acclimation can lead LHCII to be part of PSI peripheral antenna. Different PSI core subunits were reported to interact with Lhcbs (see above).

**Long term acclimation.** In higher plants, in addition to the adjustment of the RC stoichiometry (126-128), the long term acclimation involves regulation of Lhcb genes expression (129, 130). Indeed, Lhcbs were shown to function as antenna for both PSs: under continuous growing light, 40% to 65% of the PSI contains Lhcbs in its peripheral antenna (131). Less is known in C. reinhardtii.

**Short term acclimation.** After sudden change of light intensity/quality, the excitation energy is redistributed between PSs via migration of Lhcbs from one PS to another. This process is known as state transitions (132-134). State 1 occurs when all Lhcbs transfer their excitation energy to PSII and State 2 when part of the Lhcbs transfer their energy to PSI (135, 136). After low light growing conditions, we can calculate an equivalent of 1-2 “extra” LHCII trimers per monomeric PSII in higher plants (130) and 3-4 in C. reinhardtii (65) when considering the Chls a/b ratio in the cells (2.7 in A. thaliana (137) and 2.3 in C. reinhardtii (65) respectively), the PSI/PSII ratio (0.71 after growth under 20 uE.m\(^{-2}\).s\(^{-1}\) in A. thaliana (130) and 0.97 in C. reinhardtii in 20 uE.m\(^{-2}\).s\(^{-1}\) (65)) and the total number of Chls estimated in each PSs (Table 1). The pool size of the “extra” LHCI in the membrane can vary with the light intensity (130, 137). These “extra” Lhcbs were shown to be involved in state transitions in higher plants (138, 139). In C. reinhardtii, how the “extra” LHCI trimers are involved in state transitions is under debate. Several recent studies report very few Lhcbs migrating to PSI under State 2 in C. reinhardtii (140, 141) instead of 80% of the Lhcbs reported before (142). Ünlü et al. (141) found that the absorption cross section of PSI-LHCl in C. reinhardtii cells increases by less than one LHClI trimer in state 2.

**PSI-LHClI-LHCII.** The size of isolated PSI-LHClI-LHCII depends on the protocol of purification, which if too strong can dissociate possibly weakly connected Lhcbs. PSI-LHClI-LHCII from plants has been purified with only one LHCII trimer (138, 143). In partially solubilized membranes (using styrene-maleic acid copolymers), supercomplex with three trimers could be obtained in higher plants (144). In C. reinhardtii, PSI-LHClI-LHCII was isolated with different antenna size (65, 145-147). The largest PSI-LHClI-LHCII isolated so far ((57), Figure 11) contains seven Lhcbs (two LHCII trimers and one monomer located on the PsaH/L side of the core), in addition to the nine Lhcas located on the other side of the core (48).
Figure 11: EM picture of PSI-LHCI-LHCII in *C. reinhardtii* (57) overimposed with the crystal structure of PSI-LHCI in higher plants ((49), stromal view) extended by five Lhcas in a second outer ring (light brown) and seven Lhcbs on the other side (green and yellow). Scale bar 10 nm. Figure modified from (57).

The PSI-LHCI-LHCII supercomplex of *C. reinhardtii* PSI binds 322 Chls and 76 Cars (see SI of Chapter 3 for details on this estimation) and thus 45% more pigments than PSI-LHCI resulting in a significant increase of light harvesting capacities.

**Time resolved fluorescence with the streak camera**

To study the EET and trapping kinetics of PSI complexes as a function of the antenna composition size and organization, we used time-resolved fluorescence measurements at a picosecond time-scale. Because of the pigment composition of the core antenna (with Chl *a* only) is different from the peripheral antenna (Chl *a* and *b*), it was possible to excite differently the two parts of the supercomplex by setting excitation wavelengths corresponding to preferential absorption of one pigment or another (117).

In this thesis, picosecond-time-resolved fluorescence measurements were performed mainly with a streak camera setup described previously in (90, 148) with some adaptations as in Figure 12.
Chapter 1

A Coherent Vitesse Duo contains an integrated 10W Verdi CW laser (output wavelength 532 nm) that seeds the Vitesse solid-state ultrafast Ti:S oscillator (output wavelength 800nm, average power ≈100 mW, pulse width ≈100 fs, repetition rate 80 MHz) and pumps the regenerative amplifier Coherent RegA 9000 (output wavelength 800 nm, average power ≈1W, pulse width 180-200 fs, tunable repetition rate between 10 kHz and 300 kHz).

The output of the RegA feeds the optical parametric amplifier Coherent OPA 9400 (output wavelength from 470 nm to 770 nm, average power up to a few mW). The frequency-doubled light (400 nm) in the OPA could also be used as an output. The repetition rate was set to 250 kHz and the OPA was set to generate either the 400 nm or the 475 nm excitation wavelength in all experiments reported in this thesis.

The light intensity was modulated and residual 800 nm light and white light from the OPA was removed. The excitation polarization was set vertical with a Berek polarization compensator (New Focus, model 5540). The light was focused in the sample with a 15 cm focal length lens resulting in a spot diameter of 50 μm in the sample. Fluorescence emission was collected at right angle by two identical achromatic lenses (B. Halle UV-Achromat f=100 mm) to collimate the light and then focus it on the input slit (100 μm) of a spectrograph (Chromex 250IS, 50 grooves/mm ruling, blaze wavelength 600 nm, spectral resolution of 2nm). Scattered excitation light was removed with an optical long-pass filter. A polarizing filter (Spindler & Hoyer, Type 10K) was placed in between the two achromatic lenses to...
collect light polarized at magic angle (54.7°) with respect to the excitation polarization. After the spectrograph, the light was focused on the input slit (40 μm) then on the photo-cathode of the streak camera Hamamatsu C5680 mounted with the M5675 Synchroscan unit (triggered by the Vitesse oscillator) and the Digital CCD Camera Hamamatsu Orca R2 (read out speed 8.5 frame/s). From the photo-cathode of the Syncroscan unit, photo-electrons will go through an electric field whose amplitude increases linearly with time: early electron will be less deviated than late electrons. Electrons will impact the phosphor screen (and then the CDD) at different spots depending on their time of generation at the photo-cathode. In other words, the delay of emission from the sample, i.e. the fluorescence decay, is mapped along the vertical dimension. An example of a streak camera image obtained through measurement is given in Figure 12.

Spectral calibration was done with an Argon lamp (Oriel Instruments Argon lamp model 6030) and spectrotemporal sensitivity (shading) correction (148) with a homogeneous white light source (Xenon lamp, Osram HLX 64642 24V 150W GER i 028). The time resolution depends on optics, filters used after the sample but predominately on the grating ruling, the spectrograph input slit (100 μm), the streak camera input slit (40 μm) and on the streak camera detector. The streak camera set-up used in this thesis has a final instrument response function (IRF) of 3-4 ps corresponding to a time resolution of ~2 ps.
CHAPTER 2

PSI-LHCI of Chlamydomonas reinhardtii: increasing the absorption cross section without losing efficiency

Clotilde Le Quiniou, Lijin Tian, Bartłomiej Drop, Emilie Wientjes, Ivo van Stokkum, Bart van Oort, Roberta Croce

This chapter is based on:

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ABSTRACT
Photosystem I (PSI) is an essential component of photosynthetic membranes. Despite the high sequence and structural homology, its absorption properties differ substantially in algae, plants and cyanobacteria. In particular it is characterized by the presence of low-energy chlorophylls (red forms), the number and the energy of which vary in different organisms. The PSI-LHCI (PSI-light harvesting complex I) complex of the green alga Chlamydomonas reinhardtii is significantly larger than that of plants, containing five additional light-harvesting complexes (together binding ≈65 chlorophylls), and contains red forms with higher energy than plants. To understand how these differences influence excitation energy transfer and trapping in the system, we studied two PSI-LHCI particles of C. reinhardtii, differing in antenna size and red form content, using time-resolved fluorescence and compared them to plant PSI-LHCI. The excited state kinetics in C. reinhardtii shows the same average lifetime (50ps) as in plants suggesting that the effect of antenna enlargement is compensated by higher energy red forms. The system equilibrates very fast, indicating that all LHClIs are well-connected, despite their long distance to the core. The differences between C. reinhardtii PSI-LHCI with and without Lhca2 and Lhca9 show that these LHClIs bind red forms, although not the red-most. Those are in (or functionally close to) LHClIs and slow down the trapping, but hardly affect the quantum efficiency, which remains as high as 97% even in a complex that contains 235 chlorophylls.

INTRODUCTION
Photosynthesis provides energy for nearly all life on Earth. In the first step of photosynthesis, light is harvested by photosystem (PS) I and II, which are multi-protein complexes embedded in the thylakoid membrane. Their light-harvesting system is composed of a large number of pigments coordinated by proteins. In both PSs the excitation energy is efficiently transferred to the reaction center (RC) where charge separation (CS) occurs (3). In eukaryotic organisms, as the unicellular green alga Chlamydomonas reinhardtii, PSI can be divided in two main parts: the core and the peripheral antennae. The core complex is composed of 14 protein subunits (PsaA-PsaL, PsaN and PsaO), together binding ≈100 chlorophylls (Chls) and ≈20 carotenoids (Cars)(24). The core coordinates only Chls a, a few of which constitute the RC. The peripheral antenna, called the light harvesting complex I (LHCI) or Lhcas, is composed of different Lhca gene products that in addition to Chls a and Car, coordinate Chls b (46, 69). In the following we will refer to the PSI supercomplex, composed of the core and peripheral antennae, as PSI-LHCI.

The PSI core complex is very similar among oxygenic photosynthetic organisms (149). Highly conserved structures of PSI core have been observed for cyanobacteria (24, 30) and
higher plants (28, 49). The primary structure of the core proteins of C. *reinhardtii* shows high homology with that of plants and cyanobacteria (31, 32), suggesting a conserved structure. At variance with the core, the peripheral antenna of higher plants and of C. *reinhardtii* shows major differences in the number of genes (44, 150, 151) and in the biochemical/spectroscopic properties of the complexes (63). PSI-LHCI of C. *reinhardtii* contains nine Lhca proteins (Lhca1-9), located on one side of the core, forming two parallel concentric half rings (48), by contrast to higher plant PSI-LHCI that is formed of one half ring made of only four Lhca proteins (28, 152).

Another major difference between PSI-LHCI of C. *reinhardtii*, higher plant and cyanobacteria is the content of red forms. These red forms are Chls with red-shifted absorption and emission, a large Stokes shift and a large bandwidth compared to bulk Chls (e.g. (76, 80, 82, 153)). The red forms dominate the low temperature fluorescence emission and are responsible for the 712-717 nm emission in C. *reinhardtii* cells and isolated C. *reinhardtii* PSI-LHCI (48, 99, 154-156). This maximum is at higher energy than that of *Arabidopsis thaliana* PSI-LHCI (maximum at 735 nm, (107)) and of most cyanobacteria PSI trimers (e.g. 760 nm in *Arthrospira platensis*, previously called *Spirulina platensis*, (77, 90)). Red forms can be associated with the core and/or with the peripheral antenna of PSI-LHCI. Interestingly, despite the high structural homology of PSI core complexes, the red forms present in the core of different organisms can substantially differ in energy (90). In plants the red-most forms are associated with Lhca3 and Lhca4 (108, 157), while the core emits at 720 nm (86). In C. *reinhardtii* red forms are proposed to be associated with both core and peripheral antennae (47, 87, 99, 156, 158-160). More recently, an in vitro study of C. *reinhardtii* Lhcas has shown that Lhca2, Lhca4 and Lhca9 display the most red-shifted emission (63). An oligomer of Lhcas, lacking Lhca2, Lhca3 and Lhca9, isolated from a PSI core-minus mutant, has an emission maximum at 708nm at 77K (47), supporting the attribution of the red-most forms to Lhca2 and Lhca9. Nevertheless, the loss of Lhca2 and Lhca9 did not lead to a blue shift of the emission maximum of PSI-LHCl (48), indicating that those complexes are not the (only) responsible for the red-most emission.

Excitation energy transfer (EET) from the red forms to the RC needs to be thermally activated (161) and in higher plants and cyanobacteria, the red forms slow down the trapping kinetics (90, 114). The average decay time is 23 ps for the PSI core of higher plants (e.g. (98, 114)) and ranges from 23 ps to ≈37 ps in different cyanobacteria species with increasing red form content (e.g. (90)). In *A.thaliana* PSI-LHCl the “blue” Lhca complexes (Lhca1 and Lhca2) transfer excitation energy to the core four times faster than the “red” Lhca (Lhca3 and Lhca4) (114).
Several time-resolved measurements have been performed on isolated PSI-LHCI of *C. reinhardtii* (160, 162-164) but they have led to different results. This is probably due to differences in the protein composition of the preparations because *C. reinhardtii* PSI-LHCI easily loses part of the Lhcas (48). Furthermore, in these measurements, phenazine methosulfate (PMS) was used to maintain the PSI RC in its open state, whereas PMS was recently shown to quench chlorophyll emission (121). Since it was also demonstrated that the trapping kinetics of PSI in open and closed states differ for less than 4% [42], all the measurements reported here are performed in the absence of PMS or other reducing agents. In this work we measure the fluorescence decay kinetics of two biochemically and structurally well characterized *C. reinhardtii* PSI-LHCI complexes obtained in homogeneous preparations (48). These two PSI particles have different antenna composition containing either nine Lhcas (named the “Full PSI-LHCI” in the following) or seven Lhcas (named the “Small PSI-LHCI” in the following) lacking two red-form containing antennae Lhca2 and Lhca9. Comparing these particles allows us to study how red forms and antenna size influence EET and trapping kinetics of *C. reinhardtii* PSI-LHCI.

**MATERIALS AND METHODS**

*Sample preparation* - PSI-LHCI particles from *C. reinhardtii* were prepared as in Drop et al. (2011) (48). Small PSI-LHCI was prepared by solubilizing Full PSI-LHCI (final chlorophyll concentration of 0.2 mg/ml) with 0.5% n-Dodecyl-β-D-maltoside (β-DM) and 0.2% Zwittergent 3-16 (CalbioChem), and centrifuged overnight (41000 rpm, 17h at 4°C). After centrifugation, two fractions were collected: the upper one was Small PSI-LHCI obtained after solubilization and the lower one was Full PSI-LHCI (still not solubilized). Estimating the Chl *a*, Chl *b* and Car content in the *C. reinhardtii* PSI-LHCI particles (Table 1) requires several estimations.

<table>
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<th></th>
<th>Cyanobacteria core PSI (24)</th>
<th>A.t. PSI-LHCI</th>
<th>C.r. Small PSI-LHCI</th>
<th>C.r. Full PSI-LHCI</th>
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<tr>
<td>Number of Lhcas</td>
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<td>7</td>
<td>9</td>
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<tr>
<td>Chls <em>a</em>+Chls <em>b</em></td>
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<td>209</td>
<td>235</td>
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<tr>
<td>Chls <em>a</em>/Chls <em>b</em></td>
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<td>4.8±0.2 (48)</td>
<td>4.4±0.1 (48)</td>
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<tr>
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<td>173</td>
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<td>Carotenoids</td>
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</table>

Table 1: Estimation of the number of Chls and Cars in different PSI-LHCI supercomplexes of *A. thaliana* (A.t.) and *C. reinhardtii* (C.r.) (see Materials and Methods).
We estimate the total number of Chls in *Chlamydomonas reinhardtii* by adding the total number of Chls found in higher plant PSI-LHCI (170, (28)) with the total number of Chls in the additional Lhcas found in *Chlamydomonas reinhardtii* PSI-LHCI particles (48). For that, we assume the total number of Chls per Lhca to be 13, between the 10-12 estimated in purified Lhca (43, 62) and the 14-17 found in the crystal structure (49). The Chl *a/b* ratio of the PSI-LHCI reported in (48) was obtained by fitting the absorption spectra of 80% acetone pigment extracts with the spectra of the individual pigments (165). From the total number of Chls and the Chl *a/b* ratio, we estimate the Chl *a* and Chl *b* content. The number of Cars per Lhca is calculated from the Chl/Car ratio of the PSI-LHCI of higher plant (4.8 (±0.1), (107)) assuming 100 Chls and 22 Cars per core, as in cyanobacteria (24). It follows that in higher plants, the peripheral antenna contains 13.4 Car, corresponding to 3.4 (±0.2) Cars per Lhca. We assume the number of Cars per Lhca to be the same in Lhcas of *Chlamydomonas reinhardtii*. From the Chl and Car content, we can estimate the fraction of excitation in the core and the peripheral antennae (see Appendix A and Table A1).

**Steady state measurements** - Absorption spectra were measured on a Varian Cary 4000UV-Vis spectrophotometer and fluorescence spectra on a Fluorolog spectrofluorimeter (Jobin Yvon Horiba). All spectra were measured at room temperature (RT) and at 77K (by using a liquid N₂ Horiba FL-1013 LN dewar with a home build cuvette holder) in a plastic cuvette 10mm*3mm. To avoid self-absorption, samples were diluted to an OD of 0.07 at the Q₉ maximum (1cm path length) in a buffer containing 10 mM Tricine (pH 7.8) and 0.03% n-Dodecyl-α-D-maltoside (α-DM). Circular-Dichroism (CD) spectra were measured at 10°C on a Chirascan-Plus CD Spectrometer (Applied Photophysics, Surrey). All presented spectra are scaled to the Chl content: the absorption spectra were integrated between 630 nm and 750 nm and scaled according to the oscillator strength of the particle, by using the estimated number of Chls in the particle (Table 1) and the ratio of 0.7 for the oscillator strength of Chl *b* and Chl *a* in the Q₉ region. The CD spectra were scaled to the integrated area under the absorption between 630 nm and 750 nm (which were scaled to the particles’ oscillator strengths as described above).

**Time-resolved measurements** - The picosecond-time-resolved fluorescence measurement were performed with a streak camera setup as described previously (90, 148) adapted with a Coherent Vitesse Duo containing an integrated 10W Verdi CW laser (output wavelength 532 nm) both seeding the Vitesse ultra-fast oscillator (output wavelength 800nm, average power ≈100 mW, pulse width =100 fs, repetition rate 80 MHz) and pumping the regenerative amplifier Coherent RegA 9000 (output wavelength 800 nm, average power
≈1W, pulse width 180-200 fs, tunable repetition rate between 10 kHz and 300 kHz). The output of the RegA fed the optical parametric amplifier Coherent OPA 9400 (output wavelength from 470 nm to 770 nm, average power up to a few mW). The frequency-doubled light (400 nm) in the OPA could also be used as an output. The repetition rate was set to 250 kHz and the OPA set to generate either the 400 nm or the 475 nm excitation wavelength. The light intensity was modulated with neutral density filters, and residual 800 nm light and white light from the OPA was removed with an interference filter. The excitation polarization was set vertical with a Berek polarization compensator (New Focus, model 5540). The light was focused in the sample with a 15 cm focal length lens resulting in a spot diameter of 50 μm in the sample. Fluorescence emission was collected at right angle by two identical achromatic lenses (B. Halle UV-Achromat f=100 mm) to collimate the light and then focus it on the input slit (100 μm) of a spectrograph (Chromex 250IS, 50 grooves/mm ruling, blaze wavelength 600 nm, spectral resolution of 2nm). Scattered excitation light was removed with an optical long-pass filter. A polarizing filter (Spindler & Hoyer, Type 10K) was placed in between the two achromatic lenses to collect light polarized at magic angle (54.7°) with respect to the excitation polarization. After the spectrograph, the light was focused on the input slit (40 μm) and then on the photo-cathode of the streak camera Hamamatsu C5680 mounted with the M5675 Synchroscan unit (triggered by the Vitesse oscillator) and the Digital CCD Camera Hamamatsu Orca R2 (read out speed 8.5 frame/s). Spectral calibration was done with an Argon lamp (Oriel Instruments Argon lamp model 6030) and spectrottemporal sensitivity (shading) correction (148) with a homogeneous white light source (Xenon lamp, Osram HLX 64642 24V 150W GER i 028).

Fluorescence was detected from 590 nm to 860 nm and 0 to 155 ps (time range 1, TR1, temporal response: 4-5 ps) and 0 to 1500 ps (TR4, temporal response: 18 ps). Each dataset consisted of a sequence of images: 10 images of 5 mins at TR1 and 15 images of 1 min at TR4. Image sequences were corrected for background, shading and jitter (temporal drift between images within an image sequence) and finally averaged in HPD-TA 8.4.0 (Hamamatsu). These corrected datasets were binned to 2 nm, and zoomed between 640 nm and 800 nm in Glotaran 1.3 (166).

Samples were in 10 mM Tricine (pH 7.8), 0.03% α-DM, 0.5 M sucrose, and measured in a 10mm*10mm quartz cuvette at room temperature (RT). OD at the Qy maximum was 0.98 (Full PSI-LHCI) and 0.86 (Small PSI-LHCI). To avoid self-absorption, the laser beam was focused in the sample close to the cuvette wall and emission was collected at right angle close to the entry point of the laser beam into the cuvette. To avoid singlet-singlet annihilation by multiple simultaneous excitation of PSI-LHCI complex, the pulse energy was
reduced to 0.6 nJ. To avoid singlet-triplet annihilation, the sample was stirred with a magnet bar. A power study confirms the absence of annihilation (results not shown).

**Data analysis of time-resolved measurements** - The streak camera datasets were analyzed globally with a sequential model in order to extract a minimum number of exponential components \( n \) (with increasing lifetimes \( \tau_n \)) that can satisfactorily describe the data (no structure in the residuals). The two datasets from the same experimental condition (corresponding to the two TRs) were fitted simultaneously with the same kinetic scheme and spectra. The fit yields Evolution Associated Spectra (EAS) characterizing the spectral evolution (e.g. the third EAS rises with the second lifetime and decays with the third lifetime). Decay Associated Spectra (DAS) corresponding to a loss or a gain of emission at specific lifetimes, were calculated from the EAS (148, 167, 168). The raw decay measured at one detection wavelength \( (\lambda) \) can be written as a sum of exponential decays (convoluted by the IRF) weighted by their DAS (148, 167, 168):

\[
\text{Decay}(\lambda, t) = \sum_n [\text{DAS}_n(\lambda).\text{IRF} \otimes \exp\left(-t/\tau_n\right)]
\]

The IRF was modeled as the sum of two Gaussians, with full-widths at half maximum (FWHM) of 4-5 ps (94.5% relative integrated area) and 21 ps (5.5 %) for TR1 and 18 ps (92.6%) and 288 ps (8.4%) for TR4.

The average decay time \( \tau_{av \, CS} \) (Equation 1) characterizes the time until CS occurs (for open RCs) and is calculated by considering only the components attributed to the PSI-LHCI kinetics (excluding components attributed to e.g. disconnected species).

\[
\tau_{av \, CS} = \frac{\sum_n (\tau_n A_n)}{\sum_n A_n} \quad \text{(Equation 1)}
\]

In Equation 1, \( A_n \) is the area under the DAS of the \( n \)-th component (i.e. its total amplitude). This approach excludes all components associated with energy transfer in the average decay time calculation, since their positive and negative contributions will cancel (assuming no superradiant or dark states). In cases where transfer components yielded \( A_n < 0 \) this was attributed to noise, and \( A_n \) was set to zero.

The data were further analyzed by a target fit (167-169) of all eight streak camera datasets simultaneously with the model described in Scheme 1. The model consists of two compartments for Small PSI-LHCI and three for Full PSI-LHCI. The additional compartment in Full PSI-LHCI (green box) represents the difference between the two PSI particles and can be structurally associated with the two antennae Lhca2 and Lhca9. Species Associated Spectra (SAS), characterizing the emission properties of each species, were estimated by means of a non-negative least-squares variable projection algorithm (170). Equilibria could be estimated under the assumption of equal oscillator strength of all species (equal area under the SAS, 171)). All rate constants were estimated (uncertainties ranging from 0.8% to 3%) as well as the free-energy difference between the different compartments (uncertainties
ranging from 0.2% and 1.3%). Some kinetics faster than the time-resolution were visible around $t_0$ (Figure 2). These were modeled as precursors of the other compartments (see Appendix B.1 for more details). Additionally the model contained two functionally disconnected species with small population (between 1.0% and 6.8%) and ns-lifetimes. The full kinetic scheme is presented in Appendix B.1.

RESULTS

Steady state characterization

The steady state absorption and fluorescence emission spectra of Full and Small PSI-LHCI are presented in Figure 1 a and b. The absorption maximum in the $Q_y$ region at room temperature (RT) is at 679.5 nm for both complexes, as previously observed (48). The estimated number of Chls in the PSI-LHCI particles (Table 1) was used to scale the absorption spectra to their Chl content (see Materials and Methods). The absorption difference spectrum between the two PSI-LHCI particles (Figure 1a, green) matches the average absorption of reconstituted Lhca2 and Lhca9 (Figure 1a, blue), indicating that the presence/absence of these two complexes is the main difference between the two preparations.

The maxima of the fluorescence emission spectra at RT and 77K for both particles were 687 nm and 714.5 nm respectively (Figure 1b) in agreement with previous results (48).
To check whether the dissociation of Lhca2 and 9 affects the overall organization of PSI-LHCI, circular dichroism (CD) spectra were measured (Figure 1c). The high similarity between the CD spectra of the two particles indicates that the loss of the two Lhca subunits does not have large secondary effects on the supercomplex organization.

**Time-resolved fluorescence and global sequential analysis**

To get insights into the processes of excitation energy transfer and trapping in large systems, it is useful to compare the decay kinetics upon selectively excitation of the core and the peripheral antennae (117, 172). The difference in absorption between the core and the peripheral antennae is emphasized at 400 nm and 475 nm (Figure 1d): The largest absorption from the peripheral antenna is visible at 475 nm (≈80 %, see Table A.1) whereas
the core and the peripheral antennae have almost the same absorption at 400 nm. Therefore these two wavelengths were used for excitation when measuring time-resolved fluorescence kinetics with a streak camera setup.

Four parallel experiments were performed: Small and Full PSI-LHCl upon 400 nm and 475 nm excitation. Each sample/excitation combination was measured at two time windows (short time range, 0-155 ps, and long time range, 0-1500 ps), yielding eight streak camera datasets. A typical dataset is presented in Figure 2a.

![Streak camera image and fluorescence decay kinetics](image)

**Figure 2**: Time resolved fluorescence results. (a) Streak camera image of Full PSI-LHCl upon 475 nm excitation for the short time range. Colors represent the fluorescence emission intensity from zero (orange) to high fluorescence (red); (b) Fluorescence decay kinetics (integration from 640 nm to 800 nm) upon 475 nm and 400 nm for Full PSI-LHCl (black and red, respectively) and Small PSI-LHCl (blue and pink, respectively). The decays are normalized to their maximum.

When comparing the fluorescence decay integrated over all wavelengths (Figure 2b) two preliminary observations can be made, which are in line with the expectations: (i) Full
PSI-LHCI has a slower overall decay than Small PSI-LHCI at both excitation wavelengths, in agreement with the difference in antenna size and (ii) for both particles the overall decay upon 400 nm excitation is faster than upon 475 nm, in agreement with a preferential excitation of the peripheral antenna at 475 nm.

