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Dynamic regulation of photosynthesis in *Chlamydomonas reinhardtii*

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SUMMARY

Plants and algae have acquired the ability to acclimatize to ever-changing environments to survive. During photosynthesis, light energy is converted by several membrane protein supercomplexes into electrochemical energy, which is eventually used to assimilate CO_2. The efficiency of photosynthesis is modulated by many environmental factors, including temperature, drought, CO_2 concentration, and the quality and quantity of light. Recently, our understanding of such regulators of photosynthesis and the underlying molecular mechanisms has increased considerably. The photosynthetic supercomplexes undergo supramolecular reorganizations within a short time after receiving environmental cues. These reorganizations include state transitions that balance the excitation of the two photosystems: qE quenching, which thermally dissipates excess energy at the level of the light-harvesting antenna, and cyclic electron flow, which supplies the increased ATP demanded by CO_2 assimilation and the pH gradient to activate qE quenching. This review focuses on the recent findings regarding the environmental regulation of photosynthesis in model organisms, paying particular attention to the unicellular green alga *Chlamydomonas reinhardtii*, which offer a glimpse into the dynamic behavior of photosynthetic machinery in nature.

Keywords: acclimation, light-harvesting complex, non-photochemical quenching, state transitions, cyclic electron flow, green algae, electron transfer, *Chlamydomonas reinhardtii*.

INTRODUCTION

Photosynthesis is a process that converts solar energy into electrochemical energy. It is initiated by the absorption of light, and results in reduction of $NADP^+$ in the stroma and concomitant generation of proton motive force across the thylakoid membranes. The NADPH generated with the electron flow and the ATP produced with the proton motive force are required for assimilating carbon dioxide in the Calvin–Benson cycle. Photosystem I (PSI) and photosystem II (PSII) represent charge separation devices that drive electron flow using light energy. These two photosystems originated from a common prototype, but the contemporary PSI and PSII complexes are specialized and have major differences in the organization of their light-harvesting systems, pigment compositions, electron acceptors and donors, and several other features. We begin by describing the current knowledge of the components and the structures of the two photosystems in *Chlamydomonas reinhardtii* under normal conditions, and then discuss their regulation and how they are reorganized in response to environmental cues.

PSII and its light-harvesting complex proteins (LHCIIs) constitute a large chlorophyll (Chl)–protein supercomplex that comprises more than 30 subunits. Light energy captured by LHCIIs is transferred to the central dimeric core complex, where it is trapped and used to drive electron flow from water to plastoquinone (PQ). In green plants, LHCIIs are formed of two layers, the major trimeric LHCII proteins and the minor monomeric LHCII proteins (Dekker and Boekema, 2005). In the green alga *C. reinhardtii*, there are four major LHCII proteins (types I–IV) with five, one,

two and one isoform, respectively (LHCBM3, 4, 6, 8 and 9; LHCBM5; LHCBM2 and 7; LHCBM1) (Minagawa and Takahashi, 2004), whereas in the vascular plant *Arabidopsis thaliana*, there are three major trimeric LHCII proteins (types I–III) with five, four and one isoform, respectively (LHCB1.1–1.5; LHCB2.1–2.4; LHCB3.1) (Jansson, 1999). The two minor LHCII proteins CP29 and CP26 are encoded by *LHCB4* and *LHCB5*, respectively, in *C. reinhardtii* (Teramoto *et al.*, 2001; Minagawa, 2009), whereas *A. thaliana* expresses another minor LHCII protein CP24, encoded by *LHCB6*.