The sequential analysis showed that at least four exponentially decaying components are required to describe the data. The Decay Associated Spectra (DAS) of Full PSI-LHCI measured upon 400 nm excitation are presented in Figure 3. The fastest component (1.0 ps) represents excitation energy transfer from blue Chls (a mixture of Chls b and blue Chls a) to red Chls a. In particular, Chls b emission is visible upon 475 nm excitation at ≈650 nm detection wavelength (Figure 2a) and disappears within ≈1 ps. The weak emission intensity from these Chls b prevents accurate estimation of the rate of their depopulation. We therefore fixed it to 1 ps in all the experiments ((f)=fixed in Figure 3 and Table 2), in line with energy transfers components reported in Lhca complexes (105, 173).

The second (21.4 ps) and the third component (68.2 ps) represent mainly decay processes. Above 700 nm the second DAS is smaller than the third, indicating that the second component contains less red forms emission. Additionally the expected positive vibrational band is partially missing in the second DAS, meaning that some energy transfer occurs on this time scale. This is more pronounced upon 475 nm excitation than 400 nm, suggesting that it is associated with absorption by Lhcas. The third component represents a

![Figure 3: DAS of (a) Small PSI-LHCI and (b) Full PSI-LHCI upon 400 nm (dash lines) or 475 nm (solid lines) at RT. In each experiment, the two different time windows were fitted simultaneously (see Materials and Methods). Some parameters were fixed (f) and the fourth lifetimes were linked (l) through all experimental conditions (except for Full PSI-LHCI upon 475 nm whose residuals were significantly improved with an independent fit of this lifetime).](image-url)
pure fluorescence decay and accounts for most of the trapping. The fourth component has a small amplitude (a few percent, see Table 2) and a lifetime of 1.4 ns, which is close to the lifetime of the reconstituted Lhca monomers in plants (112). The DAS of this ns-component was blue shifted respect to the other DAS. These results suggest that this component is due to a mixture of disconnected Lhcas and Chls that are not part of the PSI-LHCI dynamics.

A similar set of components describes the decay measured in the other experimental conditions (Figure 3). In all experiments, the first component represents energy transfer from blue Chls to red Chls $\alpha$. For both excitation wavelengths, the second (red) and the third components (blue) have shorter lifetimes in Small PSI-LHCI than in Full PSI-LHCI. These lifetimes are shorter upon 475 nm excitation (solid lines) than upon 400 nm excitation (dash lines). This is mainly due to a larger contribution of EET following 475 nm excitation, which indicates that EET in the range of 15-20 ps occurs between the Lhcas or from the Lhcas to the core. A fourth component representing disconnected species was present with low amplitude in all experiments (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Small PSI-LHCI</th>
<th>Full PSI-LHCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>400nm</td>
<td>475nm</td>
</tr>
<tr>
<td>$\tau_1$ (ps)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relative amplitude</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>$\tau_2$ (ps)</td>
<td>17.6</td>
<td>17.3</td>
</tr>
<tr>
<td>relative amplitude</td>
<td>38.5%</td>
<td>20.4%</td>
</tr>
<tr>
<td>$\tau_3$ (ps)</td>
<td>54.9</td>
<td>47.9</td>
</tr>
<tr>
<td>relative amplitude</td>
<td>60.0%</td>
<td>77.1%</td>
</tr>
<tr>
<td>$\tau_4$ (ns)</td>
<td>1.4 (l)</td>
<td></td>
</tr>
<tr>
<td>relative amplitude</td>
<td>1.5%</td>
<td>2.0%</td>
</tr>
<tr>
<td>Average decay time $\tau_{av, CS}$ (ps)</td>
<td>40.3</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Table 2: Lifetimes obtained from the sequential analysis of the fluorescence decays of the two PSI-LHCI particles measured upon 400 nm and 475 nm excitation with their relative amplitude (i.e. $A_n / \sum A_n$, see Materials and Methods) and average decay time $\tau_{av\, CS}$ (the ns component is not considered in the calculation, see details and Equation 1 in Materials and Methods). The contributions were set to 0% in case the area under the DAS was negative (see Materials and Methods). Some lifetimes were fixed (f) or linked (l).

Upon 400 nm and 475 nm excitation, the average decay time $\tau_{av\, CS}$ increases from 40.3 ps to 41.5 ps for Small PSI-LHCI and from 49.7 ps to 51.4 ps for Full PSI-LHCI (Table 2). The presence of Lhca2 and Lhca9 in Full PSI-LHCI increases the average decay time by 9.4 ps at 400 nm and by 9.9 ps at 475 nm compared to Small PSI-LHCI.
**Target analysis**

To understand the EET and trapping processes in more detail, a target analysis was used to fit all datasets simultaneously. This enables estimation of rate constants, and free energy differences, between different compartments within a chosen kinetic model.

The target model (Scheme 1) consists of two compartments (Red and Bulk) for both particles, and an additional compartment for Full PSI-LHCI, representing the ensemble of Lhca2 and Lhca9 (Lhca2/a9). On a picosecond time scale the compartments are populated from precursors, with relative amounts according to Table 3 (see Appendix B.1 for details).

<table>
<thead>
<tr>
<th></th>
<th>Red</th>
<th>Bulk</th>
<th>Lhca2/a9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small PSI-LHCI upon 400 nm</td>
<td>8.8%</td>
<td>91.2%</td>
<td>n.a.</td>
</tr>
<tr>
<td>Small PSI-LHCI upon 475 nm</td>
<td>11.5%</td>
<td>88.5%</td>
<td>n.a.</td>
</tr>
<tr>
<td>Full PSI-LHCI upon 400 nm</td>
<td>8.0%</td>
<td>82.7%</td>
<td>9.3%</td>
</tr>
<tr>
<td>Full PSI-LHCI upon 475 nm</td>
<td>10.4%</td>
<td>79.9%</td>
<td>9.7%</td>
</tr>
</tbody>
</table>

Table 3: Estimated relative initial populations (error ± 0.5%) of the compartments of the target model (Scheme 1). The energy inputs ratio of the Red and Bulk compartments is linked between the two samples when measured upon the same wavelength.

The compartments have the same natural decay rate constant $k_0$, except for Bulk where the charge separation occurs in the RC (trapping with specific rate constant $k_B$). Energy is transferred between Red and Bulk, and between Bulk and Lhca2/a9.

The Species Associated Spectra (SAS) estimated from the target analysis for all the datasets, are shown in Figure 4. The Bulk SAS (blue) corresponds to bulk Chl a emission. The Red SAS is red-shifted compared to the Bulk SAS and represents red Chls and a few normal Chls in fast equilibrium with them. The Lhca2/a9 SAS (green) shows a more red-shifted spectrum compared to Bulk but clearly less than Red.
Figure 4: SAS estimated for all four experimental conditions (eight datasets) simultaneously fitted with the kinetics model of Scheme 1. The SAS are normalized to their maximum.

Two additional compartments account for the very low population of disconnected species, either disconnected Lhcas or disconnected Chls (see Appendix B.1 for details).

The relative initial populations of the compartments of Scheme 1 are given in Table 3. The Chl \(a/b\) ratio differs between compartments, as follows from the relative initial populations at the two excitation wavelengths (Table 3). Bulk is more core-like with the input decreasing upon 475 nm compared to 400 nm excitation (less Chl \(b\)), whereas Red and Lhca2/a9 are more Lhca-like with the input increasing upon 475 nm compared to 400 nm excitation (more Chl \(b\)). Bulk receives more than \(\approx 80\%\) of the excitations, which indicates that it contains Chls from the peripheral antenna in addition to those of the core.

The equilibration between Bulk and Lhca2/a9 is four times faster than the equilibration between Red and Bulk \((k_1+k_2)^{-1} \approx 4*(k_3+k_4)^{-1}\), see Scheme 1). This is explained by the further red-shifted SAS of Red compared to the Lhca2/a9 SAS which leads to a smaller spectral overlap and thus a slower back transfer to the Bulk compartment.

The differences in free energy \(\Delta G\), enthalpy \(\Delta H\) and entropy \(\Delta S\) between the compartments enable estimation of the number of Chls in each of them (see Appendix B.2 for details). From the forward and backward rate constants in Scheme 1, we calculate that the free energy of Red and Lhca2/a9 is respectively 26 and 36 meV higher than Bulk. The enthalpy, approximated as the average spectral position of the SAS yields 13930 \pm 50 cm\(^{-1}\) (1727 \pm 6 meV) for Red, 14255 \pm 10 cm\(^{-1}\) (1767 \pm 2 meV) for Bulk and 14076 \pm 20 cm\(^{-1}\) (1745 \pm 3 meV ) for Lhca2/a9. So the enthalpies of Red and Lhca2/a9 are respectively 40 \pm 5 and 22 \pm 5 meV lower than Bulk. The entropy difference (multiplied by the temperature \(T=293K\)) of Red and Lhca2/a9 with Bulk corresponds to 66 \pm 5 and 58 \pm 2 meV respectively. This gives a Chls \(a\) ratio of 0.07 \pm 0.02 between Red and Bulk and of 0.100 \pm 0.005 between Lhca2/a9.
and Bulk (see Appendix B.2). Combining this with the estimated total number of Chls $a$ (Table 1), it follows that Red contains $12 \pm 2$ Chls $a$, Bulk $163 \pm 3$ and Lhca2/a9 $16 \pm 1$.

The temporal concentration profiles of each compartment in the target model are described by the amplitude matrices (see Scheme B.3 for details). The amplitude matrices for measurements upon 400 nm excitation are presented in Table 4 (see Table B.3.1 for measurements upon 475 nm).

<table>
<thead>
<tr>
<th>Small PSI-LHCI</th>
<th>Full PSI-LHCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red</td>
</tr>
<tr>
<td>6.6 ps</td>
<td></td>
</tr>
<tr>
<td>18.3 ps</td>
<td>-0.227</td>
</tr>
<tr>
<td>55.8 ps</td>
<td>0.306</td>
</tr>
</tbody>
</table>

Table 4: Amplitude matrices in the case of Small PSI-LHCI and Full PSI-LHCI upon 400 nm.

The 6.6 ps component is only present in Full PSI-LHCI and mainly represents the energy transfer from Bulk to Lhca2/a9. In both particles, the $\approx 20$ ps component shows ingrowths of Red and trapping from Bulk. During the last component, the population of all the compartments decreases because of the trapping from Bulk. Similar observations can be made for the kinetics upon 475 nm excitation (Table B.3.1). The two trapping times are slowed down in the presence of Lhca2 and Lhca9 antennae: from 18.3 ps to 22.6 ps for the first component of the trapping and from 55.8 ps to 60.4 ps for the second one.

**DISCUSSION**

The average decay time of the PSI-LHCI complex of *C. reinhardtii* containing nine Lhcas per core complex (Full PSI-LHCI) is 49.7 ps upon 400 nm and 51.4 ps upon 475 nm, very close to those of higher plant PSI-LHCI (48 ps upon 440nm excitation and 53 ps upon 475 nm excitation for *A. thaliana* (114)). This strongly suggests that the antenna enlargement in *C. reinhardtii* PSI-LHCI compared to PSI-LHCI of higher plant is compensated by the presence of red forms with higher energy than in higher plants, resulting in a similar average decay time of PSI-LHCI in the two organisms.

Excitation dynamics of isolated Full PSI-LHCI of *C. reinhardtii* were measured previously by several groups, with reported average decay times varying from 24 ps to 59 ps (160, 162-164). The shorter lifetimes might have arisen from differences in protein composition, especially considering that instability of the large PSI-LHCI complex that easily loses part of the antenna (48). Indeed, Giera et al. (2014) (164) studied a PSI-LHCI preparation with a small antenna size (Chl $a/b$ 7.5 vs. 4.4 of our PSI-LHCI complex) observing an average decay time of 24.3 ps, a value close to that of the core complex (114). The longer
lifetimes in (160, 162) are probably due to some photosystem II impurity present in the preparations as indicated by the authors (106). These impurities are absent in our preparation (48) thanks to the presence of a His tag on PsaA (174) which facilitates the purification.

A more detailed insight in EET and trapping in the complexes is obtained from target analysis. Most of the Chls associated with PSI are present in the Bulk compartment. This compartment contains 163 Bulk Chls, including the RC, and shows a trapping rate $k_B$ of (29.2 ps)$^{-1}$. $k_B$ was estimated at (18.9 ps)$^{-1}$ in higher plants (114). The difference is well explained by the larger amount of Chls in Bulk relative to the “Core Bulk” compartment used in the target model for higher plants ($\approx$100 Chls $\alpha$): the average population of $C. reinhardtii$ RC is $\approx$1.63 times lower than in higher plants, which (in a simple trapping limited approach, (15)) leads to a $\approx$1.63 times slower trapping from Bulk as observed here. The good similarity between higher plants and $C. reinhardtii$ indicates that the Bulk Chls are equally well-connected in the two species, including the 63 Bulk Chls $\alpha$ associated with the peripheral antenna.

The Red compartment contains 12 Chls $\alpha$ including the red-most forms. Two trapping components of 18.3 and 55.8 ps are resolved because of the ingrowth process occurring in Red (directly from the precursors or from Bulk after energy transfer). The trapping is slowed down relative to the hypothetical particle composed of only Bulk. Red is preferentially excited at 475 nm, indicating that (part of) the red-most forms are located in or close to the peripheral antenna, with Lhca4 being the best candidate as indicated by the analysis of reconstituted complexes (63).

The simultaneous target analysis of the samples with and without Lhca2 and Lhca9 enabled us to quantitatively describe the role of these LhcAs in the EET and trapping kinetics of PSI-LHCI. The estimated number of Chls $\alpha$ in the Lhca2/a9 compartment (16) is consistent with the estimated difference in Chls $\alpha$ between Full and Small PSI-LHCI (18, see Table 1). The Lhca2/a9 SAS agrees well with the sum of the emission spectra of Lhca2 and Lhca9 (Figure 5) thus confirming that these complexes have a red-shifted spectrum compared to the Bulk Chls (Figure 4) but they do not contain the most-red forms (which are present in the Red compartment). In the analysis, the presence of Lhca2/a9 results in an additional energy transfer step with a 6.6 ps lifetime and a slowdown of the two main trapping components by 4.3 ps for the first one and by 4.6 ps for the second one.
From the average decay time $\tau_{av,CS}$, the trapping efficiency, i.e. the quantum yield of CS ($\Phi_{CS}$), can be calculated using $\Phi_{CS} = 1 - \tau_{av,CS} / \tau_{no,CS}$ (15), where $\tau_{no,CS}$ is the average decay time in the (hypothetical) case that CS cannot occur. $\tau_{no,CS}$ is approximated as the average decay time of plant Lhca monomer (~2 ns, (112)), the closest system of PSI-LHCI where CS does not occur. In Photosystem II, it was shown that $\Phi_{CS}$ decreases with increasing antenna size (130). Nevertheless, this loss in efficiency is compensated by a larger absorption cross section that enhances the absorption capacity enough to increase the product of the number of Chls $a$ in PSII times the quantum yield $\Phi_{CS}$. For PSI-LHCI the calculations show that even with an increasing average decay time, the trapping efficiency remains very high (Table 5).

<table>
<thead>
<tr>
<th></th>
<th>A.t. PSI-LHCI</th>
<th>C.r. Full PSI-LHCI</th>
<th>C.r. Small PSI-LHCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average decay time (ps)</td>
<td>$\tau_{av,CS} = \Sigma A_i \tau_i$</td>
<td>48ps-53ps (114)</td>
<td>51.4ps</td>
</tr>
<tr>
<td>Trapping Efficiency</td>
<td>$\Phi_{CS} = 1 - \tau_{av,CS} / \tau_{no,CS}$</td>
<td>97.3% - 97.6%</td>
<td>97.4%</td>
</tr>
</tbody>
</table>

Table 5: Comparison of average decay times and trapping efficiency between A. thaliana (A.t.) and the two C. reinhardtii (C.r.) PSI-LHCI.

The increased absorption cross-section of Full PSI-LHCI of C. reinhardtii enables it to harvest $\approx41\%$ more photons than PSI-LHCI of A. thaliana (area under their scaled absorption spectra), without affecting trapping efficiencies. This is very advantageous in low light environments. Full PSI-LHCI is slower than Small PSI-LHCI in C. reinhardtii but their trapping
efficiency are comparable: the red forms contained in Lhca2 and Lhca9 slow down the average decay time but have almost no effect on the trapping efficiency.

Acknowledgement: This work was supported by the ERC consolidator grant 281341 (ASAP) to RC and by the Netherlands Organization for Scientific Research (NWO) through a Vici grant to RC and a Veni grant to BvO. We acknowledge Jos Thieme for technical support with the initial streak camera experiments.

APPENDICES

Appendix A: Fractions of excitation in the core and the peripheral antennae

Fractions of excitation in either the core or the peripheral antennae were calculated (see Method 1 and Method 2 below, and the results in Table A.1) to choose the excitation wavelengths where the fraction differences were the largest.

<table>
<thead>
<tr>
<th>Excitation wavelength</th>
<th>Small PSI-LHCI</th>
<th>Full PSI-LHCI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>400nm</td>
<td>475nm</td>
</tr>
<tr>
<td>Percentage of excitation in the core antenna pigments</td>
<td>51%/53%</td>
<td>24%/23%</td>
</tr>
<tr>
<td>Percentage of excitation in the peripheral antenna pigments</td>
<td>49%/47%</td>
<td>76%/78%</td>
</tr>
</tbody>
</table>

Table A.1: Initial distribution of excitations over core and peripheral antennae pigments (Chls and Cars) calculated with two methods (Method 1/Method 2) to estimate the uncertainties.

Method 1: The absorption spectrum of the peripheral antenna ($A_{LHCI}$, including gap Chls) is obtained from the difference absorption spectrum PSI-LHCI ($A_{PSI-LHCI}$, from C. reinhardtii, this study) minus core ($A_{core}$, from A. thaliana (114)): $A_{LHCI} = A_{PSI-LHCI} - A_{core}$ (Figure A.1). $A_{PSI-LHCI}$ and $A_{core}$ were scaled to their Chl content (see Materials and Methods). PSI core absorption from A. thaliana was used in this study because no conclusive biochemical isolation of C. reinhardtii PSI core have been done so far (presence of disconnected antenna oligomers in the PSI core preparation visible in time-resolved data, (103-105, 160, 162)). The percentage of photons absorbed in the peripheral antenna was calculated as $A_{LHCI} / A_{PSI-LHCI}$ (Figure 1d of the main text).

Method 2: The extinction coefficients of the different pigments were assumed equal to those of pigments extracted in 80% acetone (considering the absorption in acetone to be 15 nm blue-shifted compared to the absorption in proteic environment) and were multiplied by the estimated number of each pigment (Table 1 of the main text). The energy transfer
from Cars to Chls occurs faster than our time resolution and is assumed to be 70% efficient as observed in Lhcas of higher plants (114) and in the PSI core of cyanobacteria (175).

Figure A.1: RT Absorption spectra of the core complex from *A. thaliana* (A.t.) (black) and the peripheral antenna (calculated as in Method 1 below) of *C. reinhardtii* (C.r.) in Full PSI-LHCI (red) and Small PSI-LHCI (blue), scaled to their Chl content.

Appendix B: Target analysis

**Appendix B.1: Kinetic scheme**

The complete kinetic model used for the target analysis is presented in Scheme B.1.1.

![Scheme B.1.1: Complete kinetic model including precursors and compartments associated with the disconnected species. Rates are indicated in ns⁻¹, with fit uncertainties.](image)

All datasets were fitted with the same set of rate constants and SAS (except for one rate, see below). The only difference between the data obtained from the two excitation
wavelengths was the relative population of the Red, Bulk and Lhca2/a9 compartments from the Precursors compartment (Table 3 of the main text). The precursor compartment contains emission from pigments that transfer energy among themselves faster than the time resolution of our instrument (≈2 ps). Its spectrum contains Chl a and Chl b (especially visible after 475 nm excitation). It rapidly (1-1.3ps) depopulates into Red, Bulk and Lhca2/a9.

The simultaneous target analysis of all datasets enabled the differentiation of the disconnected species into two compartments. The disconnected species are either disconnected Lhcas (named “Disc. Lhcas”) whose rate constant \( k_{DL} \) is \((1.29 \text{ ns})^{-1}\), or disconnected Chls (named “Disc. Chls.”) whose rate constant \( k_{DC} \) is \((3.8 \text{ ns})^{-1}\). The Disc. Lhcas SAS (cyan in Figure B.1.1) is indeed Lhca-like as indicated by its higher red emission compared to the Bulk SAS (blue). The Disc. Chls SAS (dark green in Figure B.1.1) has the typical blue-shifted spectrum of free Chls with the expected emission maximum around 675 nm.

![Figure B.1.1: SAS of the disconnected species compared to Bulk (normalized to their maximum).](image)

The decay rates of Disc. Lhcas were linked for all experimental conditions except for Full PSI-LHCI upon 475 nm whose lifetime was fitted independently to improve significantly the residuals at long time scale. The other fit parameters were not affecting by “unlinking” this decay rate. This difference in decay rates for Disc. Lhcas between the two excitation wavelengths may suggest subpopulations of Lhcas present in the membrane. Further quenched Lhcas could be preferentially excited upon 475 nm.

**Appendix B.2: The differences in free energy \( \Delta G \), enthalpy \( \Delta H \) and entropy \( \Delta S \)**

The free energy difference \( \Delta G \) between the compartments and Bulk are presented in Scheme B.2.1. From the forward and backward rate constants, \( \Delta G \) is calculated as
ΔG_{ij} = G_j - G_i = -k_B T \ln \left( \frac{k_{i\to j}}{k_{j\to i}} \right). The enthalpy H corresponds to the energy level of the Chls contained in one compartment and is approximated in this study as the average spectral position of the SAS. ΔH between the compartments and Bulk is represented with dashed lines in Scheme B.2.1.

The entropy difference, calculated from the two previous energy differences with TΔS = ΔH - ΔG, represents the thermal activation that enables up-hill EET from the red forms. The ratio of the number of Chls a between compartments is obtained with $\frac{N_i}{N_j} = \exp \left( -\frac{\Delta S_{ij}}{k_B} \right)$, assuming all excitations on Chl a and none on Chl b. Knowing the total number of Chls, we can estimate the number of Chls in each compartment (see main text).

**Appendix B.3: Amplitude matrices at 475 nm**

In amplitude matrices, each compartment populates (negative amplitudes) and depopulates (positive amplitudes) at indicated lifetimes (inverse rates). The amplitude matrices for measurements with 400 nm excitation are presented in the main text (Table 4) and with 475 nm in Table B.3.1.

<table>
<thead>
<tr>
<th>Small PSI-LHCI</th>
<th>Full PSI-LHCI</th>
<th>Lhca2/a9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Red</td>
<td>Bulk</td>
</tr>
<tr>
<td>6.6 ps</td>
<td>-0.212</td>
<td>0.560</td>
</tr>
<tr>
<td>18.3 ps</td>
<td>0.318</td>
<td>0.335</td>
</tr>
</tbody>
</table>

Table B.3.1: Amplitude matrices in the case of Small PSI-LHCI and Full PSI-LHCI upon 475 nm
CHAPTER 3

The high efficiency of Photosystem I in the green alga *Chlamydomonas reinhardtii* is maintained after the antenna size is substantially increased by the association of Light-Harvesting Complexes II

Clotilde Le Quiniou, Bart van Oort, Bartlomiej Drop, Ivo H. M. van Stokkum and Roberta Croce

This chapter is based on:

ABSTRACT

Photosystems (PS) I and II activities depend on their light-harvesting capacity and trapping efficiency, which vary in different environmental conditions. For optimal functioning, these activities need to be balanced. This is achieved by redistribution of excitation energy between the two photosystems via the association and disassociation of Light Harvesting Complexes (LHC) II, in a process known as state transitions.

Here we study the effect of LHCII binding to PSI on its absorption properties and trapping efficiency by comparing time-resolved fluorescence kinetics of PSI-LHCI and PSI-LHCI-LHCII complexes of *C. reinhardtii*. PSI-LHCI-LHCII of *C. reinhardtii* is the largest PSI supercomplex isolated so far and contains seven Lhcb s, in addition to the PSI core and the nine Lhcas that compose PSI-LHCI, together binding ~320 chlorophylls. The average decay time for PSI-LHCI-LHCII is ~65 ps upon 400 nm excitation (15 ps slower than PSI-LHCI) and ~78 ps upon 475 nm excitation (27 ps slower). The transfer of excitation energy from LHCII to PSI-LHCI occurs in ~60 ps. This relatively slow transfer compared to that from LHCI to the PSI core, suggests loose connectivity between LHCII and PSI-LHCI.

Despite the relatively slow transfer, the overall decay time of PSI-LHCI-LHCII remains fast enough to assure a 96% trapping efficiency, which is only 1.4% lower than that of PSI-LHCI, concomitant with an increase of the absorption cross section of 47%. This indicates that, at variance with PSII, the design of PSI allows for a large increase of its light-harvesting capacities.

INTRODUCTION

In eukaryotic organisms, the photosynthetic apparatus contains two main pigment-protein complexes, embedded in the thylakoid membrane, Photosystems (PS) I and II, that harvest light energy and convert it into chemical energy (1). The PS performances are largely determined by their capacities to harvest light and transfer excitation energy to the reaction center (RC) where charge separation occurs (3). To efficiently harvest light, PSI and PSII are equipped with two types of antennae. The inner antenna is composed of chlorophyll (Chl) a-binding complexes that together with the RC form the core complex. The core complexes of PSI and PSII bind respectively 98 and 35 Chls, respectively (24, 26) and are highly conserved in eukaryotic organisms (40). The outer antenna, by contrast, varies in the different organisms as it is optimized for the absorption in their light-growth conditions (3). In plants and green algae it is composed of members of the light-harvesting complex (Lhc) multigenic family, each coordinating between 10 and 14 Chls (*a* and *b*) (176, 177).

Despite the high sequence similarity, the number and properties of the Lhcs vary in plants and algae. LHCII trimers are composed of three major Lhcb s in *Arabidopsis thaliana*.
(Lhcb1-3 (41)) and of nine in the green alga *Chlamydomonas reinhardtii*. (LhcbM1-9 (51)). Three monomeric antenna, CP29 (Lhcb4), CP26 (Lhcb5) and CP24 (Lhcb6) located between the core and the LHCII trimers, are present in *A. thaliana* PSII-LHCII (178), whereas CP24 is absent in *C. reinhardtii* resulting in a different organization of the PSII-LHCII supercomplex in the two organisms (56, 65). Concerning PSI-LHCII, in *A. thaliana* four Lhcas (Lhca1-4) are associated with the PSI core (28), forming a PSI-LHCII complex that contains 155 Chls (5, 29). In *C. reinhardtii*, nine Lhcas compose the antenna of PSI (47), which is far larger than that of plants (48, 179).

The amount of excitation energy delivered to the RCs by the Lhcs does not only depend on their number but also on their biochemical and spectroscopic properties (69). The different affinity for Chl *a* and *b* between Lhcas and Lhcbs and the presence of far-red absorbing forms in Lhcas (58, 62) result in differences in the wavelength-dependent light-harvesting capacities of the two photosystems. This creates an excitation imbalance that can affect electron transport and induce photo-damage (125). To avoid this and maintain a maximal photosynthetic efficiency, plants and algae regulate the association of Lhcbs with PSI and PSII in a process known as state transitions (135, 136), in which mobile LHCII is associated with PSII in state 1 and with PSI in state 2. In plants, the mobile LHCII represents ~15% of the total LHCII population, and under sunlight is mainly associated with PSI *i.e.* plants in light are in state 2 (131).