Single-particle image analysis of electron micrographs revealed that these LHCII proteins are bound to both sides of the central dimeric core complex, with the core and major LHCII trimers bordered by minor LHCII monomers (Dekker and Boekema, 2005) (Figure 1). When thylakoid membranes from spinach (*Spinacia oleracea*) are solubilized using *n*-dodecyl-β-D-maltoside (β-DM) (Boekema *et al.*, 1995, 1998; Hankamer *et al.*, 1997; Nield *et al.*, 2000c), one LHCII trimer is bound strongly to each side of the core (C_2S_2 PSII–LHCII supercomplex). However, when the membranes are solubilized using *n*-dodecyl-α-D-maltoside (α-DM), the PSII–LHCII supercomplexes are organized as $C_2S_2M_{1\text{-}2}L_{0\text{-}1}$ or $C_2S_2M_0L_{1\text{-}2}$, in which one or two moderately bound LHCII trimers (M-trimers) and/or one loosely bound LHCII trimer (L-trimer), or one or two loosely bound LHCII trimers, are associated with the C_2S_2-type supercomplex (Boekema *et al.*, 1999; Yakushevska *et al.*, 2001). When *A. thaliana* thylakoid membranes are solubilized with α-DM, the $C_2S_2M_2$ organization is the largest type observed (Ruban *et al.*, 2003; Caffarri *et al.*, 2009) (Figure 1c). These results from single-particle analysis were recently confirmed through direct observation of the thylakoid membranes by cryoelectron tomography (Daum *et al.*, 2010; Kouřil *et al.*, 2011). When the PSII–LHCII supercomplex from the green alga *C. reinhardtii* was prepared with a relatively high concentration of β-DM (50 mM, 2.6%), the C_2S_2 organization appeared much as it does in vascular plants (Nield *et al.*, 2000b). The lack of M- and L-trimers in *C. reinhardtii* found in an earlier study was tentatively ascribed to the absence of CP24 in this alga (Minagawa and Takahashi, 2004), which is presumed to serve as a linker between PSII core subunits and an M-trimer in *A. thaliana* (Kovács *et al.*, 2006; de Bianchi *et al.*, 2008). However, both trimers were found in a more recent single-particle analysis of the α-DM-solubilized PSII–LHCII supercomplex from *C. reinhardtii*, in which three LHCII trimers were attached to each side of the core (the $C_2S_2M_2L_2$ PSII–LHCII supercomplex) (Tokutsu *et al.*, 2012) (Figure 1a).

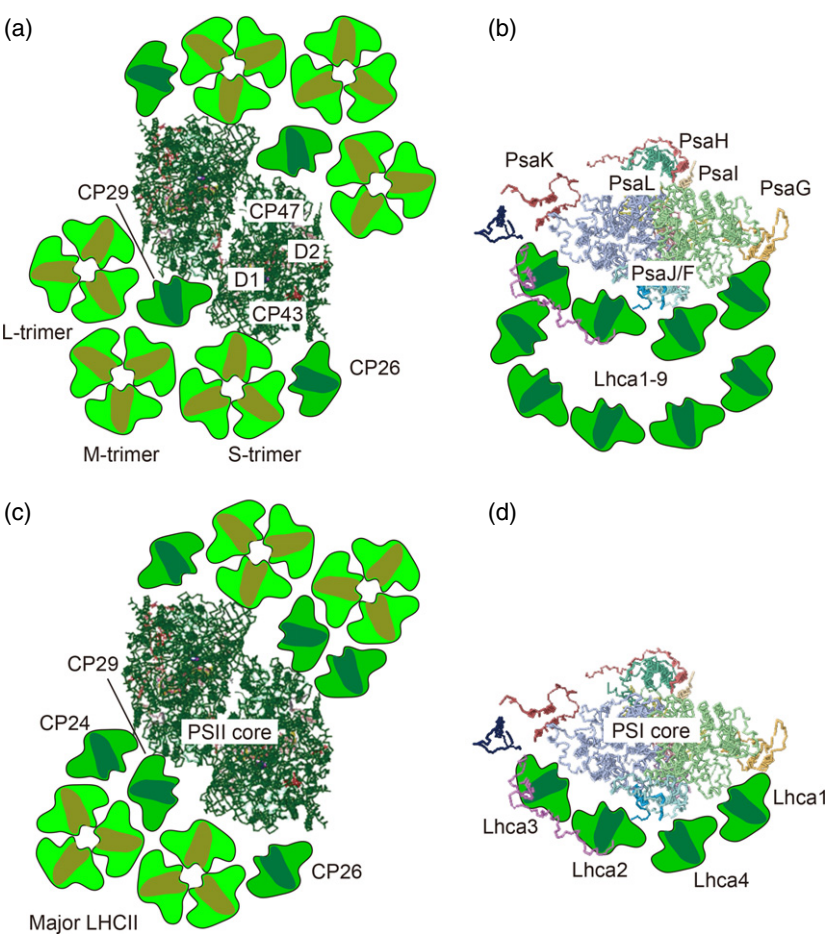

Figure 1. Supramolecular organization of PSII–LHCII and PSI–LHCI supercomplexes in green algae and vascular plants.
(a,b) Top views of the PSII–LHCII supercomplex (a) (Tokutsu *et al.*, 2012) and the PSI–LHCI supercomplex (b) (Dekker and Boekema, 2005) from *C. reinhardtii* based on single-particle image analysis.
(c,d) Top views of the PSII–LHCII supercomplex from *Spinacia oleracea* (c) (Dekker and Boekema, 2005) and the PSI–LHCI supercomplex from *Pisum sativum* (d) (Amunts *et al.*, 2010) based on single-particle image analysis and crystallography, respectively.
All top-view images are from the lumenal side. The PSII and PSI core structures were obtained from the Protein Data Bank: PSII core, PDB ID 3ARC; PSI–LHCI supercomplex, PDB ID 2WSC.

The supercomplex formed by PSI and its light-harvesting complex proteins (LHCI) is also a large Chl–protein complex comprising nearly 20 subunits. The PSI supercomplex collects light energy and drives electron flow from plastocyanin (Pc) to ferredoxin (Fd). Whereas dimeric, trimeric or tetrameric PSI cores have been reported in cyanobacteria (Boekema *et al.*, 1987; Jordan *et al.*, 2001; Watanabe *et al.*, 2011), the eukaryotic PSI cores that harbor LHCIs are monomeric (Amunts *et al.*, 2010). The association of LHCIs with a monomeric PSI core was determined based on a 3.3 Å crystal structure of the PSI–LHCI supercomplex from pea (*Pisum sativum*), in which the side of the PsaF/J subunits was occupied by a crescent-shaped LHCI belt (Amunts *et al.*, 2010) (Figure 1d). The other side of the core is unoccupied under normal conditions, exposing the PsaH/I/L subunits, but it is able to dock mobile LHCII(s) under 'state 2' conditions as described below. In vascular plants, the LHCI belt is formed by four LHCI proteins in the order LHCA1, 4, 2 and 3 (Figure 1d). In *C. reinhardtii*, however, nine LHCI proteins in total, encoded by the *LHCA1–9* genes (Stauber *et al.*, 2003; Minagawa, 2009), form a double-layered LHCI belt attached to the side of the PsaJ/F/G subunits when grown under low light conditions (Drop *et al.*, 2011) (Figure 1b). Proteomic studies demonstrated that the LHCI belt is in fact heterogeneous, and several LHCI proteins are not present stoichiometrically (Tokutsu *et al.*, 2004; Stauber *et al.*, 2009). The composition of LHCI proteins is known to be variable in response to environmental changes, under which down-regulation, degradation or processing of LHCI polypeptides have been reported, in particular under iron-deficient conditions (Moseley *et al.*, 2002; Nield *et al.*, 2004; Tokutsu *et al.*, 2004).

Because plants and algae typically do not have the means to escape adverse environments such as high light (HL) or low light (LL), cold or hot temperatures, drought or low CO_2 concentration, their ability to acclimatize to such conditions is essential for survival. The central unit of photosystems, the core complex, performs charge separations. The components of these charge separation devices are almost completely conserved among cyanobacteria, eukaryotic algae, moss and vascular plants, probably because the process was sufficiently optimized when cyanobacteria acquired the ability to oxidize water 2.7 billion years ago, employing the same system for oxygenic photosynthesis that remains in use today (Brocks *et al.*, 1999; Summons *et al.*, 1999). Furthermore, this system is invariable in changing environments. The only exception is so-called photoinhibition, which is a multi-step process of D1 protein degradation upon HL illumination and the replacement of damaged D1 by a newly synthesized polypeptide (Nixon *et al.*, 2010). In one sense, photoinhibition is a deleterious event for PSII because it loses activity at least temporarily, but, in another sense, it may save all the downstream components from even more devastating events, namely the fatal effects of reactive oxygen species produced by the Mehler reaction or photorespiration (Asada, 1999). Therefore, photoinhibition may be considered as a regulatory mechanism of photosynthetic electron transport (Sonoike, 2011). The core complex including the D1 protein is otherwise invariable.

In contrast to the core complex, the surrounding light-harvesting antenna is flexible with respect to its components. Different photosynthetic organisms generally have different antenna systems to take advantage of the optimal light-harvesting strategy for their particular niche. Moreover, the light-harvesting performance of the antenna system in a single species may be dynamically adjusted to different light regimes. The ability of the light-harvesting antenna to acclimatize are especially important for photosynthetic organisms to optimize their photosynthetic performance and to protect their photosynthetic machinery from the stress of photooxidative damage in the natural environment, where the quality and quantity of light fluctuate over time.