The mechanisms of state transitions in the green alga *C. reinhardtii* seem to differ from what is observed in plants (140, 141, 180). For a long time this difference was believed to be the pool size of LHCII involved in the transitions (80% according to (142)); however, recent results suggest that the mechanism and probably also the physiological role of this process are different from those in plants. It was indeed observed that although a large part of LHCII functionally disconnects from PSII during the state 1 to state 2 transition, only part of it associates with PSI, whereas the rest gets quenched (140, 141, 181). However, due to the complexity of the cells and the presence of different LHCII pools, a conclusion about excitation energy transfer efficiency from Lhcbs to PSI could not be made.

PSI-LHCl-LHCII supercomplexes with different antenna size have been purified (57, 145-147). The antenna size depends on the method used to induce the state transition and/or on the isolation protocol. The largest PSI-LHCl-LHCII isolated so far contains seven Lhcbs (two LHCII trimers and one monomer located on the PsaH/L side of the core complex), in addition to the nine Lhcas located on the other side of the core complex (57). This PSI-LHCl-LHCII is clearly larger than PSI-LHCl-LHCII from plants that contains only one LHCII trimer (138, 143), although recent results indicate that also in the membrane of plants more than one LHCII trimer can be associated with PSI-LHCl (144).
In this work we have performed time-resolved fluorescence measurements on the PSI-LHCl-LHClII complex of *C. reinhardtii* with or without preferential excitation of Lhcbs. We have determined the energy transfer efficiency of these Lhcbs and their influence on the trapping yield of PSI. The data indicate a loose connection between the Lhcbs and the PSI core, which is responsible for a relatively slow energy transfer step. These results are interpreted on the bases of the new PSI-LHCl structures (5, 29) and the possible energy transfer pathways in PSI-LHCl-LHClII are discussed.

**MATERIALS AND METHODS**

**Sample preparation** - PSI-LHCl-LHClII was prepared as in (57) from the PSII-lacking mutant Fl39 of *C. reinhardtii* (57, 182, 183). In short, cells were harvested in mid-logarithmic phase (growth at 25°C, 20 microeinstein s m⁻²s⁻¹) and state 2 was induced by incubating them in anaerobic conditions (*i.e.* shaken in the dark for 20 min in the presence of NaN₃). Thylakoid membranes were solubilized to a final concentrations of 0.5 mg chlorophyll /mL, 0.5% Digitonin (Sigma) and 0.2% dodecyl-α-D-maltoside, and loaded on a sucrose gradient. PSI-LHCl-LHClII was harvested with a syringe.

Light Harvesting Complexes II (Lhcbs) were prepared as in (65). Band 2 from the sucrose gradient contained Lhcb monomers such as CP26, CP29 and LhcbMs, and Band 3 contained LHCII trimers.

**Fraction of excitation in Lhcbs** - To selectively excite Lhcbs, we determine the fraction of excitation in Lhcbs and chose excitation wavelengths for the time-resolved measurements that showed the largest differences. Lhcbs were excited the least upon 400 nm with 27-29% of PSI-LHCl-LHClII excitation present in the Lhcbs, whereas Lhcbs were excited the most upon 475 nm excitation corresponding to 41-46% of the excitation. To determine the fraction, we used two different methods as described in (184). For details see SI1.

**Steady state and time-resolved measurements** - The absorption spectra were measured at room temperature with a Varian Cary 4000UV-Visible spectrophotometer. CD spectra were measured at 10°C on a Chirascan-Plus CD Spectrometer (Applied Photophysics, Surrey, UK). The 10°C steady state fluorescence spectra were measured with a spectrofluorometer (Fluorolog Tau-3 Lifetime System, Horiba Scientific) upon 500 nm excitation. To avoid self-absorption, the sample was diluted to optical density 0.07 at the Q₅ maximum (1-cm path length) in a buffer containing 20 mM Hepes (pH 7.5) and 0.02% digitonin (Sigma).

Time-resolved fluorescence measurements were performed with a streak camera setup as described in (184). In short, fs pulses were generated with a repetition rate of
250 kHz using a laser system (Coherent Vitesse Duo and Coherent RegA 9000) and were used to feed the OPA (Coherent OPA 9400) to generate excitation light at 475 nm and 400 nm. The pulse energy was reduced to 0.3 nJ to avoid singlet-singlet annihilation. The exciting light focused on the sample was 0.151 mol photons/m^2/s at 475 nm and 0.127 mol photons/m^2/s at 400 nm. Fluorescence was detected at the magic angle with respect to the excitation polarization, in the 590-860 nm range from 0 to 400 ps. The sample was measured at 15°C in its purification buffer (20 mM Hepes, pH 7.8, 0.02% digitonin, 0.7 M sucrose) in a 10 × 10-mm quartz cuvette at optical density 0.35 cm^-1 at the Q_y maximum. To avoid self-absorption, the laser beam was focused in the sample close to the cuvette wall, and emission was collected at right angle close to the entry point of the laser beam into the cuvette. The sample was stirred with a magnet bar to avoid singlet-triplet annihilation. A power study confirmed the absence of annihilation (data not shown).

The fluorescence decays measured upon 400 nm and 475 nm excitation were analyzed with a sequential model (see SI1). The average decay time \( \tau_{av} \) (Equation SI1-1) characterizes the time for the excitation energy to be used for charge separation and is calculated by considering only the components attributed to the PSI-LHCI-LHCCI kinetics (excluding ns components attributed to disconnected species). For more details on the acquisition and data analysis, see SI1.

**Target analysis** - We used the previous kinetic model describing *C. reinhardtii* PSI-LHCI (Figure 3a, (184)) and extended it with a new compartment representing the seven Lhcbs (called “Lhcb”) in equilibrium with Bulk (Figure 3b). The natural decay rate constant of Lhcb was fixed to \( k_0' \) (3.3 ns)^{-1} as found for isolated LHCCI trimers of *C. reinhardtii* (64).

The complete kinetic scheme (Scheme SI2-1) also contains precursors populating the other compartments on the ps time scale, with relative amounts varying depending on excitation wavelength (Table SI1-2), and a disconnected compartment corresponding to species with ns lifetimes. A detailed description of the procedure for the target analysis is given in SI1.

**Simulated kinetic models** - Population dynamics were simulated (MathWorks R2014b 64-bit, MATLAB) for hypothetical particles of: 1) different size (number of Chls a); 2) different connectivity; and 3) different composition (energy of Chls) (Scheme SI3-1). This enabled to independently study the effects of these three factors on trapping time and efficiency. See SI3 for details.
**Distance measurements in the reconstructed PSI-LHCI-LHCII** - PSI-LHCI-LHCII Electron Microscopy (EM) of higher plants (138) and *C. reinhardtii* (57) were used to position the apoproteins of PSI and Lhcbs. The positions were set as close as possible to the EM, but uncertainties remain concerning the exact distance that separates the different apoproteins and their relative orientation. The reconstruction was made with PyMOL Molecular graphics System (Version 1.3) to have a three-dimensional perspective and a first estimation of distances between the Chls of interest.

**RESULTS**

**Steady state characterization**

Absorption and fluorescence emission spectra of PSI-LHCI-LHCII are shown in Figure 1a and b, together with the spectra of PSI-LHCI. The maximum Qy absorption of PSI-LHCI-LHCII (at 678 nm) as well as its maximum emission (at 683 nm) are blue shifted as compared with the PSI-LHCI maxima (at 679.5 nm and 687.5 nm respectively). The absorption difference spectrum between PSI-LHCI-LHCII and PSI-LHCI (Figure 1a, green) overlaps well with the Lhcbs absorption spectrum (Figure 1a, magenta) in agreement with previous results (57).
PSI-LHCI-LHCII of Chlamydomonas reinhardtii

Figure 1: Absorption and emission and CD spectra. a, absorption at room temperature of PSI-LHCI (black) and PSI-LHCI-LHCII (blue) (scaled to their Chl content, see SI1) and their difference spectrum (green). The difference spectrum overlaps well with the absorption spectrum of Lhcb from C. reinhardtii (magenta) ((scaled LHCII trimer absorption)×2 + (scaled Lhcb monomers)×1). b, fluorescence emission spectra at 10°C of PSI-LHCI and PSI-LHCI-LHCII both upon 500 nm excitation, and of Lhcb (isolated monomers) upon 475 nm excitation. The spectra are normalized to their maximum. c, CD spectra at 10°C of PSI-LHCI and PSI-LHCI-LHCII (normalized to the absorption scaled to the Chl content). d, the difference spectrum between the CD spectra of PSI-LHCI and PSI-LHCI-LHCII is compared with the CD spectra of LHCII trimers from plants (measured after solubilization of thylakoid membranes in dodecyl-α-D-maltoside (185)). a.u., arbitrary units.

CD spectra of PSI-LHCI-LHCII and PSI-LHCI are compared in Figure 1c. They show similar features in the Qy absorption region, (negative peaks at 648 nm and 680 nm and a positive peak at 664 nm), as well as in the Soret region (similar shape below 423 nm). Major differences are visible between 423 nm and 532 nm, as shown in the PSI-LHCI-LHCII minus PSI-LHCI difference spectrum (Figure 1d, green). This spectrum strongly resembles the spectrum of LHCII trimers in detergent micelles (185) but has also some feature of aggregated LHCII trimers (distinct negative peak at 438 nm and positive peak at 483 nm (186)). This suggests that the Lhcb are involved in additional (or modified) excitonic interactions and/or that compared to the detergent, LHCII trimers associated with PSI-LHCI are present in a slightly different conformation similar to LHCII trimers in aggregates.
Excitation Energy Transfer and trapping kinetics

In this preparation, Lhcbs have been shown to be functionally associated to PSI-LHCI-LHCI (57). To characterize the excitation energy transfer and trapping kinetics of PSI-LHCI-LHCI and estimate the energy transfer rate between PSI-LHCI and Lhcbs, time-resolved fluorescence of PSI-LHCI-LHCI was measured with a streak camera set-up. In C. reinhardtii, as in higher plants, the PS core antenna contains only Chls a whereas the peripheral antenna contains also Chls b. Excitation in the Chl a and in the Chl b region can then be used to disentangle the contributions of the core and peripheral antennae (117). More specifically, upon 475 nm excitation, 41-46% of the energy was in Lhcbs, while this value dropped to 27-29% upon 400 nm excitation. These two excitation wavelengths were chosen for the time-resolved measurements.

Global sequential analysis - The fluorescence decays measured upon 400 nm and 475 nm excitation were analyzed with a sequential model. The data can be well described by a minimum of four components (no structure in the residuals, Figure 2). A clear difference between both excitation wavelengths is observed in the decay of PSI-LHCI-LHCI at an early time where fast Chl b decay is clearly visible after their preferential excitation upon 475 nm (see in particular 656 nm in Figure 2a). The decay-associated spectra (DAS) for both excitation wavelengths are shown in Figure 2b.

Figure 2: Sequential analysis of PSI-LHCI-LHCI fluorescence decays upon 400 nm or 475 nm excitation at 15°C. a, selected fluorescence decay traces after excitation at 400 nm (gray) and 475 nm (orange). Black and red lines indicate the fit curves. b, DAS of each decay component. The two different experiments were fitted simultaneously in order to link (i) the fourth lifetime associated to the disconnected species. The amplitude of each decay component (i.e. relative area= $A_n / \sum A_n$) is shown in parentheses next to the corresponding lifetime, in %. The DAS are normalized to the initial populations of excited states (i.e. the total area of PSI-LHCI-LHCI-related DAS, $\sum A_n$). See SI1 for details. a.u., arbitrary units.
The first component is a pure energy transfer component (conservative positive-negative shape of the DAS) from blue Chls ($a$ and $b$) to red Chls $a$ for both excitation wavelengths. This transfer is faster at 400 nm than at 475 nm (1.8 ps compared to 3.5 ps respectively), possibly because of an additional transfer step occurring from Chls $b$ to blue Chls $a$ upon 475 nm where Chls $b$ are preferentially excited. Indeed, the Chls $b$ emission at $\sim$650 nm appears in the DAS of this fast component upon 475 nm excitation. The next two components are mainly decay components for both excitation wavelengths, with faster lifetimes upon 400 nm excitation compared to 475 nm ($\sim$20 ps/$\sim$90 ps compared to $\sim$30 ps/100 ps respectively). The fourth component has very small amplitude ($\sim$3-4%) and represents disconnected Lhcas/Lhcbs/Chls with ns-lifetime (64, 112). Fit parameters are summarized in Figure 2b.

The average decay time $\tau_{av}$ of PSI-LHCI-LHClII is $\sim$65 ps upon 400 nm and $\sim$78 ps upon 475 nm. The association of Lhcb$s$ increases the average decay time by $\sim$15 ps at 400 nm and by $\sim$27 ps at 475 nm compared to the average decay times of PSI-LHCI (49.7 ps and 51.4 ps, respectively (184)).

**Target analysis** - To estimate the energy transfer rate between PSI-LHCI and LHCl within PSI-LHCI-LHClII, a target analysis was performed on the two datasets (400 nm and 475 nm excitation) simultaneously. The previous kinetic scheme reported in (184) (Figure 3a) was used to model PSI-LHCI related kinetics and the Lhcb$s$ were modeled by an extra compartment (called “Lhcb”) transferring energy to the “Bulk” compartment (Figure 3b). Several constraints were imposed on the fit parameters (rate constants, species-associated spectra (SAS), and initial populations) as described in SI1-iii. An additional compartment (Scheme SI2-1) accounts for a small population of disconnected species ($\sim$3%) whose lifetime (3.2 ns) is very close to that of the Lhcb$s$ in *C. reinhardtii* (64).
Figure 3: Kinetic models for target analysis of (a) PSI-LHCI and of (b) PSI-LHCI-LHClII, with the rate constants (in ns\(^{-1}\)). Rate constants are either fitted (indicated in red) or fixed (indicated in black). Both supercomplexes have similar relative initial population in Red, Bulk and Lhca2/a9 compartments. In b, there is additional initial population on Lhcb. See SI1 for details.

The kinetic model (Figure 3b) fits the data well (Figure SI2-1). Rate constants fitted from the target are given in Figure 3b (in red) and the SAS are given in Figure 4. The Lhcb SAS has a significantly lower red emission and a narrower bandwidth compared to Bulk as expected (Figure 4 and Figure SI2-2b). The red shift visible in the Lhcb SAS compared to the isolated Lhcb steady state emission spectrum (Figure 4) suggests that Lhcb associated with PSI are in a different conformation than when isolated in detergent in agreement with the CD spectra.
Figure 4: Estimated SAS of each compartment and comparison with the steady state emission spectrum of Lhcbs (isolated monomers, dashed magenta normalized to Lhcb SAS maximum). The SAS are constrained to equal area (see SI1-iii). Precursors and disconnected compartment SAS are presented in Figure SI2-2.

The free energy difference $\Delta G$ between Bulk and Lhcb is estimated at $-48$ meV, according to Equation 1.

$$\Delta G = G_{\text{Bulk}} - G_{\text{Lhcb}} = -k_B T \times \ln \left( \frac{k_5}{k_6} \right) \quad \text{(Equation 1)}$$

It corresponds to an enthalpy difference $\Delta H$ of $-21$ meV ($\Delta H = \Delta G + T \Delta S = H_{\text{Bulk}} - H_{\text{Lhcb}} = -k_B T \times \ln \left( \frac{N_{\text{Lhcb}}}{N_{\text{Bulk}}} \times \frac{k_5}{k_6} \right)$ with 147 Chls $a$ estimated in Bulk, see SI2-iv, and 55 Chls $a$ in Lhcb, Table SI1-1). This enthalpy difference corresponds to an $\sim 9$-nm blue shift of Lhcb as compared with Bulk.

The amplitude matrices (Table 1 upon 475 nm and Table SI2-1 upon 400 nm excitation) detail the extent of (de)population of each compartment for each lifetime and give the time scale at which Lhcbs transfer energy to the rest of the supercomplex.

<table>
<thead>
<tr>
<th>Lifetimes</th>
<th>Red</th>
<th>Bulk</th>
<th>Lhca2/a9</th>
<th>Lhcb</th>
<th>Overall amplitude of the trapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ps</td>
<td>0.000</td>
<td>0.008</td>
<td>0.000</td>
<td>-0.447</td>
<td>0.000</td>
</tr>
<tr>
<td>1.1 ps</td>
<td>-0.054</td>
<td>-0.491</td>
<td>0.016</td>
<td>0.001</td>
<td>-0.023</td>
</tr>
<tr>
<td>1.4 ps</td>
<td>0.000</td>
<td>0.011</td>
<td>-0.065</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>6.5 ps</td>
<td>-0.007</td>
<td>0.106</td>
<td>-0.067</td>
<td>-0.002</td>
<td>0.029</td>
</tr>
<tr>
<td>22.9 ps</td>
<td>-0.047</td>
<td>0.152</td>
<td>0.059</td>
<td>-0.013</td>
<td>0.151</td>
</tr>
<tr>
<td>63.8 ps</td>
<td>-0.308</td>
<td>0.064</td>
<td>0.018</td>
<td>0.397</td>
<td>0.171</td>
</tr>
<tr>
<td>97.3 ps</td>
<td>0.416</td>
<td>0.151</td>
<td>0.040</td>
<td>0.063</td>
<td>0.670</td>
</tr>
</tbody>
</table>

Table 1: Amplitude matrices of PSI-LHCI-LHCII upon 475 nm excitation and the overall amplitude of the trapping at each lifetime.
The three shorter lifetimes do not have large amplitudes in the trapping (overall amplitude ~0) and correspond to times where precursors transfer energy to the other four compartments. The ~23-, ~64-, and ~97-ps lifetimes represent trapping components with most of the trapping occurring with the longest lifetime. With an ~64-ps lifetime, Lhcb equilibrates (large positive amplitude) with all the other compartments. Similar observations can be made when exciting PSI-LHCI-LHCII at 400 nm (Table SI2-1) except that the ~23-ps lifetime contributes more to the trapping than upon 475 nm. The average decay times obtained by target analysis of PSI-LHCI-LHCII are 70 ps upon 400 nm and 80 ps upon 475 nm excitation (see SI1-iii), consistent with the average decay times calculated from the sequential analysis (see above).

**Contributions of antenna size, connectivity and composition to the average decay time of PSI-LHCl and PSI-LHCI-LHCII**

The equilibration time between two compartments is influenced by their size (number of Chls in each compartment), their composition (energy of the Chls) and by the connectivity between them. To disentangle these different contributions, the kinetics of hypothetical PSI-LHCl and PSI-LHCl-LHCII complexes with different compartment size, composition and/or connectivity were simulated (Scheme SI3-1), and their average decay time compared (Table SI3-1). In the simulation we have compared the effect of the presence of Lhca2 and Lhca9, the most loosely bound antenna complexes of PSI (48), with that of the seven Lhcbs. The simulations show that: 1) The addition of 16 Chls a (as many as initially contained in Lhca2 and Lhca9, see SI2-iv) when isoenergetic with the bulk, slows down the average decay time by ~2.5 ps, while the addition of 55 Chls a (as many as initially contained in the seven Lhcbs, see Table SI1-1) slows it down by 4-6 ps. 2) The connectivity of PSI with Lhca2 and Lhca9 is very good and does not influence the average decay time of PSI-LHCl, whereas the connectivity between PSI-LHCl and Lhcbs is not optimal and is responsible for an increase of the decay time of 16-25 ps as compared with the best possible case. 3) The red forms contained in Lhca2 and Lhca9 (63) slow down the kinetics of PSI-LHCl by ~5 ps, whereas the bluer Chls in Lhcbs (as compared with bulk Chls of PSI-LHCl) speed up the kinetics by 2-3 ps. A detailed description of the simulations is presented in SI3.

**DISCUSSION**

Time-resolved measurements of the PSI-LHCl-LHCII complex of *C. reinhardtii*, the largest PSI supercomplex isolated so far (57), show that the trapping time of this supercomplex is ~65 ps upon 400 nm excitation and ~78 ps upon 475 nm excitation (Table 2). This large difference between the two excitation wavelengths is attributed to the
differential excitation of Lhcbs (more Lhcb excitation at 475 nm, see Table SI1-2). This implies that the transfer from Lhcbs to PSI-LHCI is slow relative to the trapping within PSI-LHCI. The presence of seven Lhcbs slows down the overall trapping by 15 ps at 400 nm and 27 ps at 475 nm excitation (Table 2). This result helps in interpreting the time-resolved data on *C. reinhardtii* *in vivo* (141). If a large population of PSI-LHCI-LHCII particles should be expected in the cells in state 2 compared to state 1, not only a large difference in the amplitude of the PSI component should be observed but also a significant difference in its lifetime. The fact that none of these effects were observed in the time-resolved measurements in the cells indicates that the difference in the amount of LHCII associated with PSI in state 1 and 2 is relatively small as concluded by Ünlü *et al.* (141).

<table>
<thead>
<tr>
<th>Excitation wavelength</th>
<th>PSI-LHCI (184)</th>
<th>PSI-LHCI-LHCII</th>
</tr>
</thead>
<tbody>
<tr>
<td>400 nm</td>
<td>475 nm</td>
<td>400 nm</td>
</tr>
<tr>
<td>Average decay time $\tau_{av}$</td>
<td>49.7 ps</td>
<td>51.4 ps</td>
</tr>
<tr>
<td>Trapping Efficiency (15)</td>
<td>$\Phi_{CS} = 1 - \tau_{av}/\tau_{no\ CS}$</td>
<td>97.5%</td>
</tr>
</tbody>
</table>

Table 2: Average decay times and trapping efficiencies of PSI-LHCI-LHCII compared to PSI-LHCI. $\tau_{no\ CS}$ is the average decay time in the (hypothetical) case that charge separation (CS) cannot occur. $\tau_{no\ CS}$ is approximated as the average decay time of plant Lhca monomer (~2 ns), the closest system of PSI-LHCI where charge separation does not occur.

The detailed modeling of the measurements (Figure 3b) shows that the excitation energy transfer between Lhcbs and the rest of the supercomplex occurs in ~60 ps (Table 1). Several factors can influence the transfer rates: the antenna size, the energy of the pigments associated with the complexes and the connectivity between Lhcbs and PSI-LHCI. To discriminate between the contributions of these factors to the trapping time of PSI-LHCI and PSI-LHCI-LHCII, we have simulated excited state population dynamics of hypothetical particles where each factor was controlled. The connectivity of the Lhcbs was compared with that of the most loosely bound Lhcas (Lhca2 and Lhca9) (48) which also show a relatively slow equilibration (~7 ps) with the rest of the complex (184). The results indicate that the functional connectivity between Lhca2/a9 and the PSI complex is very high and that the observed relatively slow migration is due to the presence of low energy forms (63) in these Lhcas. This conclusion is in agreement with the observation that the presence of red forms slows down the trapping kinetics in different organisms (90, 114, 161, 184). On the contrary, the results show that in PSI-LHCI-LHCII the favorable down-hill energy transfer profile of Lhcbs cannot compensate their low connectivity with PSI core. This low connectivity largely explains the slow transfer from Lhcbs to the PSI core and can be due to a low number of transfer pathways.
To determine the possible transfer pathways in the PSI-LHCI-LHCII we reconstructed its three-dimensional structure by mapping the structures of LHCII trimer (58), CP29 (61) and PSI-LHCI (5, 29) of higher plant onto the EM projection map of PSI-LHCI-LHCII of C. reinhardtii (57). The structure of all subunits of C. reinhardtii is expected to be similar to that of higher plants given the sequence similarity (32, 39). Different views of the reconstruction are presented in Figure 5.

Using the reconstructed model of the supercomplex, we looked for the shortest distances between the Chls of Lhcbs and those of the PSI core (Figure 5, b and c), which should represent possible energy transfer pathways. We could not identify pairs of Chls that are separated by less than 18 Å (nearest edge-to-edge distance), in line with the slow energy transfer between Lhcbs and PSI core observed in the measurements. The shortest distances are observed between Chl a612 of one LHCII trimer (which is in a similar position as in PSI-LHCI-LHCII of higher plant, and is depicted in blue in Figure 5, nomenclature for LHCII Chls from (58)) and two PSI core Chls: Chl a1501 (nomenclature for the core Chls from (5), distance ~21 Å) bound to PsaL and the additional Chl (distance ~18 Å) associated with PsaH, which is only present in the structure of (29) (designated as H1 in this structure). The next shortest distance is ~25 Å and involves Chl a611 of the same LHCII trimer and Chl a1801, belonging to a PsaA Chl trimer that was proposed to connect PSI and LHCII trimer in plants by (29). Other pathways suggested in (5) and (29) involve Chl a1401 (coordinated to PsaA on the luminal side, Figure 5 c and d) and Chl a1403 (coordinated to PsaK, Figure 5 c and d) but are not expected to play a major role given the large distance that separate these Chls from the Chls of LHCII (> 30 Å). Similarly, PSI Chls are very far from the second LHCII trimer (depicted in green in Figure 5, with Chl a612 at a distance of ~45 Å from Chl a1501 in (5) or ~37 Å from H1 in (29)) as well as from the Lhcb monomer (Figure 5c).

The three-dimensional reconstruction should be considered with some caution as the distances between the Chls can only be approximately estimated due to the low resolution of the EM maps, and the presence of one or two additional Chls in between the complexes cannot be completely excluded. However it is clear that the gap between LHCII and the PSI core is rather large and only few energy transfer pathways seem to be available in agreement with the experimental results. The large distance between the Chls of PSI and LHCII also indicates that the observed differences in the CD signal between the sum of PSI-LHCl and LHCII spectra and the spectrum of the PSI-LHCI-LHCII complex are likely due to a different conformation assumed by LHCII when associated with PSI and not to the presence of new excitonic interactions between Chls of LHCII and PSI core.
Figure 5: a, EM images of PSI-LHCI-LHCII from *C. reinhardtii* (57) viewed from the stromal side superimposed with apoproteins of PSI-LHCI of higher plants in cyan (Protein Data Bank (PDB) 4XK8, (5)) and LHCII trimer in brown (PDB 1RWT, (58)) assembled as PSI-LHCI-LHCII of higher plants (black outline of EM of *A. thaliana*, (138)) (left); or superimposed with LHCII assembled as PSI-LHCI-LHCII of *C. reinhardtii* by slightly rotating the LHCII trimer in brown into the LHCII trimer in blue and by adding a new LHCII trimer in green, CP29 in yellow (PDB 3PL9, (61)) and the five additional Lhcas present in *C. reinhardtii* in magenta (duplicates of Lhca1 from (5)) (right). The scale bar is 10 nm. The reconstruction was made with PyMOL. b, three-dimensional views of porphyrin rings from Chls bound to the apoproteins presented in a (right) with the same color: view from the stromal side (left) rotated 45° (upper right) or 90° (lower right) along the black axis. c, selection of the Chls present at the interface of Lhcb5 and PSI core (1.8x magnification of the red frame in b): the shortest distances between the closest atoms of the porphyrin rings of PSI Chls and Lhcb5 Chls are indicated in Å for the different sides of PSI core. d, Chls a trimer (red) binding PsaA (apoprotein in gray) and Chl a1401 (pink) newly found in (5), viewed from the side indicated with red arrow in a (right). These Chls could be involved in energy transfer pathways with LHCII (apoprotein in light blue).
It should be noticed that the observed loose connectivity between LHCII and PSI core has a small influence on the trapping efficiency of PSI-LHCI-LHCII. Indeed, even with a 43% increase in the number of Chls (Table SI1-1), the trapping yield of PSI-LHCI-LHCII is as high as 96% (Table 2, (15)), only 1.4% lower than in the absence of Lhcbs. The 43% increase in Chls corresponds to an increase of the absorption cross section by 47% (averaged over the spectral range), which can make a significant difference in terms of light harvesting capacities, especially under low light conditions. In this respect, it is interesting to notice the very large difference in efficiency and flexibility between PSI and PSII. We have previously estimated that 240 Chls $a$ per RC represent a maximal antenna size for PSII since above this value the increase in light harvesting would be compensated by the slow migration towards the RC resulting in an effective loss of efficiency (130). This is clearly not the case for PSI that is able to accommodate a very large antenna, maintaining a high efficiency even in the presence of slow transfer. Indeed, the results presented here show that in PSI-LHCI-LHCII of C. reinhardtii, which contains 240 Chls $a$ per RC, 96 out of 100 photons absorbed lead to charge separation in the RC. The design of PSI seems then to represent the way to go for efficient light harvesting.