This review presents an overview of the emerging evidence that photosynthetic performance is acclimatized to environmental conditions via dynamic reorganization of photosystem supercomplexes and super-supercomplexes. These reorganizations are observed during state transitions, in the transition between light-harvesting and energy-dissipating activities in the antenna, and when switching between the two electron transfer pathways. We focus on studies in one model organism, the unicellular green alga *C. reinhardtii*, with reference to the model vascular plant *A. thaliana*.

STATE TRANSITIONS

Each of the two charge-separation devices (PSI and PSII) in the thylakoid membranes has a distinct pigment system with unique absorption characteristics. Thus, an imbalance of energy distribution between the two photosystems tends to occur in natural environments, where light quality and quantity fluctuate with time (Allen, 1992; Bellafiore *et al.*, 2005). Because the two photosystems are functionally connected in series under normal conditions, plants and algae must constantly balance their excitation levels to ensure optimal efficiency of electron flow. State transitions occur under such conditions to balance the light-harvesting capacities of the two photosystems, thereby minimizing the unequal distribution of light energy. State 1 occurs when PSI is preferentially excited and the light-harvesting capacities of PSII and PSI are increased and decreased, respectively, to adjust the excitation imbalance; this state is indicated by a higher Chl fluorescence yield at room temperature. Conversely, state 2 occurs when PSII is preferentially excited and the light-harvesting capacities of PSII and PSI are decreased and increased, respectively, to re-adjust the excitation imbalance; this state may be monitored as a lower Chl fluorescence yield at room temperature (Minagawa, 2011) (Figure 2).

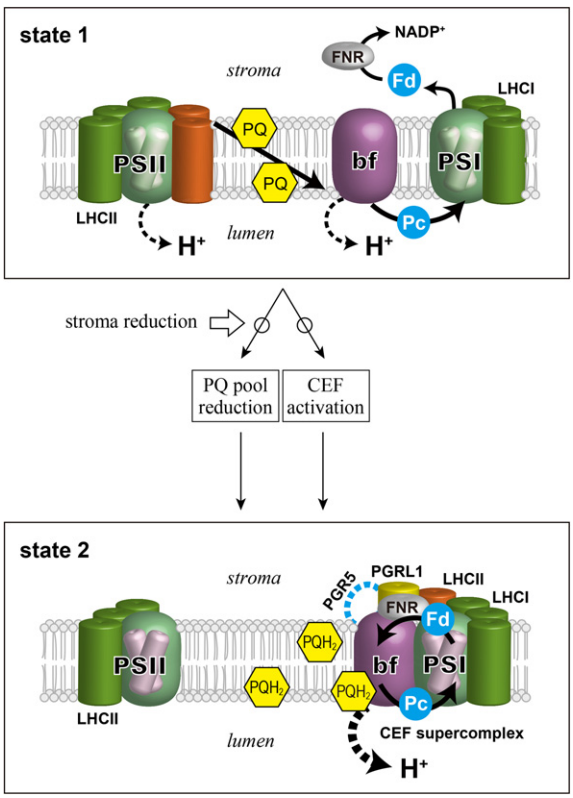

Figure 2. Schematic representation of the regulation of electron flow and state transitions in *C. reinhardtii*.
Top: when PSI is preferentially excited, the stroma of chloroplast and the PQ pool are oxidized. Under these conditions, LHCIIs are bound to PSII (state 1). The photosynthetic electron flow proceeds in LEF mode, generating NADPH as well as a proton gradient across the thylakoid membrane. Middle: when the stroma is reduced, CEF is activated by association of Cyt *bf* and FNR with PSI to form a super-supercomplex (CEF supercomplex). PGRL1 and possibly PGR5 are also associated with the CEF supercomplex. Then the PQ pool is reduced and migration of mobile LHCIIs (orange) from PSII to PSI occurs. Bottom: the cells are in state 2, and the photosynthetic electron flow proceeds in CEF mode. The stroma of chloroplast and the PQ pool are reduced. bf, Cyt *bf*.

There is clear documentation of the core concept, i.e. an instant redistribution of the light-harvesting systems of the two photosystems (Bonaventura and Myers, 1969; Murata, 1969), and of the molecular mechanisms that regulate that redistribution, including the involvement of cytochrome b_6f complex (Cyt *bf*) (Wollman and Lemaire, 1988), binding of reduced plastoquinone (PQH_2) to the Qo site of Cyt *bf* (Vener *et al.*, 1997; Zito *et al.*, 1999), and phosphorylation of LHCII by redox-dependent kinase(s) (Depège *et al.*, 2003). Moreover, data regarding the molecular events occurring at PSII and PSI are rapidly accumulating due to advances in genetic and biochemical techniques using the green alga *C. reinhardtii*. This progress is due, in part, to the fact that as much as 80% of the light-harvesting capacity is controlled by state transitions in this green alga (Delosme *et al.*, 1996), whereas, in vascular plants, only 20–25% of the light-harvesting capacity is controlled by state transitions (Allen, 1992).