Acknowledgements: This work was supported by the ERC consolidator grant 281341 (ASAP) to RC and by the Netherlands Organization for Scientific Research (NWO) through a Vici grant to RC and a Veni grant to BvO. We thank Yuval Mazor for providing the PDB file of the crystal structure of higher plant PSI-LHCI.

SUPPLEMENTARY INFORMATION

SI1: Materials and Methods

i. Methods to determine the fraction of excitation in Lhcbs

In short, the first method calculates the absorption of Lhcbs relative to PSI-LHCI-LHCII by estimating the relative difference of their absorption spectra scaled in the $Q_y$ region to their chlorophyll (Chl) content (Table SI1-1 and explanation below) and considering an oscillator strength ratio of 0.7 between Chl $b$ and Chl $a$ (7). The second method calculates the absorption cross section of both Lhcbs and PSI-LHCI-LHCII by using the extinction coefficients of the individual pigments (165) corrected by an energy transfer efficiency of 70% for the carotenoids (Cars) (114, 175).

To scale the absorption spectra of PSI-LHCI-LHCII to its Chl content and to calculate its absorption cross section, we estimate the number of pigments in C. reinhardtii (Table SI1-1). The total number of Chls in PSI-LHCI-LHCII of C. reinhardtii was obtained by adding to the 155 Chls of the PSI-LHCI of higher plant (5) (i) 70 Chls to account for the 5 additional Lhcbs
present in PSI-LHCI of *C. reinhardtii* (48) assuming an average of 14 Chls in each Lhca (5, 29) and (ii) 97 Chls to account for the 7 additional Lhcbs associated with PSI-LHCI (57) calculated by assuming 13 Chls in the monomer (61) and 42 Chls per LHCII trimer (58). The number of Car per Lhcas and Lhcbs of *C. reinhardtii* were assumed as in higher plant: with 3 in Lhca (5, 107), 3 in Lhcb monomer (61) and 12 in LHCII trimer (58), for a total of 76 Cars present in PSI-LHCI-LHCII of *C. reinhardtii* (Table SI1-1).

<table>
<thead>
<tr>
<th></th>
<th>PSI-LHCl</th>
<th>Lhcbs</th>
<th>PSI-LHCI-LHCII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Lhcas</td>
<td>9</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td>Number of Lhcbs</td>
<td>-</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1 monomer + 2 trimers)</td>
</tr>
<tr>
<td>Chls a+Chls b</td>
<td>225</td>
<td>97</td>
<td>322</td>
</tr>
<tr>
<td>Chls a/Chls b</td>
<td>4.4±0.1 (48)</td>
<td>Monomer:1.29±0.06 Trimer:1.28±0.02 (65)</td>
<td>2.95 ±0.05 (57)</td>
</tr>
<tr>
<td>Chls a</td>
<td>183</td>
<td>55</td>
<td>241</td>
</tr>
<tr>
<td>Chls b</td>
<td>42</td>
<td>42</td>
<td>81</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>49</td>
<td>27</td>
<td>76</td>
</tr>
</tbody>
</table>

Table SI1-1: Estimation of the number of Chls and Cars in PSI-LHCl, PSI-LHCI-LHCII and Lhcbs.

The initial excitation energy in Lhcbs ranges between 27-29% upon 400 nm excitation and 41-46% upon 475 nm excitation.

**ii. Sequential analysis of the time-resolved measurements**

Each experiment (one at each excitation wavelength) consists of a sequence of 20 images of 3 min each, acquired in HPD-TA 8.4.0 (Hamamatsu). Image sequences were corrected for background, shading and jitter (temporal drift between images within an image sequence) and finally averaged in HPD-TA 8.4.0 (Hamamatsu). These corrected datasets were binned to 2 nm, and zoomed between 640 nm and 800 nm in Glotaran 1.3 (166).

Each dataset was analyzed globally with a sequential model (148, 167, 168) in Glotaran 1.3. The Decay Associated Spectra (DAS) were calculated from the Evolution Associated Spectra (EAS) directly obtained after sequential analysis. The IRF was modeled as the sum of two gaussians (14.2% relative integrated area of the second gaussian) whose full-widths at half maximum (FWHM) were 6-7 ps and 22 ps for the first and the second gaussians respectively. Lifetimes associated with disconnected species were expected to be the same for both excitation wavelengths and therefore linked during the sequential analysis.
Chapter 3

The average decay time $\tau_{av}$ (Equation SI1-1) results from the sum of each lifetime weighted by the relative amplitudes of their DAS:

$$\tau_{av} = \frac{\sum_n (\tau_n A_n)}{\sum_n A_n} \quad \text{(Equation SI1-1)}$$

In Equation SI1-1, $A_n$ is the area under the DAS of the $n$-th component. Only the components attributed to the PSI-LHCI-LHCII-related DAS are used in the calculation.

iii. Target analysis of the time-resolved measurements

On the basis of our PSI-LHCI kinetic model (Figure 3a, (184)), we used several constraints in the analysis of PSI-LHCI-LHCII (Figure 3b): we fixed the relative initial populations of the compartments associated to PSI-LHCI (Red, Bulk and Lhca2/a9) and the rate constants between Bulk and Lhca2/a9 given the limited information on Lhca2/a9 specifically in the two PSI-LHCI-LHCII datasets. Previous fitted Species Associated Spectra (SAS) of Red, Bulk and Lhca2/a9 (184) were used as additional information to guide the target analysis. The relative initial population of Lhcb (Table SI1-2) was fixed in the range as calculated above.

<table>
<thead>
<tr>
<th></th>
<th>Red</th>
<th>Bulk</th>
<th>Lhca2/a9</th>
<th>Lhcb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSI-LHCI-LHCII upon 400 nm</td>
<td>5.8%</td>
<td>59.5%</td>
<td>6.7%</td>
<td>28.1%</td>
</tr>
<tr>
<td>PSI-LHCI-LHCII upon 475 nm</td>
<td>5.8%</td>
<td>44.8%</td>
<td>5.4%</td>
<td>44.0%</td>
</tr>
</tbody>
</table>

Table SI1-2: Relative initial populations of each compartment fixed during the target analysis.

Using these constraints and assuming equal oscillator strength for each compartment species (equal area under the SAS, (171)), the forward and backward rate constants between Bulk and Lhcb as well as their SAS were fitted by means of a non-negative least-squares variable projection algorithm (167, 168, 170). The target analysis was performed on the two PSI-LHCI-LHCII datasets (upon 400 nm and 475 nm excitation) simultaneously. The IRF was modeled as a single gaussian (FWHM of 7-8 ps).

To compare its consistency with the sequential analysis, the average decay time from the target analysis was calculated by weighting the lifetimes (including the precursors lifetimes) by the corresponding overall amplitude of the trapping (last column in Table SI2-1). The overall amplitude of the trapping is obtained for each lifetime by summing all the amplitudes of the different compartments (Table 1 and Table SI2-1) with the amplitude of the precursors (Table SI1-2).
**SI2: Results from the target analysis**

i. **Complete kinetic scheme**

The complete kinetic model used for the target analysis is presented in Scheme SI2-1. The precursors contain emission from pigments transferring their energy faster (1-1.4 ps) than the time resolution of our instrument (~3 ps).

![Scheme SI2-1: Complete kinetic model including precursors and compartments associated with the disconnected species (“Disc. Lhcs”, rate constant \( k_D \)). Rate constants are indicated in ns\(^{-1}\), with uncertainties for the fitted rates (in red). Precursors depopulate at slightly different rate upon 400 nm and 475 nm excitation (left/right respectively) with relative amounts of depopulation indicated in Table SI1-2.](image)

ii. **Selection of time-resolved emission at 70 wavelengths**

At all detection wavelengths, emission decay kinetics is captured by the kinetic model of PSI-LHCI-LHCII (Figure 4b) leading to a good overlap between the raw and the fitted traces (Figure SI2-1).
iii. **Comparison of Species Associated Spectra (SAS) of PSI-LHCI-related compartments. SAS of the precursors and the disconnected species**

The PSI-LHCI compartments Red, Bulk and Lhca2/a9 do converge toward the previously calculated SAS ([184], Figure SI2-2a) as expected (see SI1-iii). The enthalpy of each compartment, approximated as the average spectral position of the SAS, is therefore similar in both particles.
The SAS of the precursors contain Chl $a$ and Chl $b$ emission, especially visible after 475 nm excitation for the latter (Figure SI2-2c). The SAS of the 3% disconnected species, Disc. Lhcs (Figure SI2-2d) overlap well on the blue edge with the emission of the isolated Lhcbs, but also shows some additional amplitude in the red, which we attribute to the presence of Lhcas in this compartment.

iv. Estimation the number of Chls in PSI-LHCI-related compartments

The number of Chls in each compartment was obtained by a detailed balance analysis (see details of calculation in (184)).

From the target analysis of PSI-LHCI, it was previously estimated that 12 Chls were in Red, 163 in Bulk and 16 in Lhca2/a9 (184). Based on the recent crystal structure of PSI-LHCI (5), these estimations have to be adjusted. The total number of Chls in PSI-LHCI of higher plant revealed by this new crystal structure is 155, with 14 Chls per Lhca on average (5). This
corresponds now to a total number of Chls \( a \) in \( C. \ reinhardtii \) PSI-LHCl of 183 (Table SI1-1) instead of 191 previously estimated (184). It leads to a new estimation of 11 Chls in Red, 156 in Bulk and 16 in Lhca2/a9.

From the target analysis of PSI-LHCl-LHClII, the equilibrium between Bulk and Red was a bit different compared to (184) (Figure 3) leading to a new estimation of the Chl number in the PSI-LHCl-related compartments. By use of the detailed balance (see details in (184)) and considering that the enthalpy difference between the compartments is unchanged compared to previous study ((184), Figure SI2-2a), 21 Chls were estimated in Red, 147 Chls in Bulk and 15 Chls in Lhca2/a9. In PSI-LHCl-LHClII, Red seems to contain more Chls than previously, telling that a small number of Chls previously in Bulk are now in faster equilibrium with the red Chls. The difference in Chls number in Bulk between the two studies can partly explain that the trapping rate is changing from \((29.2 \, \text{ps})^{-1}\) in (184) to \((23.0 \, \text{ps})^{-1}\) in the current work.

v. Amplitude matrices and concentration profiles

The lifetimes and their amplitude for each compartment are given in the amplitude matrices (Table 1 in main text upon 475 nm and Table SI2-1 upon 400 nm).

<table>
<thead>
<tr>
<th>Lifetimes</th>
<th>Red</th>
<th>Bulk</th>
<th>Lhca2/a9</th>
<th>Lhcb</th>
<th>Overall amplitude of the trapping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ps</td>
<td>0.000</td>
<td>0.005</td>
<td>0.000</td>
<td>-0.285</td>
<td>0.000</td>
</tr>
<tr>
<td>1.0 ps</td>
<td>-0.052</td>
<td>-0.651</td>
<td>0.020</td>
<td>0.002</td>
<td>-0.029</td>
</tr>
<tr>
<td>1.3 ps</td>
<td>0.000</td>
<td>0.013</td>
<td>-0.079</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>6.5 ps</td>
<td>-0.011</td>
<td>0.162</td>
<td>-0.103</td>
<td>-0.003</td>
<td>0.045</td>
</tr>
<tr>
<td>22.9 ps</td>
<td>-0.093</td>
<td>0.300</td>
<td>0.117</td>
<td>-0.026</td>
<td>0.299</td>
</tr>
<tr>
<td>63.8 ps</td>
<td>-0.200</td>
<td>0.042</td>
<td>0.012</td>
<td>0.258</td>
<td>0.111</td>
</tr>
<tr>
<td>97.3 ps</td>
<td>0.356</td>
<td>0.129</td>
<td>0.034</td>
<td>0.054</td>
<td>0.573</td>
</tr>
</tbody>
</table>

Table SI2-1: Amplitude matrices of PSI-LHCl-LHClII upon 400 nm and the overall amplitude of the trapping at each lifetime.

The time-dependence of the population of each compartment (concentration profile) is plotted in Figure SI2-3.
**SI3: Quantifying the contributions of compartment size, composition and connectivity to the average decay time of PSI-LHCI and PSI-LHCI-LHCII**

The addition of antennae can affect the average decay time in several ways depending on the free energy difference, ΔG, and on the connectivity between the antennae and the rest of the supercomplex. To independently study the effect of each factor, the decay kinetics of hypothetical PSI-LHCI and PSI-LHCI-LHCII particles with altered compartment size and composition and/or connectivity (Scheme SI3-1) were simulated. The resulting average decay times, calculated from the amplitude matrices (in the same way as for the target analysis, see SI1-iii), are compared in Table SI3-1.

### i. Change in compartment size and composition

The increase of antenna size when adding the Lhca2/a9 compartment to “Small PSI-LHCI” (Scheme SI3-1a) or the Lhcb compartment to the original PSI-LHCI (Scheme SI3-1a&b) was studied independently from their connectivity and their energy level by simulating “Fused PSI-LHCI” (Scheme SI3-1c) and “Fused PSI-LHCI-LHCII” (Scheme SI3-1d) respectively.

---

Figure SI2-3: Concentration profiles for each compartment obtained from the target analysis of PSI-LHCI-LHCII (Figure 3b) on the two datasets simultaneously (upon 400 nm, dashed curves, and 475 nm, solid curves).
Scheme SI3-1: Simulated kinetic models of: (a) the original PSI-LHCI (184) and Figure 3a). “Small PSI-LHCI” (light blue frame) corresponds to a particle composed of Red and Bulk compartments only (184); (b) the original PSI-LHCI-LHClII (Figure 3b). Rate constant indicated in black were fixed and those indicated in red were obtained from the target analysis (see Results); (c) In Fused PSI-LHCI, the Chls of Lhca2/a9 are assumed to be isoenergetic with Bulk (named “IsoLhca2/a9”) and these two compartments are fused (i.e. energy transfer infinitely fast); (d) Fused PSI-LHCI-LHClII where Chls of Lhcb are considered isoenergetic with Bulk (named “IsoLhcb”) and these two compartments are fused; (e) Fastest PSI-LHCI corresponding to PSI-LHCI where the connectivity between Bulk and Lhca2/a9 has been improved to its best (see below); (f) Fastest PSI-LHCI-LHClII corresponding to PSI-LHCI-LHClII where the connectivity between Bulk and Lhcb has been improved to its best (see below). The rate constants are in ns⁻¹.

Before changing the compartment size, we need to specify the compartments size in the original kinetic models (Figure 3). The target analysis of PSI-LHCI, gave 11 Chls in Red, 156 in Bulk and 16 in Lhca2/a9 (up-dated estimation of the Chl number, see SI2-iv). This estimation was used as initial values in the simulated models of PSI-LHCI (Scheme SI3-1c&e). The target analysis of PSI-LHCI-LHClII, gave 21 Chls in Red, 147 Chls in Bulk and 15 Chls in
Lhca2/a9 (see SI2-iv). This estimation was used as initial values in the simulated models of PSI-LHCI-LHCII (Scheme SI3-1d&f).

In the “Fused PSI-LHCI” (Scheme SI3-1c), 16 Chls α, initially in Lhca2 and Lhca9, are now isoenergetic with Bulk Chls and perfectly coupled to them (IsoLhca2/a9). The change in Chls number in the fused compartment “Bulk+IsoLhca2/a9” modifies the trapping rate from this compartment to \(k'_{g}=31.0 \text{ ns}^{-1}\) (intrinsic charge separation rate multiplied by the number of Chls contained in the compartment = 34.2 \(\text{ns}^{-1}\times156/(156+16))\). When considering the spectral overlap between the fused “Bulk+IsoLhca2/a9” compartment and Red, we must picture the absorption spectrum of “Bulk+IsoLhca2/a9” as the absorption of Bulk increased proportionally by the additional Chls contained in Lhca2/a9 whereas “Bulk+IsoLhca2/a9” emission spectrum stays unchanged compared to Bulk given that the area under the spectrum is normalized to 1 on the frequency scale (see details in SI3-iii and Figure SI3-1). As the spectral overlap changes only when the fused compartment is the acceptor, only \(k_1\), the backward EET rate to “Bulk+IsoLhca2/a9”, is affected (Equations SI3-3 and SI3-4) and increases to \(k'_1\), the other rate \(k_2\) been unchanged (Scheme SI3-1c).

In “Fused PSI-LHCI-LHCII” (Scheme SI3-1d), 55 Chls, which in the model of Figure 3b are in the “Lhcb” compartment, are now isoenergetic with Bulk Chls and perfectly coupled to them (IsoLhcb). The trapping rate from the fused compartment “Bulk+IsoLhcb” changes to \(k'_{g}=31.6 \text{ ns}^{-1}\) (43.5 \(\text{ns}^{-1}\times147/(147+55))\). The backward EET rates to Bulk+IsoLhcb, \(k_1\) and \(k_4\), increase to \(k'_1\) and \(k'_4\), the other rates \(k_2\) and \(k_3\) been unchanged.

The initial population in the fused compartment corresponds to the sum of the initial populations of the two separated compartments.

ii. Change in compartment connectivity

Qualitatively, an increased connectivity between compartments can be interpreted as a decreased distance between them. In the frame of the Förster theory (Equation SI3-1), if \(R\) is reduced by a positive factor \(\sqrt{\alpha}\), the forward and backward rates will both increase by a factor “\(\alpha\)”, speeding up the equilibrium between donor and acceptor. Similar conclusion can be drawn in the limit of strong coupling since \(k_{\text{EET}} \propto \frac{1}{R^6}\), where \(x\) depends on the theoretical framework.

From the original kinetic model of PSI-LHCI (Scheme SI3-1a), the connectivity was improved by multiplying the forward and backward rates between Bulk and Lhca2/a9 in “Fastest PSI-LHCI” by different values of \(\alpha\) (Scheme SI3-1e). At increasing \(\alpha\), the average decay time of PSI-LHCI decreases to a minimum. From the kinetic model of PSI-LHCI-LHCII (Scheme SI3-1b), the connectivity was improved by multiplying the forward and backward
rates between Bulk and Lhcb in “Fastest PSI-LHCI-LHCII” by different values of β (Scheme SI3-1f). At increasing β, the average decay time of PSI-LHCI-LHCII decreases to a minimum.

The final “optimum” values of α or β were chosen such that the resulting average decay time was 1 ps above the value for α or β infinite.

**iii. Detailed calculation of rate constants when changing compartments size**

The changes in compartments size and connectivity are pictured here in the frame of the Förster theory but qualitatively similar conclusions could be drawn when using other approximations. The Förster theory describes weak excitonic interactions between a donor (D) that transfers its energy to an acceptor (A) with EET rate $k_{DA}$ described by the Förster equation (71, 72):

$$k_{DA} = \frac{1}{\tau D^2} \left( \frac{R_0}{R} \right)^6$$  \hspace{1cm} (Equation SI3-1)

where $R$ is the distance between the donor and the acceptor, $\tau D$ the intrinsic radiative lifetime of the donor and $R_0$ the Förster radius. $R_0^6$ is proportional to $\kappa^2$ and $J(\nu)$, where $\kappa$ is the dimensionless orientation factor between the donor and acceptor transition dipole moments and $J(\nu)$ is the spectral overlap integral

$$J(\nu) = \int_0^\infty \varepsilon_A(\nu) F_D(\nu) \frac{\nu^4}{\nu^4} d\nu$$  \hspace{1cm} (Equation SI3-2)

where $\varepsilon_A(\nu)$ the molar extinction coefficient of the acceptor and $F_D(\nu)$ the fluorescence emission of the donor, normalized to 1 when integrated on the frequency scale $\nu$.

The extinction coefficient of the acceptor compartment ($\varepsilon_A(\nu)$ in Equation SI3-2) depends on the number of Chls in this compartment whereas the emission of the donor compartment is normalized to 1 in the spectral overlap integral ($F_D(\nu)$ in Equation SI3-2) and will not vary if the donor compartment changes size. The fused compartments (Bulk+IsoLhca2/a9 and Bulk+IsoLhcb) have their absorption spectra proportionally increased compared to the spectrum of Bulk according to their Chls number whereas they have the same emission spectra as Bulk (Figure SI3-1). The overlap integral between compartments, and therefore the EET rate, is affected only when the fused compartments are acceptors. Therefore, $k_1$ in PSI-LHCI increases to $k_1'$ in Fused PSI-LHCI (Scheme SI3-1c) and $k_1$ and $k_4$ in PSI-LHCI-LHCII increase to $k_1'$ and $k_4'$ in Fused-PSI-LHCI-LHCII (Scheme SI3-1d). By contrast, all the other rates are unchanged.
To quantify the new rate constants, we used a detailed balance: the free energy difference $\Delta G$ between two compartments $i$ and $j$ can be calculated from the forward and backward rate constants: $\Delta G_{ij} = G_j - G_i = -k_B T \ln \left( \frac{k_{i\rightarrow j}}{k_{j\rightarrow i}} \right)$. With the free energy expressed in function of the enthalpy $H$ and the entropy $S$, $\Delta G_{ij} = \Delta H_{ij} - T \Delta S_{ij}$, and the entropy difference expressed in function of the number of Chls $N_i$ and $N_j$ in compartments $i$ and $j$, $\Delta S_{ij} = -k_B \ln \left( \frac{N_j}{N_i} \right)$, we can write the detailed balance:

$$\frac{k_{i\rightarrow j}}{k_{j\rightarrow i}} = \frac{N_j}{N_i} \exp \left( -\frac{(H_j - H_i)}{k_B T} \right) \quad \text{(Equation SI3-3)}$$

For instance in the case of Fused PSI-LHCI (Scheme SI3-1c), considering that $k_2$ stays unchanged (see above), $k_1'$ is calculated as:

$$k_1' = k_2 \cdot \frac{N_{\text{Bulk+Iso-Lhca2/a9}}}{N_{\text{Red}}} \exp \left( -\frac{(H_{\text{Bulk+Iso-Lhca2/a9}} - H_{\text{Red}})}{k_B T} \right) \quad \text{(Equation SI3-4)}$$

With the same approach, $k_3'$ and $k_4'$ were calculated.

**iv. Results from the simulated kinetics**

The effect of antenna size enlargement due to the addition of Lhca2 and Lhca9 to PSI can be studied independently from the connectivity and energy of these two subunits by simulating the hypothetical “Fused PSI-LHCI particle” (Scheme SI3-1c) where Bulk has been enlarged by the same number of Chls that are present in Lhca2 and Lhca9. Fused PSI-LHCI has an average decay time of 45.7 ps upon 400 nm excitation and 46.5 ps upon 475 nm...
excitation (Table SI3-1). The effect of the antenna enlargement due to the addition of two Lhcas is quantified by subtracting the average decay times of PSI-LHCI in the absence of Lhca2 and Lhca9 (Small PSI-LHCI, blue frame in Scheme SI3-1a) to that of Fused PSI-LHCI and it corresponds to a difference of 2.5 ps at 400 nm and 2.3 ps at 475 nm.

<table>
<thead>
<tr>
<th>PSI-LHCI</th>
<th>400 nm</th>
<th>475 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>50.5</td>
<td>51.4</td>
</tr>
<tr>
<td>Small PSI-LHCI</td>
<td>43.2</td>
<td>44.2</td>
</tr>
<tr>
<td>Fused</td>
<td>45.7</td>
<td>46.5</td>
</tr>
<tr>
<td>Fastest (&quot;optimum&quot; α=1)</td>
<td>50.5</td>
<td>51.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PSI-LHCI-LHCII</th>
<th>400 nm</th>
<th>475 nm</th>
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</thead>
<tbody>
<tr>
<td>Original</td>
<td>69.7</td>
<td>79.4</td>
</tr>
<tr>
<td>PSI-LHCI-related compartments</td>
<td>50.8</td>
<td>52.6</td>
</tr>
<tr>
<td>Fused</td>
<td>56.5</td>
<td>56.5</td>
</tr>
<tr>
<td>Fastest (&quot;optimum&quot; β=18)</td>
<td>53.6</td>
<td>54.1</td>
</tr>
</tbody>
</table>

Table SI3-1: Average decay time from the original and the simulated kinetic models of PSI-LHCI and PSI-LHCI-LHCII particles with modified compartments connectivity, size and composition (Scheme SI3-1).

To study the connectivity of the Lhca2/a9 compartment with PSI-LHCI, we simulate the hypothetical “Fastest PSI-LHCI” (Scheme SI3-1e) where the connectivity was improved to obtain the fastest possible average decay time of the particle (see SI3-ii). The average decay time of PSI-LHCI remains always the same ("optimum" α=1), leading to the conclusion that the connectivity between Bulk and Lhca2/a9 is very high in the original PSI-LHCI although Lhca2 and Lhca9 are the first complexes to be lost after detergent treatment of PSI-LHCI (48). Based on these results, we can then conclude that the difference between PSI-LHCI and Fused PSI-LHCI is due to the presence of red Chls in Lhca2/a9 that slow down the kinetics by 4.8 ps at 400 nm and 4.9 ps at 475 nm.

In a similar way we investigate the influence of the Lhcbs to the PSI-LHCI-LHCII average decay time in terms of connectivity, size and composition. The effect of the increase in the antenna size of PSI-LHCI by adding Lhcbs can be obtained by simulating the hypothetical particle “Fused PSI-LHCI-LHCII” (Scheme SI3-1d). Fused PSI-LHCI-LHCII has an average decay time of 56.5 ps for both excitation wavelengths (Table SI3-1), longer than in the absence of Lhcbs by 5.7 ps upon 400 nm and 3.9 ps upon 475 nm (subtraction of Fused PSI-LHCI-LHCII and a particle made of PSI-LHCI-related compartments only). By improving the original connectivity between Bulk and Lhcb, the “Fastest PSI-LHCI-LHCII” (Scheme SI3-1f) is obtained after multiplying the forward and backward rate constants between these
two compartments by an “optimum” $\beta=18$ and corresponds to a fastest average decay times of 53.6 ps upon 400 nm and 54.1 ps upon 475 nm (Table SI3-1), 16.1 ps and 25.3 ps faster than the original PSI-LHCI-LHCII average decay times upon 400 nm and 475 nm respectively. The higher energy of Lhcbs compared to the bulk is favoring the forward transfer to the bulk and partially counteracts the negative effect of the connectivity. We can directly estimate the influence of the presence of blue Chls in Lhcb by looking at the difference between Fused PSI-LHCI-LHCII and Fastest PSI-LHCI-LHCII, since the connectivity is very high in both particles. The bluer Chls of Lhcbs decreases the average decay time of PSI-LHCI-LHCII by 2.9 ps at 400 nm and 2.4 ps at 475 nm.
CHAPTER 4

The Light-Harvesting Complexes of Photosystem I in *Chlamydomonas reinhardtii*: a time-resolved fluorescence study

Clotilde Le Quiniou, Roberta Croce
ABSTRACT
Photosystems (PS) I and II form the center of the photosynthetic process since they harvest sunlight and use its energy to drive charge separation. The light harvesting capacities of both photosystems are assured by two types of antenna: the core and the peripheral antennae. The peripheral antenna is composed of Light-Harvesting complexes (LHC) that differ between organisms. In the green alga *Chlamydomonas reinhardtii*, nine LHCI compose the peripheral antenna of PSI. Despite a very similar chromophore organization, the nine monomers have different spectroscopic properties, in particular regarding the content and energy of the red forms, Chls absorbing at longer wavelengths than the bulk Chls. In this study, we have characterized the decay kinetics of each LHCI monomer by measuring time-resolved fluorescence. We observe a similar decay time of 1.9 ns for all the monomers. This lifetime is shorter than that of the antenna complexes of PSII. This difference is not due to the presence of the red forms in LHCI, as the data show that these Chls do not act as quenchers.

INTRODUCTION
During photosynthesis, organisms transform light energy into useful chemicals. The first steps of this process rely on a set of multi-protein complexes, among which are photosystems (PS) I and II (1). Both PSs are crimped with pigments that harvest light and transfer excitation energy to the reaction centers (RC) of PSI and PSII, where it is used to promote charge separation (3). Pigments are specifically organized either in the core antenna, which is directly connected to the RC, or in the peripheral antenna, which in plants and green algae can be further divided into subunits called Light-Harvesting Complexes (LHC). The LHCs differ in type and number in different organisms (177, 187). Our focus here is on the LHCs of PSI, called LHClis (or Lhcas), of the green alga *Chlamydomonas reinhardtii*. *C. reinhardtii* LHClis properties are discussed in comparison with those of the better characterized LHClis of the model plant *A. thaliana* and of the Lhc complexes of PSII (LHClII).

In *A. thaliana*, six *Lhca* genes were present (41) among which *Lhca1-4* are the ones that form the PSI peripheral antenna (28, 42, 43). These pigment-protein complexes are associated with the core in all conditions and are present in a 1 to 1 ratio with it (188). Two more gene products, Lhca5 and Lhca6, are present in sub-stoichiometric amount and their exact location has not been determined yet (41, 44, 114). Nine *Lhca* genes (1-9) have been found in *C. reinhardtii* (45, 46, 151), whose products are present in the PSI-LHCl complex (48). 127 chlorophylls (Chls) and 27 carotenoids (Cars) are estimated to be associated with the PSI peripheral antenna of *C. reinhardtii* (189), 70 more Chls and 14 more Cars than in the peripheral antenna of higher plants (5).
The structures of the LHClIs of higher plants have been recently obtained at high resolution (5, 29) revealing a highly conserved organization of both apoproteins and pigments. The remaining minor structural differences seem to have a large impact on the biochemical (e.g. pigment binding affinity) and spectroscopic properties of the complexes, which differ considerably between the four Lhcas (69, 190). In particular, each Lhca shows different absorption in the far-red part of the spectrum. This is due to a different content of the so-called red forms, which are Chls \( a \) that have their \( S_1 \) energy level lower than the one of the RC (74-77), or more generally of the bulk Chls \( a \) (63) and are typical of PSI (69). In higher plants, the excitonic pair formed by Chls \( a \, 603 \) and 609 (nomenclature Liu 2004) is responsible for the lowest energy absorption band in all LHClIs (79, 81, 157, 191). The strength of the interaction is modulated by the ligand of Chl 603 which is an asparagine (Asn) in the “red” Lhcas Lhca3 and Lhca4 (108). Changing this Asn into a histidine leads to the loss of the red absorption and fluorescence in Lhca3 and Lhca4 (108). It was shown that in Lhca4, the red forms are due to a mixing of this lowest exciton state with a charge transfer (CT) state (82, 84). A similar origin was suggested for the red forms associated with the other Lhcas (63, 80).

Monomeric LHClIs cannot be isolated to homogeneity, and reconstitution in vitro was then used to determine the biochemical and spectral properties of the individual complexes of \( C. \) reinhardtii (63). All \( C. \) reinhardtii LHClIs showed similar major pigment binding and spectroscopic properties despite a low sequence identity. As compared to the LHClIs of higher plants, the LHClIs of \( C. \) reinhardtii have higher affinity for Chl \( b \) binding (62, 63), with the exception of Lhca3 (nomenclature from (45)), which is however very unstable upon in vitro reconstitution. On average, the Chl \( a/b \) ratio of the reconstituted \( C. \) reinhardtii Lhca monomers was 2.2 (±0.9 when Lhca3 is excluded from the calculation) in agreement with the Chl \( a/b \) ratio of the PSI-LHCI supercomplex (48), considering 98 Chls \( a \) in the PSI core (5) in addition to the 85 Chls contained in the peripheral antenna. Low Chl \( a/b \) ratios were found in Lhca4, 7 and 8 complexes where a glutamine (Gln) residue is present as ligand for Chl 606. Gln residue at this site has previously been shown to increase the selectivity for Chl \( b \) of both 606 and 609 sites in plant LHClIs (192, 193). The nine LHClIs of \( C. \) reinhardtii have been clustered in three groups according to their red form content: the “blue” Lhcas (Lhca1, Lhca3 and Lhca7), the “intermediate” Lhcas (Lhca5, Lhca6 and Lhca8) and the “red” Lhcas (Lhca2, Lhca4 and Lhca9) (63). However, in \( C. \) reinhardtii, the red forms of the “red” LHClIs are at higher energy than those of the complexes of plants but they show common properties (63): (i) an Asn residue acts as ligand for Chl \( a \) 603 and (ii) they have a very broad bandwidth suggesting a CT origin. And finally, Lhca9 of \( C. \) reinhardtii has a Stokes shift of 15
nm, smaller than that of the “red” Lhcas of higher plants (80, 153) but larger than the “blue” Lhcas (191).

The presence and properties of the LHCI subunits influence the overall kinetics of PSI, slowing down the trapping time (90, 114, 163, 184). It was also shown that LHCIs have a multi-exponential decay (83, 112, 194-197), which is attributed to multiple conformations assumed by a monomer, each characterized by a specific lifetime and spectrum (112, 197). The analyses of the Lhcas of A. thaliana have shown that the conformation that harbors the reddest forms, also has the longest lifetime. However, in general the Lhcas lifetime is shorter than that of Lhcbs (197, 198).

To complete previous work on the properties of LHCIs in C. reinhardtii (63), time-resolved fluorescence of the nine Lhcas was measured, their decays were compared to those of C. reinhardtii Lhcbs and discussed on the basis of the red form content of each Lhca.

MATERIALS AND METHODS

Sample preparation - Lhcas were reconstituted in vitro as in (63). For a detailed description of the reconstitution procedure see (199). Lhcb monomers were prepared as in (65).

Spectroscopic measurements -

Steady state measurements - Absorption and fluorescence spectra were measured at room temperature (RT) on a Varian Cary 4000 UV-Vis spectrophotometer and a Fluorolog spectrofluorimeter (Jobin Yvon Horiba) respectively. For fluorescence measurement, samples were diluted below OD 0.07 cm⁻¹ at the maximum in the Qy in order to avoid self-absorption.

Time-resolved fluorescence measurement with a TCSPC (Time-Correlated-Single-Photon-Counting) set-up - The fluorescence decay kinetics of reconstituted Lhca antenna from C. reinhardtii were measured with the Lifetime Spectrometer FluoTime 200 (MCP-PMT detector) after excitation of 468 nm. The FWHM of its IRF is around 82 ps (due mainly to the pulse duration of 78 ps) and the time resolution is around 50 ps (channel spacing of 8 ps). The spectral bandwidth was 8 nm (monochromator ST-9030, 1200 lines/mm grating with 1.0 mm slits). The repetition rate was 10 MHz and the energy per pulse was 10 pJ (average power of 100 μW) which corresponds to far less than one photon per pulse per complex. Practically, the signal count rate has been set at less than 1% of the excitation repetition rate frequency. The samples were diluted at OD 0.07 cm⁻¹ at the Qy maximum to avoid self-
absorption (buffer 10 mM Tricine (pH 7.8), 0.03% α-DM, 0.5 M sucrose) and the fluorescence measured at 10°C, every 4 nm between 650 nm and 750 nm.

**Data analysis of time-resolved measurements** - The sequential analysis (148, 167, 168) was done as described in (184). The deconvolution and the fitting were made in TRFA Data Processor v 1.4 (chi² ranging between 1.033 and 1.353). The IRF was measured with pinacyanol chloride whose lifetime is 6 ps in methanol (117). The DAS were calculated manually, to correct for the sensitivity of the TCSPC detector, by the following formula: $DAS_n(\lambda) = \frac{I(\lambda) p_n(\lambda)}{\sum_n(p_n(\lambda) \tau_n)}$, with $p_n$ the amplitude of the exponential decay (not convoluted with the IRF) associated to the $n$-th component and $I$ is the total emission (equivalent to the steady state emission) at a specific detection wavelength.

The average decay time $\tau_{av}$ is calculated with the following formula: $\tau_{av} = \sum_n (\tau_n \cdot A_n) / \sum_n A_n$ with $A_n$ the area under the DAS of the $n$-th component (i.e. its total amplitude) and $A_n/\sum_n A_n$ the relative amplitude (see (184) for details). Care is taken to extract from the kinetics any influence of the disconnected species (Table 1). The relative initial population of the free Chls (%) was extracted from the amplitude of the 3rd DAS. For that, steady state fluorescence emission upon 468 nm excitation was compared to the one upon 500 nm. The emission difference upon 468 nm excitation compared to 500 nm was assumed to be related mainly to free Chls $b$. In case of a very high percentage of disconnection, the 3rd lifetime was discarded from the calculation of the average lifetime (Lhca3). Also the range considered to calculate the area under the DAS permits to exclude the region where free Chls dominate the emission (Lhca3, Lhca5).

<table>
<thead>
<tr>
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<th>Relative initial population of the free Chls (%)</th>
<th>DAS range considered to calculate the relative DAS area (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lhca1</td>
<td>3.3%</td>
<td>650-740</td>
</tr>
<tr>
<td>Lhca2</td>
<td>5.7%</td>
<td>650-750</td>
</tr>
<tr>
<td>Lhca3</td>
<td>26.0% 3rd lifetime discarded</td>
<td>674(c)-750</td>
</tr>
<tr>
<td>Lhca4</td>
<td>3.6%</td>
<td>650-760</td>
</tr>
<tr>
<td>Lhca5</td>
<td>1.9%</td>
<td>674(c)-740</td>
</tr>
<tr>
<td>Lhca6</td>
<td>3.9%</td>
<td>650-750</td>
</tr>
<tr>
<td>Lhca7</td>
<td>4.7%</td>
<td>650-750</td>
</tr>
<tr>
<td>Lhca8</td>
<td>3.4%</td>
<td>650-750</td>
</tr>
<tr>
<td>Lhca9</td>
<td>2.1%</td>
<td>650-750</td>
</tr>
</tbody>
</table>

Table 1: Percentage of free Chls and DAS range considered for the calculation in order to exclude the free Chls contribution.
RESULTS

Absorption and emission spectra at RT of all nine Lhcas are shown in Figure 1. Differences between the complexes can be observed in the ratio between the main absorption peak and the shoulder around 650 nm, which mainly reflects the differences in the binding affinity for Chl a or b. All complexes have their absorption maximum above 677 nm with the exception of Lhca3, 5 and 7. In the case of Lhca3 and Lhca5 the presence of disconnected Chls contributes to the blue shift as observed before (63). Lhca2, Lhca4 and Lhca9 have the largest absorption above 700 nm (Figure 1A) as well as the reddest emission (Figure 1B). However, the fluorescence spectra of all Lhcas have larger contributions above 700 nm than the spectrum of Lhcb monomers, indicating the presence of red-absorbing species in all nine Lhcas.

To estimate the relative amount of the red forms in the different complexes, the percentage of Qy absorption (630-750 nm) and of fluorescence emission above 700 nm were calculated. The results are reported in Table 2 and they indicate that the contribution of red forms increases in this order: Lhca1< Lhca6< Lhca3< Lhca5< Lhca8< Lhca7< Lhca4< Lhca9< Lhca2 in absorption and Lhca1< Lhca5< Lhca3< Lhca6< Lhca7< Lhca8< Lhca4< Lhca9< Lhca2 in emission.
Lhc of Chlamydomonas reinhardtii

<table>
<thead>
<tr>
<th></th>
<th>Relative absorption above 700 nm</th>
<th>Relative emission above 700 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lhca1</td>
<td>1.1%</td>
<td>34.3%</td>
</tr>
<tr>
<td>Lhca2</td>
<td>3.5%</td>
<td>54.0%</td>
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<td>Lhca3</td>
<td>1.7%</td>
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<td>Lhca4</td>
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<td>Lhca5</td>
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</tr>
<tr>
<td>Lhca9</td>
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<td>45.2%</td>
</tr>
<tr>
<td>Lhcb monomers</td>
<td>0.7%</td>
<td>25.6%</td>
</tr>
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</table>

Table 2: Percentage of absorption and emission above 700 nm

To determine the excited state lifetimes of the Lhcas, time-resolved fluorescence of each complex was measured upon 468 nm excitation by using a TCSPC set-up (see Materials and Methods). The data were globally analyzed by a sequential model (see Materials and Methods). The lifetimes and decay associated spectra (DAS) of all nine Lhcas are presented in Figures 2A and B. The decay of all complexes was well fitted by three or four components. Since four components did not significantly improve the fit, the three components description was used. The lifetimes range between 0.26-0.69 ns for the 1\textsuperscript{st} component, between 1.6-2.6 ns for the 2\textsuperscript{nd} component and between 3.6-4.8 ns for the 3\textsuperscript{rd} component. The ~2 ns component dominates the decay of all the Lhcas (Figure 2A).
When normalized to the maximum (Figure 2B), the shape of the first and second DAS are similar for most of the complexes, with the exception of Lhca2, Lhca4 and Lhca8 in which the second component is more red-shifted. In all the complexes the third DAS shows some increased emission in the blue, indicating the presence of disconnected Chls. This is particularly visible for Lhca3 and Lhca5, which also show a contribution of Chl b emission in
the second DAS. However, for most of the complexes the third component has an increased intensity in the far-red, indicating that part of the emission originates from an Lhc conformation enriched in red forms. An increased red-shift in the component characterized by a long lifetime was observed before for plant LHCl (112).

When comparing the DAS of the individual Lhcas (Figure 3), a large difference in the red form content of the complexes becomes visible. Lhca2 shows the largest amplitude in the far red for all three components, followed by Lhca9, Lhca3, Lhca4, Lhca6, Lhca7, Lhca8, Lhca5 and finally Lhca1 for the first component, which is not influenced by the presence of “free” pigments.

The percentage of disconnected Chls (i.e. Chls that do not participate in the excitation energy transfer within the complex) in each sample was evaluated (see Materials and Methods, Table 1) in order to calculate the average decay time of the individual Lhca. For most of the complexes the average decay times are in the 1.7-2.1 ns range (Table 3). Lhca9 shows a slightly longer lifetime (2.3 ns), but far shorter than that of the Lhcb complexes of C. reinhardtii (64).
Table 3: Table of lifetimes and relative amplitude (in brackets) estimated from the sequential analysis of Lhcas fluorescence decays (detection between 650 and 750 nm) compared to the Lhcb monomers (64). The relative contributions of disconnections in the amplitudes of the DAS have been estimated and excluded for the calculation on Lhcas (see Materials and Methods). However, the presence of disconnected species leads to a relatively high uncertainty (7%) of the average decay time.

DISCUSSION

The red forms of LHCI are known to have a CT character (80, 84), which is normally associated with a relative dark state and can behave as a quencher (82, 200). Studies of the fluorescence properties of LHCI of plants by time-resolved (112, 197) and single molecule spectroscopy (113), have revealed that these complexes switch between different conformations characterized by blue and red-spectra. However, differently than expected the conformation with the most red-shifted emission was shown to have the longest lifetime (112), suggesting an inverse relationship between red form content and quenched states. The nine Lhcas of *C. reinhardtii* have different contents of red forms and thus allows a systematic investigation of the effect of these forms on the excited state lifetimes of the complexes.

The fluorescence decays of the nine reconstituted LhcAs in *C. reinhardtii* are well described with three components of 0.4 (± 0.1) ns, 1.9 (± 0.3) ns and 3.8 (± 0.1) ns. The average decay times were all around 1.9 ns (± 0.2), significantly shorter than the average decay time of Lhcbs (64), but very close to that of the Lhcas of plants (112, 197).

It is interesting to observe that not only the average lifetime, but also all the three lifetimes that describe the decay of Lhcas are shorter than the three lifetimes that characterize the decay of LhcbMs of *C. reinhardtii* (64). This indicates that although all Lhcs exist in different conformations, the Lhcas are always present in a more quenched state than the Lhcbs.
In the case of Lhca2, which is the most red-shifted complex, the longest DASs also showed a higher amount of red forms, indicating that as it is the case in plants, the low energy forms are not acting as quenchers. However, a direct correlation between the energy/amount of red forms and the lifetime of the complexes could not be observed as all Lhcas have a very similar lifetime.
CHAPTER 5

The Photosystem I core of *Chlamydomonas reinhardtii*: spectral properties, excitation energy transfer and trapping kinetics

Clotilde Le Quiniou, Emine Dinc, Roberta Croce
ABSTRACT
Photosystem (PS) I is one of the two multi-protein complexes that harvest sunlight to perform charge separation and transport electrons across the photosynthetic membranes. PSI is made of two moieties, the core and the peripheral antennae. Among the hundreds of pigments associated with PSI, the majority are chlorophylls (Chls) $a$, but their spectral properties vary due to the presence of the protein and of neighboring pigments. In particular, some of these Chls $a$ (called red forms) absorb at lower energy compared to the other (“bulk”) chlorophylls present in the complex. The red forms have been well characterized in cyanobacteria and higher plants, where they have been shown to slow down the excitation energy transfer (EET) and trapping kinetics of PSI. On the contrary, the decay kinetics and the presence of red form in the PSI core of the green alga *Chlamydomonas reinhardtii* are still a matter of debate. The reason arises from the difficulty to disentangle the kinetics of the two PSs in cells, and to purify PSI core to homogeneity for *in vitro* analysis. To overcome these problems, we have used a *C. reinhardtii* mutant that lacks PSII and Chl $b$, for *in vivo* studies and for the purification of the complex. We show that red forms absorbing at 701.2 nm are present in the core and influence its EET and trapping kinetics. These red forms constitute a pool of Chls that are energetically well distinguishable from the bulk Chls and from the red forms present in the peripheral antenna. A possible location of the red forms in the core is discussed.

INTRODUCTION
Oxygenic photosynthesis relies on four major trans-membrane pigment-protein complexes that transport electrons and pump protons across the thylakoid membrane. The photosynthetic machinery produces reducing power (NADPH) and chemical energy (ATP) for the Calvin-Benson cycle where $CO_2$ is reduced to carbohydrates. Light harvesting and charge separation are assured by two of these four major complexes, photosystems (PS) PSI and PSII. Most of the subunits of the Photosystems are highly conserved among eukaryotic organisms and cyanobacteria and constitute the core complex, while the outer antenna subunits have evolved differently (40). Both core and outer antennae bind chlorophylls (Chls) and carotenoids (Cars) that increase the absorption cross section of the reaction center (RC) where charge separation occurs. The core antenna only binds Chls $a$ while the peripheral antenna binds other types of Chl. In higher plants and green algae the peripheral antenna is composed of trans-membrane proteins called Light Harvesting Complexes (LHC) that also contain Chl $b$. The subunits associated with PSI are called Lhca (or LHCI) and those principally associated with PSII Lhcb (or LHCII). The light harvesting capacity of the PSs can vary between organisms depending on the number of LHCs associated with the cores. In
Particular, four Lhcas are present in the PSI-LHCI of higher plants (28) and nine in the PSI-LHCI of the green alga *Chlamydomonas reinhardtii* (48).

Excitation energy transfer (EET) and trapping kinetics in PSI-LHCI were shown to depend not only on the size of the antenna but also on its composition (90, 114, 163, 184). PSI is enriched in the so-called “red forms”, which are Chls a that absorb at energy lower than the RC, or more generally, than the bulk Chls a and that are characterized by a broad absorption spectrum and a large Stokes shift (63, 74-77, 80, 82, 86). Red forms can be located either in the core and/or in the peripheral antennae depending on the organism and they strongly influence the trapping kinetics. The average decay time changes from ~14 ps in the PSI of a cyanobacterium without red forms (115) to ~40 ps in the PSI of *Arthrospira platensis*, a cyanobacterium that contains the most red-shifted absorption forms observed so far (90). When located far from the RC, the red forms act as competing local traps from which the excitation energy is released back to the bulk after thermal activation (161, 201-205). It was shown that the red forms located in the Lhcas introduce a diffusion-limited step in the EET and trapping kinetics in higher plants like *Arabidopsis thaliana* (114, 116, 117) and to a smaller extent in *C. reinhardtii* (184, 189), where the “red” Lhcas are higher in energy than in higher plants (63, 197).

The role and the presence of the red forms in eukaryotic PSI core have been debated for long. In *C. reinhardtii*, the PSI RC was reported to absorb at ~697 nm (100, 103, 118, 119) and no Chls absorbing above 700 nm were detected (100, 101). However, the maximum of the 77K fluorescence emission of *C. reinhardtii* PSI core peaks at 720 nm (99), suggesting the presence of red forms in this complex.

In previous works, the decay kinetics of the isolated PSI core in *C. reinhardtii* was described with two major components of ~8 ps and ~22 ps lifetimes while a minor component of ~100 ps containing red-shifted emission was considered to be due to impurities and discarded (104, 164, 206). To answer the question whether PSI core contains red forms it is thus necessary to study a stable and pure preparation of the complex. However, the purification of the PSI core from WT *C. reinhardtii* cells is difficult as the core co-migrates with some Lhcas (48, 104). To overcome this problem, a mutant of *C. reinhardtii* lacking PSII and in principle Lhcs (CC 2696) was used in previous studies on PSI core (100-105, 118, 164, 206-211). Nevertheless, remaining Lhcs were present in this mutant complicating the analysis of the PSI core kinetics (104, 164, 206). Here we have characterized PSI core isolated from a different mutant, lacking PSII and Chl b (CC 2754), whose *in vivo* decay was dominated by PSI kinetics (156, 212, 213). We studied EET and trapping kinetics of PSI core by time-resolved fluorescence, *in vivo* and upon isolation, and we analyzed the data by target analysis. We observe that a red Chl pool is present in the PSI.
core of *C. reinhardtii* and absorbs at 701.2 nm. These red forms slow down the EET and trapping kinetics of the core even though less than observed in other organisms. We also show that the red forms of PSI core are in faster equilibrium with the bulk Chls than the red forms present in the Lhcas.

**MATERIALS AND METHODS**

**Strains and growing conditions** - The strain CC 2754 (Fl 39 Pg28) is a PSII/Chl b-less mutant obtained after UV irradiation of CC 2753 (Fl 39, (182)). CC 2754 is a non-photosynthetic mutant as it does not have PSII activity. It lacks part of the Lhcas and does not produce Chl b (156, 212, 213). Cells of CC 2754 were grown in liquid Tris-Acetate-Phosphate (TAP) medium (214) at room temperature (25°C) on an incubator shaker (Orbitron, INFORS HT) at 140 rpm in the dark. Cells of CC 4402 (WT (215)) and CC 2696 (previously called A4d (207, 216)) were grown in similar conditions but under continuous normal light (50 umol photons PAR m⁻².s⁻¹, Sylvania GROLUX T8 F15W-GRO).

**Sample preparations** -

*Thylakoid preparation from CC 2754* - The cells were harvested by centrifugation (3500 rpm Rotor JA-10, 5 min, 20°C) of six liters of culture (5.1 10⁷ cells/mL). Thylakoid membranes were prepared as described in (217), with the modifications reported in (48) and additional modifications. In short, cells were disrupted by sonication (52 Watt in 15 cycles of 10s ON/30s OFF, Vibracell VCX130 6mm probe, Sonics) in 80 mL with stirring and centrifuged (10000 rpm Rotor JA-10, 25 mins, 4°C). Thylakoids were loaded on a discontinuous sucrose gradient (thylakoids in 9mL of 1.8M sucrose +5 mM Hepes+10 mM EDTA (H3)/ 3mL of 1.3M sucrose+5mM Hepes+10mM EDTA (H4)/ 3mL of 0.5M sucrose+5mM Hepes (H5)) and separated by centrifugation (24000 rpm Rotor JA-25.15, 1 h, 4°C). Two bands were collected: the upper band between H4 and H5 and the lower band between H3 and H4. Thylakoids were stored at -80°C.

*Thylakoid solubilization from CC 2754* – Isolated core complexes were prepared as in (86) and (48) with some modifications. Thylakoids were washed three times with 20 mM Hepes (pH7.5), unstacked in 20 mM Hepes with 5 mM EDTA and finally washed with 20 mM Hepes. Thylakoid were pelleted then resuspended in 20 mM Hepes (pH 7.5) to a final concentration of 0.3 mg chlorophyll /ml, 0.5% Zwittergent 3-16 and 1% n-dodecyl- β-D-maltoside (DM). Samples were vortexed for few seconds and then centrifuged (14000 rpm Sigma 1-15k, 5 mins, 4°C) to remove unsolubilized material. Supernatant was loaded on a sucrose density gradient (prepared by freezing and thawing 0.5 M sucrose, 20 mM Hepes (pH 7.5), 0.03% α-DM buffer). The complexes present in the membrane were separated by
ultracentrifugation (41000 rpm Rotor SW41 14h, 4°C). The green bands visible in the sucrose gradient were harvested with a syringe.

PSI-LHCI were prepared as in (48), PSII-LHCII and native Lhcb monomers as in (65) and PSI core of A. thaliana as in (107).

**SDS-PAGE analysis** - Proteins were analyzed by SDS-PAGE with Tris-Tricine buffer system (218) at 14.5% acrylamide concentration. The amount of samples loaded into each well was 1 µg (in Chls). The Coomassie stained gel was imaged with a ImageQuant LAS-4000 (GE Healthcare).

**Western blot analysis** - Protein extraction and immunoblot analysis were performed as described previously (219).

**Spectroscopic measurements** -

**Steady state measurements** - Absorption and fluorescence were measured on a Varian Cary 4000 UV-Vis spectrophotometer and a Fluorolog spectrofluorimeter (Jobin Yvon Horiba) respectively at room temperature (RT) and 77K. The cells absorption was measured with the Varian 900 external Diffuse Reflectance Accessory. For 77K measurements, samples were in 65% glycerol. For fluorescence measurement, samples were diluted in order to avoid self-absorption. The Circular-Dichroism (CD) spectra were measured using a Chirascan-Plus CD Spectrometer at 10°C.