Reorganization of the PSII supercomplex during state transitions

The PSII supercomplex has been hypothesized to reorganize during transition from state 1 to state 2, such that a significant number of LHCIIs forming the peripheral antenna of PSII are undocked upon phosphorylation (Haldrup *et al.*, 2001; Iwai *et al.*, 2008). However, recent studies using *in vivo* measurements and high-resolution biochemistry have presented an alternative view of state transitions at the PSII side. By measuring small-angle neutron scattering in living *C. reinhardtii* cells during state transitions, Nagy *et al.* (2014) detected a strong periodicity of the thylakoids in state 1, with characteristic repeat distances of approximately 200 Å, which was almost completely lost in state 2. However, circular dichroism corresponding to the long-range order arrangement of the Chl protein complexes, namely the array of the PSII–LHCII supercomplexes, was only 20% reduced, not 80%, upon transition to state 2 (Nagy *et al.*, 2014). Therefore, a large part of the PSII–LHCII supercomplex array was preserved during state 2, suggesting that most of the peripheral LHCIIs remain physically connected to the PSII core. Nevertheless, the maximal room temperature fluorescence from PSII decreased significantly (by approximately 70%). This decrease indicates that the energy absorbed by the LHCIIs was not trapped by the charge separation device, although the LHCIIs were physically associated with it, suggesting that these LHCIIs, which are possibly phosphorylated, may dissipate such energy within themselves (Nagy *et al.*, 2014). Using time-resolved fluorescence lifetime kinetics, Ünlü *et al.* (2014) observed that only a fraction of LHCII re-coupled to PSI under state 2 conditions; the increased amplitude of the fluorescence decay component ascribed to PSI was not as high as 40% upon transition to state 2, which corresponds to 80% movement of LHCII from PSII, and was, in fact, only 4%.

The possibility of energy quenching within phosphorylated LHCII was previously suggested following monitoring of fluorescence lifetime in living *C. reinhardtii* cells. Iwai *et al.* (2010b) observed the effects of state transitions on the lifetimes of the fluorescence from LHCII at 665–685 nm in *C. reinhardtii* cells. While the cells were in state 1 under far red light, the dominant component of the fluorescence lifetime was 170 psec, and shifted to 250 psec under blue light illumination. Because this lifetime shift was not observed in the *stt7* mutant, which is incapable of state transitions, and because the appearance of the 250 psec component was paralleled by the activation of LHCII phosphorylation and formation of LHCII aggregates, the 250 psec component was ascribed to the aggregated phospho-LHCIIs in the thylakoid membranes in state 2. If the LHCIIs were simply dissociated from the PSII core and existing as individuals, the lifetime would be > 1 nsec.

Nagy *et al.* (2014) provided further evidence that such aggregated and quenched phospho-LHCIIs remained physically associated with the core (Figure 3). In Figure 3, the two types of state transition events are illustrated in *C. reinhardtii*: one is the classical LHCII migration, and the other is the green algae-specific phosphorylation-dependent quenching of aggregated LHCIIs. Although the former type of state transition causes a reduction and an increase of the excitation levels of PSII and PSI, respectively, the latter type of state transition only causes reduction of the excitation level of PSII. The classical LHCII migration between the two photosystems explains a 10–20% reduction of the PSII light-harvesting capacity in *C. reinhardtii*, which has been previously reported several times (Kargul *et al.*, 2005; Takahashi *et al.*, 2006; Tokutsu *et al.*, 2009), and parallels that in vascular plants (Allen, 1992); thus, we propose that green algae and vascular plants share migration-type state transitions. However, only green algae have developed additional ability to perform quenching-type state transitions, which may explain why an 80% change in the light-harvesting capacity was observed in *C. reinhardtii* (Delosme *et al.*, 1996). The reason why the quenching-type state transition only developed in green algae is not clear at present. However, these variances in the molecular mechanisms may be linked to variations in the platforms for the photosynthetic machineries, namely the thylakoid supra-structures. It is known that morphological domains of the thylakoid membranes are quite different in vascular plants and most green algae (Nevo *et al.*, 2009). In vascular plants, grana and stroma lamellae regions are clearly separated. While 10–20 layers of thylakoid membranes are stacked in the grana region, only a single or a few membrane stacks are present in the stroma lamellae region (Mustárdy and Garab, 2003; Mullineaux, 2005; Shimoni *et al.*, 2005). Many green algae have areas of appressed thylakoid membranes, also called grana, but these do not have the highly structured multiple membrane layers of the grana in vascular plants (Bertos and Gibbs, 1998). It is thus plausible that variances in the molecular mechanism for state transitions, and the other photoacclimation events of photosynthetic machineries, may be due to such differences in the thylakoid supra-structures.

Reorganization of the PSI supercomplex during state transitions

Vascular plants without PsaH and PsaL (Lunde *et al.*, 2000) and those without PsaO (Jensen *et al.*, 2004) are deficient in state transitions. Because these small PSI subunits are located on the opposite side of the LHCI belt (Amunts *et al.*, 2010) (Figure 1), they were hypothesized to constitute a binding site for the mobile LHCII(s). Further information regarding the reorganization of PSI upon transition from state 1 to state 2 was provided by a study on *C. reinhardtii*, in which the PSI–LHCI–LHCII supercomplex from state 2 cells contained two minor monomeric LHCII proteins, CP26 and CP29, and one major trimeric LHCII protein, LhcbM5, suggesting a pivotal role for the minor monomeric LHCII in state transitions, at least in green algae (Takahashi *et al.*, 2006, 2014). The significance of the minor LHCIIs in state transitions in *C. reinhardtii* was further supported by an RNA interference (RNAi) study in which the levels of CP29 or CP26 were individually reduced

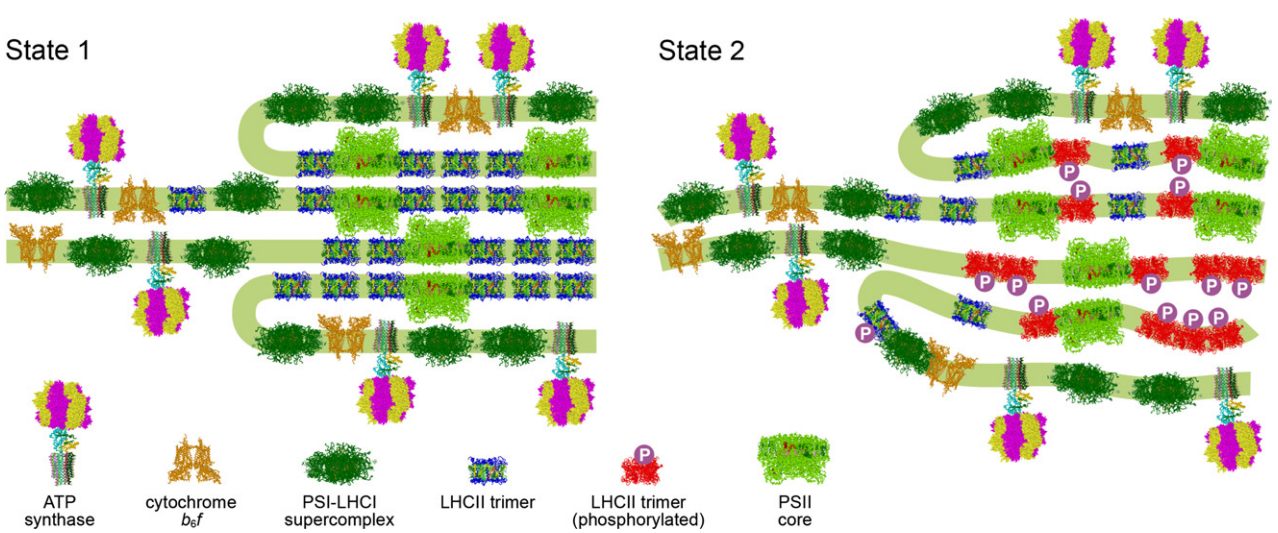

Figure 3. Model for the remodeling of the photosynthetic supercomplexes in the thylakoid membranes during state transitions in *C. reinhardtii*.
Side views of the membrane planes showing alterations in the thylakoid ultrastructure and photosystem supercomplex composition. In state 1 (left), thylakoids are more stacked, and large arrays of PSII–LHCII supercomplexes are present in the appressed regions. The periodicity of thylakoid membranes is well defined. PSI–LHCI supercomplexes, Cyt *bf* and ATP synthases are present in the non-appressed regions. In state 2 (right), a number of LHCII proteins are phosphorylated, and the thylakoids are partially unstacked and undulating. The periodicity of the thylakoid membranes is weak. Many of the phosphorylated LHCIIs (red) are in the energy-quenching state and remain associated with PSII, such that a large part of the PSII–LHCII supercomplex array is preserved. Some of the phosphorylated LHCIIs (red) are aggregated and in the energy-quenching state, whereas the unphosphorylated LHCIIs (blue) are actively harvesting light.