**Time-resolved fluorescence measurements on the streak camera set-up** – Picosecond-time-resolved fluorescence measurements were performed with a streak camera setup as previously described (90, 148, 184). The samples were measured upon 400 nm excitation at RT for the cells and 15°C for the isolated particles. The repetition rate was set at 150 kHz, the pulse energy was 0.5 nJ (0.12 mol photons/m²/s) for the cells and 1.5 nJ (0.38 mol photons/m²/s) for the isolated particles. The samples were stirred in all cases. A power study confirmed the absence of annihilation in the measuring conditions. The fluorescence was detected from 590 nm to 860 nm in two different time ranges (TR): from 0 to 155 ps (TR1 temporal response 5-6 ps) and from 0 to 890 ps (TR3 temporal response 12 ps). Sample OD was low enough to avoid self-absorption during the time-resolved measurements: the maximum OD in the Qy were 0.39 cm⁻¹ for the cells (measured with the integrating sphere), 1.2 cm⁻¹ for lb4 and 0.28 cm⁻¹ for ub5. The datasets consist of sequences of images : for the cells, 100 images of 1 min at TR3 were acquired; for lb4, 40 images of 2 mins at TR1 and 60 images of 30 s at TR3; and for ub5, 23 images of 5 mins at TR1 and 40 images of 90 s at TR3. These sequences were corrected and treated in HPD-TA 8.4.0 (Hamamatsu) as in (184).
Data analysis of time-resolved measurements -

*Sequential analysis* - The corrected datasets were globally analyzed with a sequential model (148, 167, 168) in Glotaran 1.3 (166). The instrument response function (IRF) was modeled with a simple Gaussian. From the sequential analysis, the Evolution Associated Spectra (EAS) were fitted and the Decay Associated Spectra (DAS) calculated from them. During the sequential analysis of the isolated particles, the streak camera datasets of TR1 were simultaneously fitted to link the two first lifetimes. The average decay time $\tau_{av}$ is calculated from the area under the DAS (see (184) for details) and characterizes the time until charge separation occurs. Only the components attributed to the PSI-related DAS are used in the calculation.

*Target analysis of the streak camera measurements* - Datasets from PSI core (fraction ub5) together with datasets from PSI-LHCI (previously analyzed in (184)) were simultaneously analyzed by a target fit (148, 169). Two TRs were used for each sample: TR1 and TR3 (see above for the temporal resolution) for ub5 and TR1 and TR4 (from 0 to 1500 ps, temporal response 18 ps) for PSI-LHCI. The instrument response function (IRF) parameters were fixed as obtained from the sequential analysis. Datasets from ub5 were chosen for the target analysis since this preparation was the most devoid of disconnected species (see Results).

The kinetic model, described in Scheme 1, contains four compartments: (i) two compartments common to both datasets and therefore corresponding to Core-related compartments and (ii) two compartments only used for PSI-LHCI datasets and corresponding to Lhcas-related compartments as they represent the structural difference between PSI core and PSI-LHCI. The trapping occurs from one compartment of the core made of bulk Chls (called Bulk Core).

Additionally, the model contains one functionally disconnected compartment (of different type in the different preparations) representing disconnected species with a small population (between 2.9% and 3.7%) and ns-lifetimes.

The following constraints were used during the fitting:

- The intrinsic decay rate of the PSI-related compartments was fixed to (1.9 ns)$^{-1}$, as measured on the reconstituted Lhca monomers (Chapter 4).
- Since Red Core represents red species, its Species Associated Spectrum (SAS) was set to zero below 675 nm.
- The PSI-related compartments were initially populated as in Table 1. Excitation was constrained to be equally shared between the core and the Lhcas (see Results Figure 5B inset). Red Core was initially populated as the reddest pool identified from the 77K absorption (see Results Figure 6C).
Table 1: Relative initial populations of the PSI-related compartments of the kinetic model shown in Scheme 1. Inputs on the compartments were fixed (f) and the others estimated.

<table>
<thead>
<tr>
<th></th>
<th>Bulk Core</th>
<th>Red Core</th>
<th>Bulk Lhcas</th>
<th>Red Lhcas</th>
</tr>
</thead>
<tbody>
<tr>
<td>ub5</td>
<td>96.8% (f)</td>
<td>3.2% (f)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PSI-LHCI</td>
<td>48.4% (f)</td>
<td>1.6% (f)</td>
<td>36%</td>
<td>14%</td>
</tr>
</tbody>
</table>

Using these constraints and assuming equal oscillator strength for each compartment species (equal area under the SAS (171)), the SAS and the rate constants (uncertainties ranging from 0.2% to 22%) were fitted by means of a non-negative least-squares variable projection algorithm (167, 168, 170) as well as the free-energy differences between compartments and the initial population on Lhcas-related compartments (uncertainties of 4%).

The average decay time was calculated from the amplitude matrices as the product of the overall amplitude of the trapping (last column of Table 3) with the corresponding lifetimes.

**Excitonic interaction calculation** - Interactions between excitonically coupled Chls were calculated to have a first approximation of their lowest exciton state. We consider the simple case of isoenergetic Chls (e.g. site energy at 680 nm=14706 cm⁻¹) with similar transition dipole strength d=μ² (21 Debye² in vacuum (9)). We use the dipole-dipole approximation to describe their interaction. The coupling strength of a Chl pair is thus written as

$$V = C \frac{5.04 \cdot \kappa \cdot d}{R^3} \text{ (in cm}^{-1} \text{ (15, 220))}$$

with \( \kappa \) the orientation factor

$$\kappa = \hat{\mu}_a \cdot \hat{\mu}_b - 3 \left( \hat{\mu}_a \cdot \hat{R} \right) \left( \hat{\mu}_b \cdot \hat{R} \right)$$

that describes the organization of monomer \( a \) and monomer \( b \). Their centers are relatively positioned according to \( \hat{R} \) and \( C \) is a correction factor that considers the permittivity of the medium. We take \( C \) equals to 1 here as if the Chls were in vacuum. The exciton states were calculated in different species and compared. The orientations and distances were taken from the crystal structures of *S. elongatus* (PDB 1JB0 (24)), of higher plants (PDB 4XK8 (5)) and of *Synechosystis* PCC 6803 (PDB 4KTO (30)). The interaction matrices (Table S1) were diagonalized with Matlab (MathWorks R2014b 64-bit, MATLAB).

**RESULTS**

**PSI-only mutants: Steady state and time-resolved spectroscopy on cells**

The spectroscopic properties of *C. reinhardtii* mutant CC 2754 and mutant CC 2696, which are expected to contain only PSI core, were analyzed. The RT absorption spectrum of
CC 2754 cells does not show any shoulder around 650 nm (Figure 1A) in agreement with the absence of Chls $b$ in this strain (212). On the contrary Chl $b$ absorption is clearly visible in the WT spectrum as well as in the spectrum of the CC 2696 cells although it is less pronounced than in the WT cells (Figure 1A). This indicates that the CC2696 mutant has a reduced peripheral antenna size compared to the WT but that some Lhcs are still present in the cells (216). The reddest $Q_y$ absorption of the CC 2754 cells in all the strains ($Q_y$ maximum at 680.5 nm instead of 678 nm and larger far-red absorption, Figure 1B), indicates a relatively higher amount of PSI in this mutant. Based on these results the CC 2754 mutant was selected for further analysis. The emission spectrum of the CC2754 cells has its maximum at 715 nm at 77K indicating that it originates mainly from PSI (Figure 1C). However, a shoulder around 684 nm (Figure 1C) suggests the presence of a small amount of non-PSI Chls.

Figure 1: A) Absorption spectra at room temperature (RT) of the cells CC 4402 (WT, black), CC 2696 (red) and CC 2754 (blue), normalized to 1 in the $Q_y$; B) Enlarged view of the absorption spectra shown in (A); C) steady-state fluorescence emission at RT and 77K of the CC 2754 cells upon 440 nm and 494 nm excitation

Time-resolved fluorescence of the cells was measured with a streak camera setup upon 400 nm excitation and the decays analyzed globally with a sequential model (see Materials and Methods). From the measurements of the CC 2754 cells (Figure 2), two components with lifetimes of 9.3 ps and 44.3 ps (Figure 2A) and red-shifted spectra (Figure 2B) were observed and can be attributed to PSI. The first component represents mainly a decay process mixed with some energy transfer as revealed by its Decay Associated Spectrum (DAS) whose amplitude equals zero above 720 nm (Figure 2A). The second component is a pure decay component and dominates the trapping kinetics. The average decay time of PSI (calculated from the area under the PSI-related DAS, see Materials and Methods) in the cells is ~30 ps for PSI (Table 2), very close to the shortest lifetime reported in the cells previously (213). This average decay time is longer than the PSI core lifetime of higher plants (21.3 ps (114)). This is unexpected, since the red forms of PSI in C. reinhardtii are at higher energies than those of A. thaliana PSI core and in all studied PSI core preparations, a direct relationship between the energy of the red forms and the trapping
kinetics was observed. It is thus likely that the slower PSI trapping kinetics in this mutant is due to the presence of some Lhcs.

![Graph](image1.png)

Figure 2: DAS (A) and EAS (B, normalized to the maximum) from the sequential analysis of the time-resolved fluorescence of the CC 2754 cells measured between 0 and 890 ps and upon 400 nm excitation.

Indeed, even though most of the Lhcs are not expected to fold without Chl \( b \) (165), immunoblotting analysis reveals the presence of CP29 and CP26 in the CC 2754 cells (Figure 3A). Moreover, from the time-resolved fluorescence measurements it is observed that 23.6% of the initial excitation (Table 2) populates two species that have their Evolution Associated Spectra (EAS) maxima around 680-682 nm and whose lifetimes of 268.4 ps and 3.2 ns match well with those of monomeric Lhcb (64). These results show that some antenna complexes are indeed present in this mutant, supporting the hypothesis that the lifetimes observed in the cell is due to the association of some of them with PSI.

We should therefore conclude that the composition of CC 2754 thylakoid membrane remains complex preventing the determination of the properties of PSI core directly in the cell. We therefore proceed with the isolation of the PSI core.

**Isolated PSI core: biochemical characterization and steady state spectroscopy**

Two bands containing thylakoids were obtained and called upper (u) and lower (l) depending on their mobility in the discontinuous sucrose gradient. These two bands were solubilized, with the procedure used to isolate the PSI core from plants (86) and resulted in two slightly different patterns on the successive sucrose density gradient (Figure 3B) with the u band containing complexes with higher molecular weight.
Figure 3: Thylakoid isolation and solubilization. A) Immunoblotting analysis with antibodies against CP26 and CP29. B) Sucrose density gradients showing the separation of the solubilized thylakoids from the upper (u) and lower (l) thylakoid bands. C) SDS-PAGE gels (stained by Comassie) showing the protein pattern of thylakoids of WT (CC 124) and of the mutant CC 2754 (l thylakoids), isolated PSI-LHCl (48), PSII-LHCl and trimeric Lhcb (65) and of the bands from the sucrose gradient in (B).

The protein composition of the gradient bands was analyzed by SDS-PAGE (Figure 3C). Neither LHCII nor Lhcas are present in the lb4, ub3 and ub5 bands. These fractions mainly contain PSI core, in agreement with the absorption spectra (Figure 4B). The extra bands present in the gel in the lb4, ub3 and ub5 samples are subunits of the ATP synthase (Figure 3C). The 77K fluorescence emission spectrum of these three fractions is dominated by the 715-716 nm emission, characteristic of PSI (Figure 4C). An additional band with maximum around 675 nm suggests the presence of free pigments in all the preparations, but in a smaller amount in ub5. This fraction was thus selected for the spectroscopic characterization.
Figure 4: A-B) Absorption spectra at RT of the gradients bands of the solubilized thylakoids (Figure 3C). C) Fluorescence emission at 77K of gradient bands containing PSI

The mobility in the gradient suggests that lb4 contains PSI monomers, while ub5, which is lower in the gradient, corresponds to PSI core in a higher aggregation state. However, the absorption spectra of the two bands are virtually identical, indicating that these two PSI preparations only differ for the aggregation state. In the following, we will refer to these bands as “PSI core”.

The spectroscopic properties of these PSI core preparations were compared to those of the PSI core isolated from *A. thaliana* (Figures 5A and C): the absorption and the circular dichroism spectra have similar shapes in the *Qy* region, while some differences are visible in the blue region, probably due to differences in carotenoid content.
Figure 5: A) Absorption at RT of *C. reinhardtii* (**C.r.**) PSI core (measured on fraction ub5) compared to *A. thaliana* (**A.t.**) PSI core normalized to 1 in the Qy. B) Absorption spectra from *C. reinhardtii* of PSI-LHCI (green) and PSI core (red, fraction ub5), scaled to their Chl content, and their difference (blue). Inset: percentage of absorption from the Lhcas in PSI-LHCI. C) Circular Dichroism (CD) of *C. reinhardtii* PSI core (measured only on fraction lb4 since the CD of ub5 fraction was impossible to measure considering its low OD) and *A. thaliana* PSI core, normalized to the minimum in the Qy. D) CD from *C. reinhardtii* PSI-LHCI (green) and PSI core (red, fraction lb4), scaled to the linear absorption in the Qy, and their difference spectrum (blue).

By scaling PSI-LHCI and PSI core absorption spectra to their Chl content, their difference spectrum (PSI-LHCI minus PSI core in Figure 5B, blue), which should represent the average LHCI spectrum, can be calculated. The absorption above 694 nm is more intense in the core than in the LHCI spectrum, which suggests that the content of red forms in the core is higher than in the LHCIs. The CD difference spectrum (Figure 5D, blue) shows features similar to the CD of the reconstituted Lhcas (63) with the typical “- + -” signature in the Qy region and three negative components in the blue region (around 470, 480 and 500 nm). This suggests that, within each Lhcas, the main organization of the pigments and their excitonic interactions are preserved when they are assembled with the PSI core.
Red form content and decay kinetics of the PSI core fractions

The absorption maximum of the PSI core (lb4) at 77K in the Q_y region is at 677.5 nm (Figures 6A). The second derivative of the 77K absorption spectrum in the Chl region shows the presence of absorption forms at 670.5, 678, 684.5 and 699.5 nm (Figure 6B). After gaussian deconvolution (Figure 6C), two red components with maxima at 696.5 nm and 701.2 nm were identified. They account for 3.6% and 3.2%, respectively, of the absorption in the Q_y region. In the carotenoid region, a minimum is visible at 499 nm, and it should correspond to the β-carotenes in PSI core.

Time-resolved fluorescence of the PSI core fractions was measured upon 400 nm excitation. These decays were properly fitted by three components whose DAS are presented in Figure 7. The first component (with a lifetime of 6.3 ps) has a positive/negative signature and represents energy transfer from blue Chls a to red Chls a, although it also contains some decay as indicated by the non-conservative area under the DAS. The second
component (with a lifetime of 21.6 ps) is a pure decay component and represents the time during which most of the trapping occurs. The slowest component, that accounts for 3-6% of the initial excited states, shows blue-shifted DAS and long lifetimes (4.1-5.6 ns) and corresponds to a mixture of disconnected species, mostly Lhcs (lifetime of fraction lb3 is ~4 ns) or free Chls (with a lifetime of ~6 ns (112)).

![Figure 7: DAS from the sequential analysis of the fluorescence decays of lb4 (solid) and ub5 (dashed) measured between 0 and 155 ps and upon 400 nm excitation. The two first components had their lifetimes linked.](image)

The average decay times (calculated using the area under the DAS, see Materials and Methods) of lb4 and ub5 are 18 ps and 17.2 ps, respectively, (Table 2) very close to each other despite the difference in molecular weight of the samples (Figure 3), confirming that both preparations contain PSI core complexes only differing in their aggregation state.
<table>
<thead>
<tr>
<th></th>
<th>Cells CC2754</th>
<th>lb4</th>
<th>ub5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tau_1$ (ps)</td>
<td>9.3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>relative amplitude</td>
<td>11%</td>
<td>21.9%</td>
<td>27.6%</td>
</tr>
<tr>
<td>$\tau_2$ (ps)</td>
<td>44.3</td>
<td></td>
<td>21.6</td>
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<tr>
<td>relative amplitude</td>
<td>65.4%</td>
<td>72.5%</td>
<td>69.1%</td>
</tr>
<tr>
<td>$\tau_3$ (ps)</td>
<td>268.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>relative amplitude</td>
<td>16.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\tau_4$ (ns)</td>
<td>3.2</td>
<td>4.1 ns</td>
<td>5.6 ns</td>
</tr>
<tr>
<td>relative amplitude</td>
<td>7.1%</td>
<td>5.6%</td>
<td>3.3%</td>
</tr>
<tr>
<td>Average decay time $\tau_{av}$ (ps) of PSI complex</td>
<td>30</td>
<td>18</td>
<td>17.2</td>
</tr>
</tbody>
</table>

Table 2: Lifetimes obtained from the sequential analysis of the fluorescence decays of the CC 2754 cells and the isolated PSI cores, with their relative amplitude (i.e. $A_n / \sum A_n$ see Materials and Methods) and average decay time $\tau_{av}$ (only components attributed to PSI are considered in the calculation). The lifetimes of the two first components of lb4 and ub5 were linked.

**Decay kinetics of PSI core as a building block of PSI-LHCI**

After characterizing the isolated PSI core, its contribution to the EET and trapping kinetics of PSI-LHCI was analyzed. This was done by performing a simultaneous target analysis of the time-resolved fluorescence measurements of PSI-LHCI (184) and PSI core (ub5).

The model used to fit the PSI-LHCI kinetics consists of four compartments (Scheme 1), two related to PSI core (Red Core and Bulk Core) and two to Lhcas (Bulk Lhcas and Red Lhcas). Only Red Core and Bulk Core compartments were used for the target analysis of the PSI core datasets. The trapping occurs from Bulk Core with a rate $k_T = (12.85$ ps)$^{-1}$. Initial populations of each compartment (Table 1) were based on the absorption spectra (Figure 5B inset): in PSI-LHCI upon 400 nm excitation, Lhcas and core are equally populated. The population of the Red Core is calculated based on the absorption of the 701nm-pool which is 3.2% of the core absorption (Figure 6) and 1.6% of the PSI-LHCI absorption. In addition to these four PSI-related compartments, two compartments (one for each sample) account for the small amount of disconnected species present in the preparations.
Scheme 1: Kinetic model for the simultaneous target analysis of PSI core and PSI-LHCI measured upon 400 nm. Red Core and Bulk Core compartments are similar for both particles (identical rate constants, SAS and initial population ratio) while Bulk Lhcas and Red Lhcas are only found in PSI-LHCI. All PSI compartments decay naturally with the intrinsic decay rate $k_i = (1.9 \text{ ns})^{-1}$, fixed (f) as measured on reconstituted Lhca monomers (Chapter 4), except for Bulk Core from where the trapping occurs with a rate $k_T$. Additionally the model contains functionally disconnected species of different type in the different preparations. The estimated rate constants (ns$^{-1}$) are indicated with the fit uncertainties in brackets.

The Species Associated Spectra (SAS) and the DAS estimated from the target analysis are shown in Figure 8. The SAS of the Bulk compartments (Bulk Core and Bulk Lhcas) are very similar and correspond to bulk Chls $a$ emission. Although Bulk Lhcas has a bluer maximum than Bulk Core (Table 4), both SAS have an equal average spectral position at 14255 (± 20) cm$^{-1}$ (i.e. 701 ± 1 nm). Red Core has its maximum at 708 nm, more than 20 nm red-shifted as compared to all the other compartments (Figure 8A). Red Lhcas contains red Chls and a few bulk Chls in fast equilibrium with them (Figure 8A).
The disconnected species with ns-lifetimes (Figure 8C-D) are present in small amount (area under the DAS between 2.9% and 3.7%) and represent mostly disconnected Chls in the PSI core preparation and disconnected Lhcas in the PSI-LHCI preparation (mixed with very few free Chls (184)). Bulk compartments’ SAS are redder than the SAS of the disconnected species (Figure 8B).

DAS of PSI core and PSI-LHCI (Figure 8C-D) can be interpreted with the help of the amplitude matrices (Table 3) which permit to follow the (de)population of each compartment at each lifetime.
Chapter 5

<table>
<thead>
<tr>
<th></th>
<th>Bulk Core</th>
<th>Red Core</th>
<th>Bulk Lhcas</th>
<th>Red Lhcas</th>
<th>Overall amplitude of the trapping (%)</th>
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</thead>
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<tr>
<td><strong>PSI Core</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7.6 ps</td>
<td>0.642</td>
<td>-0.265</td>
<td></td>
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<td>38%</td>
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<tr>
<td>24.4 ps</td>
<td>0.326</td>
<td>0.297</td>
<td></td>
<td></td>
<td>62%</td>
</tr>
<tr>
<td><strong>PSI-LHCI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2 ps</td>
<td>0.155</td>
<td>-0.005</td>
<td>-0.135</td>
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<td>11.9 ps</td>
<td>0.073</td>
<td>-0.131</td>
<td>0.127</td>
<td>-0.002</td>
<td>7%</td>
</tr>
<tr>
<td>37.0 ps</td>
<td>0.233</td>
<td>0.143</td>
<td>0.329</td>
<td>-0.024</td>
<td>68%</td>
</tr>
<tr>
<td>127.4 ps</td>
<td>0.022</td>
<td>0.009</td>
<td>0.034</td>
<td>0.170</td>
<td>24%</td>
</tr>
</tbody>
</table>

Table 3: Amplitude matrices of PSI core and PSI-LHCI upon 400 nm excitation.

For PSI core, the DAS estimated from the target analysis (Figure 8C) have very similar shape and lifetimes as those from the sequential analysis (Figure 7). In 7.6 ps, 41% of the energy in *Bulk Core* is transferred to *Red Core* (negative amplitude which corresponds to population of the compartment) and the rest is trapped, representing 38% of the overall trapping. In 24.4 ps, 62% of the trapping occurs from *Bulk Core* and *Red Core* to the same extent (equal positive amplitudes). For PSI-LHCI, the 1.2 ps lifetime involves energy transfer from red Chls $a$ to blue Chls $a$ related to the depopulation of *Bulk Core* into *Bulk Lhcas* (Table 3). This lifetime is within the time resolution of the experiment (~10% uncertainties) and has to be interpreted carefully. In 11.9 ps, *Red Core* is populated by both *Bulk* compartments and the trapping starts (7% of the overall trapping, Table 3). In 37.0 ps, most of the trapping occurs (68%, Table 3) from the core (both *Bulk Core* and *Red Core*) and *Bulk Lhcas*, while *Red Lhcas* is still getting populated. Finally, 24% of the trapping occurs after a slow transfer from *Red Lhcas* (on a time scale of ~130 ps). The average decay times (calculated from the amplitude matrices, see Materials and Methods) are 18 ps for PSI core and 56 ps for PSI-LHCI, in agreement with previous results (184).

Using the free energy difference, $\Delta G$, and the enthalpy difference, $\Delta H$, between compartments, we can estimate the number of Chls in each compartment from the $\Delta S$ value in the detailed balance (184) for details). From the ratio of the estimated rate constants, we observed the following differences in the free energy of the compartments: (i) *Red Core* is higher than *Bulk Core* by 25 meV; (ii) *Bulk Core* is higher than *Bulk Lhcas* by 7 meV and (iii) *Bulk Lhcas* is lower than *Red Lhcas* by 43 meV. The enthalpy is approximated either as the maximum or as the average spectral position of the SAS (Table 4) to consider the possible heterogeneity in the compartments. In particular, the “red” compartments can also contain few blue Chls in fast equilibrium with the red forms. The SAS maximum in the “red” compartment is closer to the zero-phonon line of the blue Chls (considering Boltzman equilibrium at RT) while the average spectral position is closer to the zero-phonon line of the red forms since the vibrational band of the blue Chls contribute to the red wing of the SAS.
By using the two approximations, we characterize the extremes of the compartment enthalpy. The associated number of Chls for the compartments is actually in between the two numbers calculated in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>SAS Maximum (nm)</th>
<th>Number of Chls a (% of Chls a)</th>
<th>SAS Average Spectral Position in cm⁻¹ (corresponding wavelength in nm)</th>
<th>Number of Chls a (% of Chls a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk Core</td>
<td>687</td>
<td>94 (51.3%)</td>
<td>14260 (701)</td>
<td>95 (51.7%)</td>
</tr>
<tr>
<td>Red Core</td>
<td>708</td>
<td>4 (2.3%)</td>
<td>13780 (726)</td>
<td>3 (1.8%)</td>
</tr>
<tr>
<td>Bulk Lhcas</td>
<td>685</td>
<td>73 (39.4%)</td>
<td>14250 (702)</td>
<td>80 (43.8%)</td>
</tr>
<tr>
<td>Red Lhcas</td>
<td>686</td>
<td>12 (7.1%)</td>
<td>14030 (713)</td>
<td>5 (2.6%)</td>
</tr>
<tr>
<td>sum PSI Core</td>
<td></td>
<td></td>
<td></td>
<td>98 (53.6%)</td>
</tr>
<tr>
<td>sum PSI-LHCI</td>
<td></td>
<td></td>
<td></td>
<td>183 (100%)</td>
</tr>
</tbody>
</table>

Table 4: Maxima (± 0.5 nm) and average spectral positions of the SAS of the PSI-related compartments used as approximation of the enthalpy in the detailed balance (see main text) in order to estimate the number of Chls a in each compartment. Uncertainty: (i) on the Average Spectral position has been estimated according to the SAS noise and ranges from 20 to 50 cm⁻¹ (i.e. 1-3 nm); (ii) on the percentage is ±0.3%.

Considering 98 Chls a in PSI core as in higher plants (5), 183 Chls a in PSI-LHCI of C. reinhardtii (189) and the two different approximations for the enthalpy, the number of Chls a in each compartment is calculated (Table 4): the Bulk compartments contain indeed most of the Chls a (more than 91%) and the Red compartments contain between 8 and 16 Chls. Red Core, which has energetically well distinct Chls (see above), contains only 3-4 Chls.

Excitonic calculations were performed on the Chls that were proposed to be responsible for the red forms in the PSI core (Chls B31, B32 and B33 (24)). We compared the spatial organization of the Chls in the PSI cores of S. elongatus, Synechosystis PCC 6803 and higher plants, for which the crystal structures are available. Eigen values of the interaction matrices (Table S1) correspond to the energy levels of the exciton state. Eigen values are 14265.6, 14740.9 and 15111.2 cm⁻¹ for S. elongatus, 14419.8 and 14992.0 cm⁻¹ for Synechosystis PCC 6803 (B33 absent) and 14324.9, 14725.8 and 15067.0 cm⁻¹ for higher plants. The lowest exciton state of the Chl trimers in S. elongatus and in higher plants are both lower in energy than the lowest state of the Chl dimer in Synechosystis PCC 6803. This indicates that the presence of B33 in S. elongatus and in higher plant does not disrupt the interaction between B31 and B32.
DISCUSSION

The analysis of *C. reinhardtii* mutants that were supposed to contain only PSI core, has shown that this is not the case. Even the CC2754 cells, that have no Chl *b*, still contain some antenna complexes that complicate the study of PSI core *in vivo*. To overcome this problem we thus purified PSI core from these cells. PSI core was isolated in monomeric and oligomeric forms. Both preparations showed identical absorption spectra and emission maxima at 715 nm at 77K. Part of the reddest emission observed in *C. reinhardtii* cells at 77K can thus safely be attributed to PSI core. Pigment organization of PSI core, as revealed by circular dichroism, is very similar to that of the *A. thaliana* complex in agreement with the highly conserved primary structures of the PSI core proteins of the two organisms. The results also indicate that the oligomerization of the PSI core does no influence the spectroscopic properties of the sample.