(Tokutsu *et al.*, 2009). Both the CP29 and CP26 RNAi mutants underwent reductions in PSII antenna size during transition from state 1 to state 2. However, the LHCIIs undocked from PSII did not re-associate with PSI in the CP29 RNAi mutant. By contrast, the mobile LHCIIs in the CP26 RNAi mutant did re-associate with PSI, such that a PSI–LHCI–LHCII supercomplex was visualized on a sucrose density gradient (Tokutsu *et al.*, 2009).

These results thus clarify that CP29, but not CP26, is crucial for the classical migration-type state transitions in *C. reinhardtii*. As mentioned above, most LHCII molecules remain bound to PSII as an energy-dissipative complex, but the remainder, which may be as low as 9% (Nagy *et al.*, 2014), migrate to PSI. CP29 appears to be crucial in this minor event. Two-dimensional structures of such a PSI–LHCI–LHCII supercomplex were visualized by single-particle analysis of electron micrographs in *C. reinhardtii* (Drop *et al.*, 2014) and *A. thaliana* (Galka *et al.*, 2012), and two LHCII trimers/one LHCII monomer (in *C. reinhardtii*) and one LHCII trimer (in *A. thaliana*) were found to be located near the PsaH/I/L subunits.

QE QUENCHING

In nature, unexpected changes in light intensity may lead to over-excitation of the photosystems, resulting in accumulation of harmful reactive oxygen species (Li *et al.*, 2009). Plants and algae have developed protective non-photochemical quenching (NPQ) mechanisms that alleviate such photooxidative stress. Among these mechanisms, rapid down-regulation of the light-harvesting capacity of PSII that thermally dissipates excess light energy as a negative feedback mechanism is called qE quenching (Govindjee *et al.*, 1967; Murata and Sugahara, 1969). This type of NPQ is dependent upon the ΔpH across the thylakoid membranes (Wraight and Crofts, 1971), and its extent and capacity are correlated with accumulation of the xanthophyll zeaxanthin (Zx) (Demmig *et al.*, 1987). Niyogi and co-workers further paved the way for elucidation of the site and molecular mechanism of qE by studying and providing various *npq* mutants (Horton *et al.*, 2008; Li *et al.*, 2009; de Bianchi *et al.*, 2010).

Xanthophylls

Zx accumulates in thylakoid membranes under HL conditions by reversible de-epoxidation of violaxanthin (Vx) in a process called the xanthophyll cycle (Yamamoto *et al.*, 1962), which is activated by acidification of the thylakoid lumen (Demmig-Adams and Adams, 1992; Demmig-Adams *et al.*, 1996). The *npq1* mutant, which is deficient in conversion of Vx into Zx, was first reported in *C. reinhardtii* (Niyogi *et al.*, 1997a) and then in *A. thaliana* (Niyogi *et al.*, 1998). The locus was mapped to a gene encoding Vx de-epoxidase (VDE) in *A. thaliana* (Niyogi *et al.*, 1998), whereas it was mapped to a different gene in *C. reinhardtii* (Anwaruzzaman *et al.*, 2004).

Because over-accumulation of Zx increases the level of qE in vascular plants (Niyogi *et al.*, 1998; de Bianchi *et al.*, 2010), Zx has been attributed to a quenching site for qE. Fleming and co-workers reported that energy dissipation occurs via charge transfer between Chl–Zx in the thylakoids (Holt *et al.*, 2005) and in the reconstituted CP29 protein (Ahn *et al.*, 2008). The increased Chl–Zx interaction was directly correlated with NPQ in LHCII (Bode *et al.*, 2009). The triplet-*minus*-singlet spectra of carotenoids in leaves and the isolated LHC complexes showed a characteristic red shift in the presence of Zx (Dall'Osto *et al.*, 2012). However, Amarie *et al.* (2007) demonstrated that the presence of Vx or Zx cation radicals in LHCII made no difference to the excitation lifetimes of Chls. Alternatively, Zx (Johnson *et al.*, 2011), together with PSBS (Kereiche *et al.*, 2010), was hypothesized to decrease the size of the ordered semi-crystalline arrays of PSII supercomplexes, leading to an increase in the fluidity of the thylakoid membranes and promotion of the energy-dissipative aggregation of LHCIIs (Goral *et al.*, 2012).