EET and trapping kinetics of both monomeric and oligomeric cores are well described by ~6 ps and ~22 ps lifetimes. Both particles have a similar average decay time of ~18 ps upon 400 nm excitation, faster than the decay time of higher plant PSI core (21.3 ps in *A. thaliana* (114)). This is in agreement with the presence of red forms that absorb at 701.2 nm and are thus blue shifted compared to the red pool in the PSI core of plants (76). This 701.2 nm pool is responsible for the 77K emission at 715 nm, having thus a Stokes shift of 13.5 nm (269 cm⁻¹) and optical reorganization energy (Sₐ) of 135 cm⁻¹. This Sₐ is close to that observed in *C. reinhardtii* “red” Lhcas (150 cm⁻¹ in Lhca9 (63)) and smaller than that of the “red” Lhcas of higher plants (240 cm⁻¹ in Lhca4, (80)), but larger than Sᵥ of the bulk Chls *a* (15-25 cm⁻¹ (86)) and of the “blue” Lhcas of higher plant (120 cm⁻¹ in Lhca2 (191)).

Given the presence of red forms in PSI core, we considered them separately from the RC in the kinetic model (Scheme 1). In the previous studies, PSI core kinetics was modelled either as trap-limited (120, 123) or diffusion-limited (90, 122, 221). Which model is correct is still an on-going discussion since several kinetic models can well describe the time-resolved data (69, 124). For instance, the “charge recombination model” of PSI core describes PSI RC as a shallow trap. In this model, the red emission of PSI core is ascribed to recombination of the first radical pair that repopulates the exciton state of the RC Chls (98, 104). This model discards the possible presence of red forms in eukaryotic PSI core. However, our results show that red forms are present in *C. reinhardtii* PSI core and that the kinetics is not purely trap-limited. The trapping occurs from the “bulk” Chls of the core with a rate of (12.6 ps)⁻¹. Considering that, in this compartment, an excitation equilibrates very fast (no diffusion limiting step) and that there are 94-95 Chls (Table 4), the intrinsic charge separation rate kᵢCS is ~(134 fs)⁻¹, comparable to (189 fs)⁻¹ estimated in PSI of higher plants (trapping rate estimated at (18.9 ps)⁻¹ from a “Core Bulk” compartment containing ~100 Chls *a* (114)).
In parallel to PSI core analysis, EET and trapping kinetics of PSI-LHCl was also modelled by performing target analysis of both PSI complexes simultaneously. The detailed analysis of PSI-LHCl when using the structural constraint of its core, gives access to the kinetics of the different PSI building blocks. The core and the Lhcas can both be modelled with a “bulk” compartment containing most of the Chls, and a “red” compartment containing mainly the reddest Chls (Scheme 1). The “bulk” compartments are almost isoenergetic and equilibrate very fast (~1.2 ps, Scheme 1 and Table 3), in agreement with previous results (184). Therefore, decays from Bulk Core and Bulk Lhcas are comparable (Figure 9).

![Figure 9: Concentration profiles of PSI-related compartments in the kinetic model shown in Scheme 1.](image)

In conclusion, both core and Lhcas of PSI-LHCl in C. reinhardtii contain red forms that influence the EET and trapping kinetics in different ways. The “red” compartment of the Lhcas is more populated over time than the “red” compartment of the core (Figure 9). This has multiple explanations: (i) the larger number of Chls in Red Lhcas (Table 4) that also contains some bulk Chls in very fast equilibrium with the red forms (Figure 8A), (ii) the larger initial population of Red Lhcas (Table 1) and (iii) the slower equilibrium of Red Lhcas with its related “bulk” (Scheme 1). Indeed the equilibrium between Bulk Lhcas and Red Lhcas is 10 times slower than the equilibrium between Bulk Core and Red Core ((1.4+7.6)^1 ≈ 10*(68.9+25.9)^1).

From the target analysis, we could also calculate the average decay time of a hypothetical PSI core without red forms (trapping time from Bulk Core compartment). The value obtained is 13 ps, which is very close to ~14 ps found in a cyanobacterial PSI without red forms (115).
The red forms of PSI core in different organisms

Despite the very similar structures of PSI from various cyanobacteria (24, 30) and higher plants (5, 28, 29), the red forms composition and their influence on EET and trapping kinetics vary greatly between organisms (76, 90, 114). These variations are thus probably the result of local differences in the organization of some Chl clusters within the cores. In *Synechococcus elongatus*, several Chls show relative short ring-to-ring distances, and transition dipole orientations that can lead to strong excitonic interactions (24). In particular, the Chl trimer B31-B32-B33 (nomenclature from (24)), on the luminal side of PSI core, was proposed to be responsible for the reddest forms in *S. elongatus* (24), with absorption at 719 nm (75). In *Synechocystis* PCC 6803, B33 is missing because the PsaB luminal loop is shorter (30). Red forms in *Synechocystis* PCC 6803 are then expected to only involve Chl dimers (including the remaining dimer B31-B32) (30) with the most red pools absorbing at 708 nm (23, 76). In higher plants, the PsaB loop extension on the luminal side of PSI core is also absent (28). Nevertheless, a new Chl bound to PsaG and Lhca1 was recently resolved (5, 29) resulting in the formation of a stacked Chl trimer at about the same place as in *S. elongatus* (Figure 10).

![Figure 10: Luminal Chl trimer B31-B32-B33 (magenta) in *Synechococcus elongatus* (PDB 1JB0 (24)) and its equivalent in higher plants (marine): B33 is replaced with the newly resolved Chl bound to PsaG and Lhca1 (light green and dark blue respectively, PDB 4XK8 (5)).](image)

Despite the “restored” Chl trimer, the reddest absorption of higher plants is blue shifted as compared with *S. elongatus* possibly because of the different geometry of the Chl trimer in the two organisms (Figure 10). To test this possibility, simple excitonic calculations were performed: we considered isonergetic Chls with similar transition dipole strength with their interaction described by the dipole-dipole approximation. The lowest state of the Chl
trimers in *S. elongatus* and in higher plants are both lower in energy (14266 cm\(^{-1}\) and 14325 cm\(^{-1}\) respectively, in our example that does not include displacement energy) than the lowest state of the Chl dimer in *Synechosystis* PCC 6803 (14420 cm\(^{-1}\)). However, the reddest absorption of higher plant PSI core has been shown to have the maximum at 705 nm (76), thus at higher energy than the maxima for both *Synechosystis* PCC 6803 and *S. elongatus*. A difference in the Chl environment between the organisms can in principle explain this difference. We can speculate that in higher plants the B31-B32-(B33) cluster might still be responsible for the 705 nm-absorption of the isolated PSI core as, in this complex, Chl B33 has most probably a different environment/geometry than in the PSI-LHCl structure due to the absence of Lhca1. This would also imply that B313-B32-B33 have a lower energy absorption in PSI-LHCl (Figure 1) than in the isolated core, explaining the difference in red form content between PSI-LHCl and the sum PSI core + LHCl observed in plants (86).

The “restored” Chl trimer in higher plant is suggested to be crucial for the association of Lhca1 to the core (29). In *C. reinhardtii* PSI-LHCl, this position is occupied by either Lhca2 or Lhca9 (nomenclature by (45), (48)). These two Lhcas were shown to be loosely bound to the core as they are easily lost upon detergent treatment (48). We speculate that in *C. reinhardtii*, the Chl trimer close to Lhca2/Lhca9 might be organized differently than in higher plant PSI core explaining the higher energy of the red forms in the PSI core of *C. reinhardtii*. 
### SUPPLEMENTARY INFORMATION

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<thead>
<tr>
<th>Species</th>
<th>Chls</th>
<th>B31</th>
<th>B32</th>
<th>B33</th>
</tr>
</thead>
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<tr>
<td>S. elongatus</td>
<td>B31</td>
<td>14705.9</td>
<td>-302.3</td>
<td>-35.0</td>
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<td>-22.4</td>
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</tr>
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Table S1: Interaction matrices between the Chls B31, B32 and B33 (nomenclature (24)) in different organisms. The site energy of Chls are represented inside the diagonal and their coupling strength outside the diagonal (in cm$^{-1}$). See Materials and Methods for details.
**CHAPTER 6**

Conservation of core complex subunits shaped structure and function of photosystem I in the secondary endosymbiont alga *Nannochloropsis gaditana*

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This chapter has been *submitted*
ABSTRACT
Photosystem I (PSI) is a pigment protein complex catalyzing the light driven electron transport from plastocyanin to ferredoxin in oxygenic photosynthetic organisms. Several PSI subunits are highly conserved in cyanobacteria, algae and plants, while others are differentially distributed in the various organisms. Here we characterized structural and functional properties of PSI purified from the heterokont alga Nannochloropsis gaditana, showing that it is organized as a supercomplex including a core complex and an outer antenna, as in plants and other eukaryotic algae. Differently from all known organisms, Nannochloropsis gaditana PSI supercomplex contains five peripheral antenna proteins, identified by proteome analysis as type-R Light Harvesting Complexes (LHCr4-8). Two antenna subunits are bound in a conserved position, as in PSI of plants, while three additional antennae are associated with the core on the other side. This peculiar antenna association correlates with the presence of PsaF/J and the absence of PsaH, G and K in N. gaditana genome and proteome. Excitation energy transfer in the supercomplex is highly efficient, leading to a very high trapping efficiency as observed in all other eukaryotic PSIs, showing that although the supramolecular organization of PSI changed during evolution, fundamental functional properties as trapping efficiency were maintained.

INTRODUCTION
In organisms performing oxygenic photosynthesis Photosystem I (PSI) is responsible for the light-driven electron transport from plastocyanin/cytochrome 6 to ferredoxin (27, 69, 222, 223). In eukaryotes, PSI is organized as a supercomplex composed of two moieties, the core and the peripheral antenna system. PSI core is composed of 12-14 subunits (46). PsaA and PsaB bind the reaction center P700 as well as most of the cofactors involved in electron transport and approximately 100 chlorophylls (Chls) active in light harvesting (5, 24). PsaA and PsaB are highly conserved in cyanobacteria, algae and plants (32). Other core subunits are instead differently distributed in various phylogenetic groups. For instance PsaG and PsaH are only found in plants and green algae (46, 224) where they serve as docking site for the association of the peripheral antenna (28, 36).

The peripheral antenna is composed of light harvesting complexes (LHC) whose sequences vary in different organisms: LHCa1-6 are present in plants (41), LHCa1-9 in the green alga Chlamydomonas reinhardtii (63), while LHCr proteins are believed to compose the peripheral antenna in red algae and diatoms (46, 225, 226). The number of LHCs associated with PSI is also variable: four subunits are associated with the PSI core in higher plants, in the moss Physcomitrella patens and in some red algae and diatoms (28, 225, 227,
Differently, nine antenna subunits are found associated with PSI in other red algae (226, 229) as in the green alga *C. reinhardtii* (48).

Finally, the oligomeric state of PSI also varies in different organisms. Cyanobacterial PSI has been often reported to be a trimer (24, 230), although PSI tetramers are being identified in a growing number of species (231-233). On the contrary PSI was found to be monomeric in all eukaryotes analyzed so far, including plants (28, 234), diatoms (227, 235), green and red algae (48, 229).

A peculiar feature of PSI is the presence of Chls absorbing at energy lower than the primary electron donor P700, called red forms (69). Although these far red-absorbing Chls account only for a small fraction of the total absorption, they have a strong influence in excitation energy transfer and trapping, slowing down the trapping time as they introduce uphill steps in the energy transfer process (90, 114, 116, 161). The presence of low energy absorbing Chls is ubiquitous in the PSI, but their energy appears to be highly species-dependent (69, 76). In plants the most red forms are associated with the outer antenna complexes and in particular with LHCa3 and LHCa4 (236, 237), although the core also contains low energy forms (76, 86).

In this work we investigated the structural and functional properties of the Photosystem I supercomplex (PSI-LHC) of the eustigmatophycea *Nannochloropsis gaditana*. This microalga belongs to the phylum of Heterokonta, which also includes diatoms and brown algae (238, 239), that originated from a secondary endosymbiotic event where an eukaryotic host cell engulfed a red alga (240). In the last few years, species belonging to the *Nannochloropsis* genus have gained increasing attention for their evolutionary position but also for their ability to accumulate a large amount of lipids (241-243). The *N. gaditana* photosynthetic apparatus presents distinct features with respect to other algae like the presence of only Chl a and an atypical carotenoid composition with violaxanthin and vaucheriaxanthin esters as the most abundant xanthophylls (244-246). A deeper characterization of this organism thus contributes to a better understanding of the variability of photosynthetic organisms in an evolutionary context.

**MATERIALS AND METHODS**

**Cell growth and thylakoid isolation** - *N. gaditana* from the Culture Collection of Algae and Protozoa (CCAP), strain 849/5, was grown in sterile F/2 medium using 32 g/l sea salts (Sigma-Aldrich), 40 mM TRIS-HCl (pH 8), and Guillard’s (F/2) marine water enrichment solution (Sigma-Aldrich). Cells were grown with 100 μmol of photons.m⁻².s⁻¹ of illumination using fluorescent light tubes and air enriched with 5% CO₂. The temperature was set at 22 ± 1°C. Thylakoid membranes were isolated as previously described (246).
Thylakoid solubilization and PSI isolation - Thylakoid membranes (corresponding to 500 μg of Chl) were solubilized with 0.6% α-DM or 1% β-DM as described in (246) and then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient containing 0.06% α-DM and 10 mM HEPES (pH 7.5) (280000 x g, 18 hours, 4°C). Fractions of the sucrose gradient were then harvested with a syringe. PSI samples isolated after α-DM or β-DM solubilization were named PSI-LHC$_\alpha$ and PSI-LHC$_\beta$ respectively.

Electron microscopy and Image analysis - Four μL of purified sample was absorbed onto glow discharged carbon coated grids and subsequently stained with 2% uranyl acetate for contrast. Imaging was performed on a Tecnai T20 equipped with a LaB6 tip operating at 200 kV. The “GRACE” system for semi-automated specimen selection and data acquisition (247) was used to record 2048×2048 pixel images at 133000 × magnifications using a Gatan 4000 SP 4K slow-scan CCD camera with a pixel size of 0.224 nm. 200000 particles were picked from 16000 raw images and single particles were analyzed with XMIPP software (including alignments, statistical analysis and classification, as in (248) and RELION software (249). The best of the class members were taken for the final class-sums.

Sequence analysis - Emiliania huxleyi, Phaeodactylum tricornutum, Thalassiosira pseudonana, Ectocarpus siliculosus, N. gaditana, C. reinhardtii, Physcomitrella patens, Arabidopsis thaliana, Oryza sativa, Cyanidioschyzon merolae, Galdieria sulphuraria genome databases were accessed online via the National Center for Biotechnology Information (NCBI) portal using TBLASTN and BLASTP. Additional data were collected from http://bioinformatics.psb.ugent.be/genomes/view/Ectocarpus-siliculosus for E siliculosus genome, Department of Energy Joint Genome Institute (JGI) for E. huxleyi sequences. N. gaditana sequences were retrieved from www.nannochloropsis.org using TBLASTN. The multiple sequence alignments (MAS) of protein were performed using clustalW in BioEdit and MUSCLE (MUltiple Sequence Comparison by Log- Expectation). Sequence similarities and secondary structure information were obtained by ESPript, 'Easy Sequencing in PostScript', using crystal structure of spinach major light-harvesting complex as a reference (1RWT pdb code, (58)).

MS analysis - The green bands visible on the sucrose gradient were harvested with a syringe and then loaded on SDS-PAGE (precast 12% polyacrylamide SDS gel (C.B.S. Scientific)). In-gel tryptic digestion was performed as described in (250), with minor modification with acetonitrile as the organic phase. The MS measurements were performed as described by (251) using an Ultimate 3000 nanoflow HPLC system (Dionex) coupled with an LTQ Orbitrap
XL mass spectrometer (Thermo Finnigan) device for autosampling, column switching and nano-HPLC. For the identification of peptides, OMSSA (version 2.1.4) (252). A new database was created downloading *N. gaditana* sequences from www.nannochloropsis.org/page/ftp to assign the peptides resulting from MS analysis to *N. gaditana* protein.

**Label-free quantification** - For peptide identification and determination of protein intensities mass spectrometry raw data were processed using MaxQuant 1.5.1.2 (253). The Uniprot reference proteome of *N. gaditana* (downloaded 12/12/2014) concatenated with randomized sequences of all entries was used for database search, with default settings for high-resolution MS1 (Orbitrap) and low-resolution MS2 (ion trap). Oxidation of methionine and acetylation of the protein N-terminus were allowed as variable modifications. False discovery rate (FDR) was set to 1 % on peptide and protein level. The feature ‘match between runs’ was activated. Due to the fundamentally different protein composition of each fraction, intensity normalization by MaxQuant was omitted. Instead, normalization factors were calculated based on the sums of all non-normalized protein intensities of each fraction.

**Pigment analysis** - Chlorophylls and carotenoids were extracted using 80% acetone, and pigment content was determined by HPLC (Beckman System Gold) as described in (254). The vaucheriavxanthin retention factor was estimated from the one of violaxanthin, with a 10% correction accounting for the different absorption spectra.

**Spectroscopic steady state measurements** - Absorption of isolated PSI-LHC particles was measured at 77K and room temperature (RT) with a Varian Cary 4000 UV-Vis spectrophotometer. For 77K measurements, samples were in 65% glycerol. Fluorescence spectra were measured at 77K and RT on a Fluorolog spectrofluorimeter (Jobin Yvon Horiba). Samples were diluted in order to avoid self-absorption in a buffer containing 10 mM Hepes (pH 7.5) and 0.06% α-DM. The circular dichroism (CD) spectra were measured using a Chirascan-Plus CD Spectrometer at RT.

**Time-resolved measurements** –

*Streak camera set-up.* Picosecond-time-resolved fluorescence measurements were performed with a streak camera setup as previously described (90, 148, 184). The samples were measured upon 400 nm excitation. The repetition rate was set at 250 kHz, the pulse energy was below 0.4 nJ. The samples were stirred in all cases. A power study confirmed the absence of annihilation in the measuring conditions.
Fluorescence was detected from 590 nm to 860 nm in three different time ranges (TR): from 0 to 155 ps (TR1 temporal response 4-5 ps), 0 to 400 ps (TR2 temporal response 6-7 ps) and from 0 to 1500 ps (TR4 temporal response 20 ps). The streak camera data were treated in HPD-TA 8.4.0 (Hamamatsu) as described in detail in (184). The instrument response function (IRF) was modeled with a simple Gaussian for all the TRs. PSI-LHC particles were diluted at an OD of 0.6 cm\(^{-1}\) for PSI-LHC\(_{\alpha}\) and 1.2 cm\(^{-1}\) for PSI-LHC\(_{\beta}\) at the Qy maximum with a buffer containing 10mM HEPES (pH 7.5) and 0.06% α-DM. The chosen OD enabled time-resolved measurements without self-absorption.

Data analysis of time-resolved measurements – The streak camera measurements were analyzed globally in Glotaran (166) with a sequential model. The average decay time \(\tau_{av,CS}\) (Equation 1) characterizes the time until CS occurs (for open RCs) with \(A_n\) the area under the DAS of the \(n\)-th component (i.e. its total amplitude) and \(A_n/\sum A_n\) the relative amplitude.

\[
\tau_{av,CS} = \frac{\sum_n (\tau_n A_n)}{\sum_n A_n} \quad \text{Equation 1}
\]

See (184) for more details.

RESULTS

EM reveals peculiar association between core and peripheral antennae in the PSI-LHC complexes from N. gaditana

PSI-LHC can be purified from \(N. \) gaditana thylakoids in supercomplexes containing the core complex with its associated antenna (246). The structure of PSI-LHC from \(N. \) gaditana purified after solubilization with either α- or β-DM (PSI-LHC\(_{\alpha}\) and PSI-LHC\(_{\beta}\)) was investigated using electron microscopy (EM). Projection maps of negatively stained PSI-LHC\(_{\alpha}\) and PSI-LHC\(_{\beta}\) were obtained by single particle analysis (248, 249, 255). In PSI-LHC\(_{\alpha}\) we found two types of particles: a monomeric complex (Figure 1A-D) and a dimeric complex (Figure 1E), in a ratio 8:2 (≈230000 and 58000 particles, respectively). The monomers have a size comparable to that of the PSI-LHCI isolated from \(A. \) thaliana (Figure 1F-G). β-DM gives harsher solubilization than α-DM (246) and PSI-LHC\(_{\beta}\) samples showed the presence of monomers with less than 1% of the particles that could be classified as dimers. An equivalent P700/Chl ratio was measured in PSI-LHC\(_{\alpha}\) and PSI-LHC\(_{\beta}\) supporting the hypothesis that monomers and dimers have similar composition (Figure S1).
Figure 1: Electron microscopy analysis of PSI-LHC complexes from *N. gaditana*. A-D) Projection maps of the *N. gaditana* monomer, sums of four best classes after processing 200000 particles, see text. The red arrowhead in Frame B shows extra density, absent in Frame A. E) Projection map of the *N. gaditana* dimer, after processing 50000 projections. F-G) Maps of *A. thaliana* PSI-LHCl-LHCII and PSI-LHCII, re-analyzed from (143). H) Aligned contours of *N. gaditana* (Frame A in green, upper monomer of Frame E in blue) and *A. thaliana* (Frame G in orange). The “reversed J” motif (in orange) and other densities were helpful to align the core parts and also to establish that the dimers of *N. gaditana* (Frame E) consist of up and down oriented monomers, as indicated.

In PSI-LHC\(_\alpha\) four main types of monomers were found upon classification. The largest ones have a triangular shape (Figure 1A) and some of them show an extra density on the upper left side (indicated with a red arrow in Figure 1B). In other particles, this density is less pronounced, which can partly be caused by a tilt of the particles on the carbon support film (Figures 1C and D). All projection maps show clear densities that in the *A. thaliana* map (Figure 1G) correspond to the extrinsic subunits of PSI core. These densities can then be used for the alignment for *N. gaditana* particles (green contour in Figure 1H) with the one of *A. thaliana* (orange contour in Figure 1H). This comparison shows that the *N. gaditana* particles have a different shape than those of *A. thaliana*, being substantially smaller at the bottom but larger at the top.

In the dimeric complex the upper monomer has exactly the same outline as the monomer of Figure 1D, indicating a small loss of density at the tip (blue contour in Figures 1E and H). The lower monomer of the dimer is, however, not related by 2-fold symmetry to the upper one, as the blue contours plus the orange-marked density clearly demonstrate. This is only compatible with a flipping plus a rotation, indicating that monomers are oriented upside-up and upside-down. Such an opposite orientation of the two monomers suggests that the dimers are artificial, which reminds the earlier observed artificial dimers in plants (234).
The best two maps of *N. gaditana* (Figure 2) were overlaid with the atomic model of the plant PSI-LHCl structure (5, 28). In plants like pea and *A. thaliana* the LHCa1-4 monomers constitute a belt at one side of the core while in *N. gaditana* there appears to be space for only two LHCs, in a position corresponding to the plant LHCa2/3 dimer, at the lower right tip (Figure 2). The model further shows space for at least three other LHCs at the other side of the core complex, especially in the more bulky map of Figure 2B. This modeling clearly shows that *N. gaditana* PSI-LHC has a unique peripheral antenna structure, different from plants and green algae. It is likely composed of five LHC subunits associated to two opposite sides of the core complex.

Figure 2: Model for subunit positions in *N. gaditana* PSI-LHC. A-B) Projection maps of the *N. gaditana* monomer respectively from Figures 1A and B overlaid with wire models of the components of the plant high-resolution PDB structure 4XK8 from (5). From the plant model subunits G, H, K and LHCa1-4 were removed and all other subunits have been overlaid as a fixed structure, including LHCa2-3 (in cyan/purple). There is no space left for the two additional LHCs at the lower left positions next to LHCa2. Instead, additional LHCs (green) have been positioned at the top, to indicate ample space at this site for three extra LHCs, especially in Frame B.

**PSI-LHC composition in *N. gaditana***

PSI core complex subunits were identified in *N. gaditana* genome using similarity searches with blastp tool at NCBI (using as query sequences from *A. thaliana, Cyanidioschizon merolae* and *Pheodactylum tricornutum*, Table 1). Sequences of PSI subunits from various additional species from *Viridiplantae* (*A. thaliana thaliana, Oryza sativa, Physcomitrella patens, C. reinhardtii*), red algae (*Galdieria sulphuraria*), heterokonts (diatoms and *Ectocarpus siliculosus*) and haptophyta (*Emiliania huxleyi*) were also searched to build a comprehensive view of the subunit distribution in different photosynthetic eukaryotes (Tables 1 and S1).
Table 1: PSI core complex subunits. The table shows the identification of PSI core subunits in model species from different taxonomic groups. Presence/absence in cyanobacteria was retrieved from literature (256), while for the other species sequences were identified (+) or not (ND) with blastp tool at NCBI (using as query sequences from A. thaliana or C. merolae and P. tricornutum for the subunits absent in A. thaliana). Sequence identification numbers are all reported in supplementary material (Table S1).

Some subunits (PsaA-E), known to be involved in charge separation and electron transport, are highly conserved in all photosynthetic organisms including cyanobacteria. Noteworthy, their conservation is not only limited to the presence/absence of the subunits but also extends to their amino-acid sequence (Table S2). Other PSI core subunits (PsaF, Psal, PsaJ and PsaL) are also conserved in all the species analyzed, suggesting they play relevant role in PSI structure and function.

Several other core complex subunits are instead differently distributed among the species analyzed. N. gaditana genome lacks several PSI subunits identified in other organisms, namely PsaG, PsaH, PsaK, Psam, PsaN and PsaO. As shown in Table 1, PsaG, PsaH and PsaN are present exclusively in plants and green algae (257). PsaO is instead only present in primary endosymbiotic groups (plants and green/red algae). PsaM, typical of cyanobacteria is also found in some algae species while missing in others including N. gaditana (256). PsaK showed instead a peculiar distribution since it is conserved from...
cyanobacteria to red algae and plants but is absent from all heterokonts and haptophytes analyzed, including diatoms and *N. gaditana*. These organisms are all secondary endosymbionts originated from a red alga, thus suggesting that PsaK was present in these organisms ancestors but was later lost.

A similarity search for antenna sequences allowed identifying three subgroups of LHCs in *N. gaditana*, which were classified as LHCf, LHCr and LHCx (Table 2), as previously described (246, 258). Within the three subgroups, proteins were named according to their RNA expression levels, starting from the most actively transcribed (Alboresi, Perin et al, submitted).

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<td>W7TI84</td>
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Table 2: LHC proteins of *N. gaditana*. LHC are grouped according to different subgroups as in diatoms (258). Sequences of the same subgroup were numbered based on their gene expression levels, starting from the most abundantly transcribed (Alboresi, Perin et al, submitted). Gene ID is taken from [http://www.nannochloropsis.org](http://www.nannochloropsis.org) (259). Proteins (UniProt ID) were identified by mass spectrometry using the UniProt reference proteome of *N. gaditana* (UP000019335) for database search.

* indicates enrichment in PSI-LHC. The correspondence with proteins from *N. oceanica* is shown in Supplementary Material (Table S3).
N. gaditana thylakoids were solubilized with α-DM and pigment binding protein complexes were first separated in sucrose gradients by centrifugation and then analyzed by tandem mass spectrometry (MS/MS) (Figure 3). All PSI core and LHC subunits reported in Tables 1 and 2 with the exception of PsaC were detected by mass spectrometry confirming their presence in N. gaditana thylakoids. Although it does not provide an absolute quantification, MS analysis allowed determining the distribution of each polypeptide between the different bands of the sucrose gradient (i.e. LHC monomers, LHC trimers, PSII core and PSI-LHC). All putative PSI core complex proteins listed in Table 1 showed a strong enrichment (75-100% of the total amount of each peptide) in the PSI-LHC fraction, confirming that they are indeed components of this supercomplex (Figure 3B). Five antenna subunits, LHCr4-8, showed a distribution along the sucrose gradient bands similar to that of PSI core subunits with a strong enrichment in the PSI-LHC fraction, thus suggesting they are also specific components of this supercomplex (Figure 3C). One additional LHC-like subunit (Naga_100030g5), recently attributed to the group of red lineage chlorophyll binding-like proteins (RedCAP1) (260), was also enriched in the PSI-LHC fraction. All other LHCs, instead, presented a different distribution with higher abundance in other bands (Figure S2), suggesting a different localization in the thylakoids membranes. It is interesting to point out that three other LHCr proteins (LHCr1-3) were detected in this experiment but not found specifically enriched in the PSI-LHC fraction (Figure S2). In particular, LHCr1 was mainly present as a monomer, LHCr2 was accumulated in the band of LHC mono/oligomers (≈35%), PSII core (≈40%) and PSI-LHC (≈35%) while LHCr3 was mainly accumulated as a monomer/oligomer (≈80%). MS analysis thus shows that these subunits are not specifically associated to PSI or at least not as strong as LHCr4-8.
Figure 3: Protein distribution in the different fractions harvested after sucrose gradient sedimentation of solubilized thylakoids. *N. gaditana* thylakoid membranes were first solubilized with α-DM and then separated by sucrose density gradient ultracentrifugation and subsequently characterized by MS/MS analysis. A) Example of sucrose density gradient after centrifugation of thylakoid membranes of *N. gaditana*. B) Distribution of subunits of PSI core complex in the gradient fractions. C) LHC complexes showing a relative enrichment in PSI fractions, others are shown in supplementary material (Figure S2). All *N. gaditana* PSI core subunits and LHCs are listed in Tables 1 and 2. D) Structure-based sequence alignment of the crystallized spinach LHCII ((58) pdb code 1RWT) with PSI associated LHCs of *N. gaditana* and *A. thaliana*. The secondary structure of the spinach LHCII trimer is shown above the alignment together with the names of the helices. Conserved amino acids highlighted by a red background are identical and those in red letters are similar. Alpha helices are represented as helices and the β-turns are marked with TT. Conserved residues involved in the binding of Chl α molecules are labeled under the alignment.
Protein sequences of LHCr4-8 from *N. gaditana* were compared with the ones from LHCa1-4 of *A. thaliana* (Figure 3D). The α-helices A and B are well conserved including the key residues involved in the binding of Chl *a* molecules (Chl 602, 603, 610, 612, and 613). The α-helix C is more variable and a clear ligand for Chl 606 is missing while a glutamic acid likely coordinating Chl 609 can be identified. With the exception of LHCr7, α-helix D is not identifiable in *N. gaditana* antennae and thus the ligand for Chl a614 is not conserved as previously observed for LHCb6 of *A. thaliana* (59). In LHCa complexes Chl 603 is coordinated either to an asparagine or a histidine in the helix B. The presence of an asparagine, as in the case of LHCa3 and LHCa4, was shown to be responsible of a red-shift in the fluorescence spectrum (108). LHCr5-8 have a histidine in that position while LHCr4 has an asparagine suggesting the possible existence of red-shifted forms in the peripheral antenna of *N. gaditana* PSI-LHC.

**Functional properties of PSI-LHC from *N. gaditana***

As expected Chl *a* is the only Chl species present in PSI-LHC (Table 3). The Chl/Car ratio of PSI-LHC$_a$ is lower than that of PSI-LHC$_b$ suggesting that some of the xanthophylls (mainly violaxanthin) are loosely bound to the complex and lost with the stronger solubilization (Table 3). The Chl/Car ratio of PSI-LHC$_b$ is lower than that of *A. thaliana* (4.8±0.1, (107)) and *C. reinhardtii* PSI-LHCs (5.0±0.2, (48)). When normalized to the same number of Chls, however, the amount of β-carotene (12.4± 2.1 mol/100 Chls) is comparable to that of *A. thaliana* (13.1±0.3 mol/100 Chls), while the value of xanthophylls, especially violaxanthin is higher. Since the latter are preferentially bound to the antenna proteins, this suggests a relatively higher carotenoids content in this moiety, in agreement with the low Chl/Car ratio of the LHs of *N. gaditana* (246) compared to higher plants (107) and *C. reinhardtii* (48).

<table>
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<th>Vaucheria-xanthin</th>
<th>Antera-xanthin</th>
<th>Zeaxanthin</th>
<th>β-carotene</th>
<th>Chl/Car</th>
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<td>1.2 ± 0.8</td>
<td>12.4 ± 2.1</td>
<td>4.3 ± 0.7</td>
</tr>
</tbody>
</table>

Table 3: PSI-LHC pigments content. Pigments content of PSI-LHC fractions purified after different thylakoids solubilization is reported, expressed in mol/100 Chls. Values are reported as mean ± Standard Deviation (n > 3).

The absorption spectra of the PSI particles at 77K and room temperature (RT) as well as their second derivatives are presented in Figure 4, Figure S3 and Table S4.
Figure 4: Spectroscopic characterization of *N. gaditana* PSI-LHC. A) Absorption spectra of PSI-LHC<sub>α</sub> (black) and PSI-LHC<sub>β</sub> (red) at 77K and their difference (blue). The absorption spectra are normalized to the red absorption from 705 nm and above. B) Second derivative of the 77K absorption spectra shown in (A). C) Enlarged view in the Q<sub>y</sub> region of the 2nd derivative shown in (B). See Figures S3A and B for absorption and second derivatives at room temperature (RT). D) Circular dichroism compared to *A. thaliana* PSI-LHCl (green, normalized to the maximum absorption in the Q<sub>y</sub>. E) RT Fluorescence emission upon 500 nm excitation, normalized to the maximum emission. See Figures S3C and D for RT emission upon 400 nm. F) 77K fluorescence emission upon 400 nm excitation, normalized to the maximum in the red region.

The second derivative of the absorption spectra in the Chl region shows the presence of absorption forms around 669 and 680 nm at RT (Figure S3 and Table S4) and at 669, 679.5, 685 and 697.5 nm at 77K (Figures 4B and C and Table S4) for both PSI particles. In the carotenoid region, minima of the second derivative are visible at 486.5 nm and 503 nm at 77K (Figure 4B and Table S4). The wavelength of the second minimum and the fact that it is similar in the two preparations suggest that it is the signature of β-carotene in the PSI core. On the contrary, the large difference in the 486 nm signal between PSI-LHC<sub>α</sub> and PSI-LHC<sub>β</sub> suggests that it originates from xanthophylls (mainly violaxanthin), that are strongly reduced in PSI-LHC<sub>β</sub>. The circular dichroism spectra of the two preparations are shown in Figure 4D.
together with the spectrum of *A. thaliana*. Although small differences can be observed, the main components of the spectra are present in all complexes indicating that the overall pigment organization is conserved, confirming that the two preparations contain the same complex.

The fluorescence emission spectra at RT have maxima at 681.5 and 683 nm for PSI-LHC$_\alpha$ and PSI-LHC$_\beta$ respectively (Figure 4E). The emission spectra at 77K (Figure 4F) of both preparations show an intense band with maximum at 722 nm, typical of the red forms. A second emission band with maximum at 675-680 nm is visible at 77K, indicating the presence of Chls that do not transfer energy to PSI-LHC (see the shift in emission upon 400 nm and 500 nm excitation in Figures S3C and D at RT).

To study excitation energy transfer and trapping, the fluorescence decay kinetics of PSI-LHC$_\alpha$ and PSI-LHC$_\beta$ were measured with a streak camera set-up. The fluorescence was imaged along wavelengths (from 640 and 800 nm) and time (for three different time ranges up to 1500 ps). An example of streak image is shown in Figure 5A. After sequential analysis, the global decay of the two samples is described as a sum of decay components (see Materials and Methods) and the Decay Associated Spectra (DAS) are shown in Figure 5B.

A minimum of four components was needed for a good description of the kinetics of both samples. The first component (with a lifetime of 10.5 ps in PSI-LHC$_\alpha$ and 13.0 ps in PSI-LHC$_\beta$) is mainly a decay component although it still contains some energy transfer features as it can be inferred by the partial absence of the expected positive vibrational band. The second component (with a lifetime of 45.5 ps in PSI-LHC$_\alpha$ and 45.1 ps in PSI-LHC$_\beta$) is a pure decay component and represents the time when most of the trapping occurs. The two
slowest components show blue shifted DAS and long lifetimes (1.7-1.6 ns and 6 ns), and correspond to disconnected species. Indeed ~1.7 ns is close to the lifetime of LHCas of higher plants (112, 197). The average decay times are calculated by using Equation 1 (see Materials and Methods, Table 4) considering only the components attributed to the PSI-LHC kinetics (thus 1 and 2) and are 31 ps for PSI-LHCa and 32 ps for PSI-LHCb (±3 ps). The overall trapping time is thus much shorter than that of higher plants (48 ps, (114)) or C. reinhardtii PSI (50 ps, (184)).

<table>
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<td>Average decay time (\tau_{avCS}) (ps)</td>
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Table 4: PSI-LHC fluorescence lifetimes. Lifetimes were obtained from the sequential analysis of the fluorescence decays of PSI-LHCa and PSI-LHCb of N. gaditana measured upon 400 nm excitation with their relative amplitude (see Materials and Methods) and average decay time. The longest lifetime of both samples is attributed to free Chls a visible in the steady state emission spectra (Figures S3C and D) and fixed (f) to 6 ns which corresponds to the free Chls a lifetime in acetone (261).

**DISCUSSION**

In this work the combination of structural, proteomic and functional analysis provides a comprehensive picture of the structure and composition of PSI-LHC from *N. gaditana*. EM analysis of PSI purified from *N. gaditana* shows that this is a supercomplex composed by a core complex and a peripheral antenna system, as in all eukaryotes analyzed so far (Figure 1). At variance with plants and other algae (5, 48, 226), however, five LHCs, identified by MS analysis as LHCr4-8, are found associated with *N. gaditana* PSI-LHC (Table 2, Figure 3C). MS analysis detected three more LHCr-type LHCs, eight LHCf, three LHCx, thus covering the entire LHC superfamily identified in the genome of *N. gaditana* (Table 2). These additional LHCs, however, were not enriched in PSI-LHC suggesting that they are not associated to this supercomplex, although it is not possible to exclude that some additional LHCs are loosely associated to PSI *in vivo* but lost during purification.

The superimposition of *N. gaditana* PSI supercomplex EM map with the high resolution structures of plant PSI evidenced a peculiar arrangement of antenna complexes
with two LHCs bound in a highly conserved position, the same occupied by LHCa2/3 in plants (Figure 2). Three additional LHCs are instead found at the other side of the core complex, where PsaL is located.

As schematized in Figure 6, this peculiar structural organization correlates with differences in the composition of the PSI core complex derived from genome and proteome analysis (Tables 1 and 2, Figure 3).

Figure 6: Schematic comparison of PSI-LHC structural organization in N. gaditana with plants and other algae. Dark-green are subunits conserved in all eukaryotes (see Tables 1 and 2), red subunits are present in plants, light-green only in N. gaditana.

In plants the association of LHCa1/4 with the core complex is partly mediated by PsaG (5, 28). PsaG is absent in N. gaditana and indeed no LHC was observed in the position corresponding to plant LHCa1/4. PsaF and J have been suggested to mediate interactions with LHCa2/3 in plants (5). Consistently with the conservation of PsaF and PsaJ in N. gaditana, two LHCs are found in the same position also in this species. Considering that the core complex subunits are well conserved in different organisms (Table 1) this observation also suggests that two LHCs are likely bound in this position in PSI from all photosynthetic eukaryotes, including diatoms.

In plants, psak knock-down plants showed destabilized LHCa2/3 association (262) suggesting that PsaK contributes to their binding to PSI. The recent structure of plant PSI supercomplex, however, showed that PsaK does not directly interact with the peripheral antennae (5, 29). PsaK is not found in N. gaditana or in any other heterokont analyzed so far (Table 1) (263, 264) likely because of a loss that occurred during or after the secondary endosymbiosis. In these organisms the absence of PsaK does not prevent the association of two LHCs on this side of the core, which confirms that PsaK is not strictly necessary for the association of the peripheral antenna.
Additional PSI core subunits identified in plants are not conserved in heterokonts. PsaN in plants is found associated with PsaF in the docking site for plastocyanin (265), the soluble PSI electron donor. In *N. gaditana* and other heterokonts only PsaF is present, suggesting PsaN is dispensable for efficient electron transfer to PSI, consistent with its absence in cyanobacteria. This is likely correlated with a difference in electron transport chain, since *N. gaditana* genome lacks a plastocyanin encoding gene (259) and the PSI luminal electron donor is likely cytochrome *c*6 as previously suggested for the red alga *Galdieria sulphuraria* (224) and diatoms (266).

It is also worth underlining the absence of PsaH, a subunit essential for the association of LHCII during state transitions in plants (36). In *N. gaditana* other LHCs associate to the core complex in the region generally occupied by PsaH (Figure 6). This picture suggests that state transitions, if present in *N. gaditana*, most likely involve different structural interactions between antenna and PSI complexes than the ones described in plants and green algae (57, 143).

The functional data show that, despite this different organization, energy transfer and trapping in the PSI complex of *N. gaditana* is very fast. Indeed, the average decay time of *N. gaditana* PSI-LHC monomer is even shorter (32±3 ps) than that of both *A. thaliana* and *C. reinhardtii* PSI-LHCI (~50 ps, (114, 184)). This difference can be due to a smaller number of pigments associated with the PSI of *N. gaditana* and/or to a difference in the red forms. It is well documented that the number and the energy of these forms influence the excitation energy migration towards the reaction center in PSI (i.e. more red forms, slower transfer) (90, 114, 184). Although the number of Chls associated with the LHCs in *N. gaditana* is not known and we can thus not exclude that the antenna size of PSI in *N. gaditana* is smaller, it is very likely that part of the observed difference in trapping time is due to the diversity in red forms. Indeed, the red forms of PSI-LHC of *N. gaditana* are at a higher energy than those of *A. thaliana* as indicated by their emission maximum (722 nm for *N. gaditana* vs. 735 nm for *A. thaliana*) and absorption maximum (~697 nm in *N. gaditana* vs. 705-710 nm in *A. thaliana* (80, 108) and are then expected to have a smaller influence on the trapping time than the red forms of plants. Independently from the exact origin of this difference, the very fast trapping time observed for PSI-LHC of *N. gaditana* also indicates that all the LHC subunits are functionally well connected with the core, allowing for fast excitation energy transfer and high quantum efficiency of energy conversion. This high efficiency is common to all PSI complexes analyzed so far (114, 184, 189) and thus appears to be independent of the organization of the antenna around the core since the position of the additional LHCs in *N. gaditana* differs from that of both LHCI and LHCII in plants and *C. reinhardtii* (5, 48, 57, 143). This suggests that the design of the PSI core allows the functional association of
additional subunits to different part of the complex such that even a PSI core completely surrounded by antennae can maintain a very high quantum efficiency.

**Acknowledgements:** We thank dr. Gert Oostergetel for critical discussion and Stefania Basso for the preparation of thylakoids from *N. gaditana*. KNSY and EJB acknowledge a grant from NWO-ALW. TM acknowledges support from ERC starting grant BIOLEAP nr. 309485. RC acknowledges support from the ERC consolidator grant ASAP nr. 281341. M.H. acknowledges support from the German Science foundation (DFG).

**SUPPLEMENTARY MATERIAL**

*Figure S1: Quantification of P700 content in PSI-LHC isolated from Nannochloropsis gaditana.*

The ΔOD_{705} signal measured with a JTS10 spectrophotometer (Biologic, France) was used as a quantification of the total P700 amount present in the sample after normalization to the precise Chl content of each sample (=10 µg of Chl). To obtain the maximal P700 oxidation, P700 of isolated PSI-LHC was fully reduced by incubating the samples in the dark for 5 minutes with 375 µM Methylviologen and 2 mM Ascorbate. After incubation, the sample was illuminated with an actinic red light (630 nm). A) Signal from PSI-LHCα (black squares) and PSI-LHCβ (red circles) depending on the actinic light intensity (n = 3). B) Average over the different light intensities (150-2050 µmol photons m^{-2} s^{-1}). The average values are not statistically different (n = 12).
### Table S1. NCBI Sequences AC numbers of identified PSI core subunits.

* indicates that more than one isoform has been identified in the genome (228, 256).

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<th>C. reinhardtii</th>
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**Table S2: Identity matrix for PsaA protein obtained with MUSCLE tool (EMBL-EBI).**

Conservation versus *A. thaliana* and *N. gaditana* are highlighted in green and yellow, respectively.

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Table S3: LHC proteins correspondence between N. gaditana and N. oceanica.

The UniProt ID allowed the identification of the corresponding N. oceanica Gene ID. Proposed names of N. oceanica CCMP1779 are also reported (260).

* indicates proposed attribution to PSI.

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**Figure S2:** Protein distribution in the different fractions collected from a sucrose gradient loaded with thylakoids of *N. gaditana* solubilized with α-DM.

Only LHC subunits not specifically enriched in PSI fraction are reported here, the corresponding sucrose density gradient is reported in Figure 3A.

**Figure S3:** Spectroscopic characterization of *N. gaditana* PSI-LHC at room temperature.

Absorption spectra (A) of PSI-LHCα (black) and PSI-LHCβ (red), normalized to the maximum in the $Q_y$ region, and their second derivative (B). Fluorescence emission spectra upon 400 nm excitation (narrow line) or 500 nm excitation (thick line) of PSI-LHCα (C) and PSI-LHCβ (D). Spectra are normalized to the maximum emission.
Table S4: Maximum absorption of PSI-LHC$_\alpha$ and PSI-LHC$_\beta$ in the $Q_y$ and absorption forms visible in the second derivative of the absorption spectra, at 77K and RT.

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<th>2nd Derivative in the Car region</th>
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<td>669, 679.5, 685, 697.5</td>
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Bibliography

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Abbreviations

β-DM n-Dodecyl β-D-maltoside
α-DM n-Dodecyl α-D-maltoside
ATP Adenosine Triphosphate
Car Carotenoid
CD Circular Dichroism
Chl Chlorophyll
CS Charge Separation
CT Charge Transfer
DAS Decay Associated Spectrum
EAS Evolution Associated Spectrum
EET Excitation Energy Transfer
EM Electron Microscopy
ETC Electron Transport Chain
Fd Ferredoxin
FNR Ferredoxin-NADP⁺ Reductase
FWHM Full Width at Half Maximum
IC Internal Conversion
ISC Inter-system Crossing
LHC Light-Harvesting Complex
LHCl, Lhca LHC of PSI
LHCl II, Lhcb LHC of PSII
NADP Nicotinamide Adenine Dinucleotide Phosphate
PC Plastocyanin
PMS Phenazine Metasulfate
PQH₂ Plastoquinone
PS Photosystem
Psa PSI core subunit
RC Reaction Center
ROS Reactive Oxygen Species
RT Room Temperature
SAS Species Associated Spectrum
SI Supplementary Information
TR Time Range
Summary

Oxygenic photosynthesis is not only fundamental because it enables some living organisms to meet their energy needs, but also because it plays a major role in oxygenizing the atmosphere which supports much of life on earth. The photosynthetic process uses photons from sunlight to produce intermediate chemicals involved in oxidation-reduction (redox) reactions. In higher plants and algae, light is harvested by two photosystems (PS) PSI and PSII, located in the thylakoid membrane of the chloroplast. These multi-protein complexes, together with the cytochrome b6f complex, form an electron transport chain that also creates a proton motive force across the photosynthetic membrane which drives the ATP synthase. In the end, water and CO$_2$ are consumed, and oxygen and sugars are produced.

The light harvesting function of PSs is assured by two types of antenna: the core antenna, which only coordinates chlorophylls (Chls) $a$ and carotenoids (Cars), and the peripheral antenna, which also coordinates Chls $b$. The core antenna contains the reaction center (RC) where charge separation (CS) occurs. The peripheral antennae are also called Light-harvesting Complexes (LHC). Structure and pigment organization of the core antenna is highly conserved among species, while the peripheral antenna varies in size and composition. This thesis describes the capacity of PSI to harvest light energy and promote charge separation in two algae *Chlamydomonas reinhardtii* and *Nannochloropsis gaditana*.

PSI in *C. reinhardtii* contains nine LHCIs in its peripheral antenna. This is five additional LHCIs compared to higher plants. They form an outer half ring on the same side of the PSI core as the four LHCIs also present in higher plants. A characteristic of PSI is the presence of low-energy chlorophylls called red forms. Red forms are Chls $a$ that absorb (and emit) at lower energy than other Chls, and have a broad absorption bandwidth and a large stoke shift. Concurrent with its comparatively larger size, PSI-LHCI in *C. reinhardtii* has red forms with higher energy (less red-shifted) compared to PSI of higher plants. The lower in energy the red forms are, the more they act as local traps and slow the excitation energy transfer (EET). The larger the antenna size, the more photons are harvested, but the migration time of the excitation energy in the antennae is longer. From time-resolved fluorescence decay measured on isolated PSI particles, we related PSI EET and trapping kinetics with the antenna size and red forms content. In the second chapter we detail how the significant antenna enlargement in *C. reinhardtii* PSI-LHCI compared to plants is compensated by less red-shifted red forms, resulting in similar average decay time of ~50 ps in both organisms. We show that most of the Chls are in very fast equilibrium (~150 Chls $a$ over 183 Chls $a$) including Chls in the LHCIs most distant from the core antenna. *C.
**reinhardtii** PSI-LHCI efficiency is as high as 97% despite slower EET migration due to the larger number of Chls in its antennae (estimated at 225 Chls \( a \) and \( b \) vs 155 Chls higher plants). Another PSI particle was isolated from *C. reinhardtii* without Lhca2 and Lhca9, two LHCIs that were previously characterized as containing red forms. By comparing both five- and nine-LHCI PSI particles of *C. reinhardtii*, we show that the presence of Lhca2 and Lhca9 slows down the overall kinetics mainly because of their red forms (details in **Annex of Chapter 3**). However, it is shown that the red-most forms were in, or functionally close to, other LHCIs.

In response to changes in light intensity and quality, cells can modify peripheral antenna’s size and composition in a process known as state transition. For example, in response to changes in light quality, some LHCII can detach from PSII and associate with PSI (state 2) in order to balance the energy distribution. From cells of *C. reinhardtii* induced in state 2, PSI-LHCI was isolated with seven LHCIIIs (one monomer and two trimers) in addition to the nine LHCls. The LHCls remain associated to PSI on the Psaf/J side while the LHCIIIs attach opposite on the Psah side. The **third chapter** determines the energy transfer efficiency of LHCIIIs to PSI core and their influence on the trapping yield of the photosystem. Energy transfer between LHCIIIs and PSI core (~60 ps lifetime) is slower than between LHCls and PSI core (~7ps) mainly because of a looser connectivity (details in **Annex of Chapter 3**). From a reconstructed 3D model of PSI-LHCI-LHCI structure scaled from EM images, we observed that the distance of the closest Chls (nearest edge-to-edge distance) between LHCIIs and PSI Chls is 18 Å. Despite the increase in Chls number by ~43% (322 Chls) and the relatively slow EET step between LHCIIIs and PSI core, the overall kinetics of PSI-LHCl-LHCI has an average decay time of 78 ps (or less depending on the excitation wavelength) which is fast enough to maintain a very high trapping efficiency (above 96%).

In parallel with characterizing the EET and trapping kinetics of PSI-LHCI in *C. reinhardtii*, we studied the LHCls and the core antenna separately. LHCls in higher plants show highly conserved proteins structure and pigment organization but there are a few notable biochemical and spectroscopic properties that vary between the monomers. For example, some LHCls are enriched in red forms of Chl \( a \), increasing their absorption in the far red. *C. reinhardtii* LHCls also contain varying red form content. The **fourth chapter** characterizes the decay kinetics of excitation energy in the nine LHCls of *C. reinhardtii*. We observe a decay time of 1.9 ns for all the monomers. LHCls have shorter lifetime than LHCIIIs indicating that they are in a more quenched state. Since no correlation was observed between LHCl lifetimes and their red form contents, we conclude that red forms do not act as quenchers. This is in agreement with previous observation made in plant LHCls.
After the peripheral antenna, the second moiety analyzed in PSI-LHCI was the core antenna. In the fifth chapter, we have purified *C. reinhardtii* PSI core to homogeneity to characterize its spectral and excitation energy trapping properties. In PSI core, red forms absorb at 701.2 nm and can be related to an average decay time of ~18 ps. This is slower than cyanobacterial PSI devoid of red forms (~14 ps) but faster than PSI core of higher plants (21.3 ps) or other cyanobacterial PSIs (up to 40 ps) whose red forms are lower in energy compared to *C. reinhardtii*. By analyzing PSI core kinetics in parallel with PSI-LHCI kinetics, we show that red forms in the core are functionally distinct from the bulk Chls, in contrast to red forms of the peripheral antenna which are always found in fast equilibrium with bulk Chls. Despite highly conserved structure between species, red form content in PSI core varies tremendously and small conformational changes of PSI core subunits could considerably affect the red form properties. The best candidate for the reddest red form in cyanobacteria is a Chl trimer located at the periphery of the core, on the luminal side of PsaG. In higher plants and *C. reinhardtii*, the presence of LHCIs at this position was proposed to change the environment and thus the Chl trimer organization.

The sixth chapter characterizes PSI-LHC in the heterokont alga *Nannochloropsis gaditana*. Differently from higher plants and *C. reinhardtii*, all LHCs were not located on the same side of the core. Two LHCs are located at the same position as Lhca2 and Lhca3 of higher plants while three others are located on the Psal/L side of the core. PSI-LHC of *N. gaditana* has an average decay time of ~30 ps which is much faster than in higher plants. Despite the antenna enlargement by one additional LHC in *N. gaditana*, the quicker trapping kinetics can be explained by higher energy (bluer) red forms than in plants, acting less as local traps. From an evolutionary point of view, *N. gaditana* gives another example of supramolecular organization of PSI-LHC that results in a very high trapping yield.

In this thesis, we have explored PSI in *C. reinhardtii* and *N. gaditana* and compared its composition and energy kinetics to higher plants. Despite evolutionary conserved components, remarkable differences in antennae size and composition, as well as tremendous variety in the location, number and spectral properties of red forms of Chl a, were observed. Nevertheless, PSI maintains an almost perfect trapping efficiency, even in a context of loose connectivity.
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