In contrast to the VDE mutant of vascular plants, the *npq1* mutant of *C. reinhardtii* showed only a minor effect on qE, indicating a possibility that the majority of photoprotection is achieved without Zx in this alga (Niyogi *et al.*, 1997a,b). Although the lack of VDE activity induced little photoinhibition under HL conditions, an additional mutation in *npq1* that inhibits lutein synthesis (*lor1*) caused severe photoinhibition, suggesting that lutein, a central structural component of LHCIIs (Minagawa, 2009), greatly contributes to qE quenching in *C. reinhardtii* (Niyogi *et al.*, 1997b).

qE effectors

npq4 mutants, deficient in rapid induction of qE despite a normal xanthophyll cycle and normal lumenal acidification, have been reported in both *A. thaliana* and *C. reinhardtii*, but the alleles mapped to different genes: the four-helix LHC family protein PSBS in *A. thaliana* (Li *et al.*, 2000) and the three-helix LHC family protein LHCSR3 in *C. reinhardtii* (Peers *et al.*, 2009). Again, the existence of the same phenotype in different genotypes indicates that plants and algae perform qE quenching in different ways. PSBS was originally described as a 22 kDa intrinsic membrane protein binding Chls and carotenoids, and was therefore designated CP22 (Funk *et al.*, 1994). However, subsequent studies did not confirm the protein's pigment-binding ability (Dominici *et al.*, 2002). Indeed, for PSBS, most of the Chl ligands identified from the LHCII crystal structure are not conserved. PSBS interacts weakly with PSII (Funk *et al.*, 1995), but was not stably associated with the PSII–LHCII supercomplex (Caffarri *et al.*, 2009); instead, it was observed in LHCII-rich regions that interconnect the supercomplexes in the membrane (Nield *et al.*, 2000a).

The putative PSBS protein encoded in the genome of *C. reinhardtii* shares 45% identity over 180 amino acids with *A. thaliana* PSBS (Minagawa and Takahashi, 2004), including the putative pH-sensing Glu residues (Li *et al.*, 2004). There are strong lines of evidence suggesting that the PSBS protein in *A. thaliana* controls the macro-organization of the thylakoid membranes through acidification of the lumen (Kiss *et al.*, 2008; Kereiche *et al.*, 2010; Goral *et al.*, 2012). Such a membrane 'phase transition' is thought to facilitate the dissociation of several LHCII proteins, including CP24, CP29 and the LHCII M-trimer from PSII (Betterle *et al.*, 2009); moreover, their aggregation (Kiss *et al.*, 2008; Johnson *et al.*, 2011) probably allows for the conformational change within major LHCIIs (Ruban *et al.*, 2007) and/or minor LHCIIs (Ahn *et al.*, 2008) to generate energy-quenching site(s). Alternatively, a recent report has claimed that PSBS directly interacts with LHCII and induces energy dissipation (Wilk *et al.*, 2013). A further study measured Chl lifetimes, and showed that PSBS does not affect the relaxation dynamics of the Chl excited state, but changes the amount and the initial induction rate of quenching (Sylak-Glassman *et al.*, 2014).

Although both Zx and PSBS are thought to be crucial in qE quenching in vascular plants, *C. reinhardtii* still shows qE quenching in the absence of VDE activity (Niyogi et al., 1997a,b). This green alga does not express the PSBS protein, either (Finazzi *et al.*, 2006; Bonente *et al.*, 2008) despite the presence of the *PSBS* gene (Anwaruzzaman *et al.*, 2004). Although PSBS in *A. thaliana* is constitutively present even in LL-grown plants (Demmig-Adams *et al.*, 2006) to provide instant photoprotection, qE in *C. reinhardtii* cannot be activated immediately upon exposure to HL. The activation of qE in *C. reinhardtii* requires prolonged exposure to HL (Niyogi *et al.*, 1997a), suggesting that algae have a distinct mechanism for qE induction.

The *C. reinhardtii npq4* mutant was mapped to an 'ancient' LHC protein, LHCSR3 (Peers *et al.*, 2009). The genes for LHCSR3 (*LHCSR3.1* and *LHCSR3.2*), formerly known as LI818 (Gagne and Guertin, 1992), encode a 25–26 kDa integral membrane protein whose expression was induced under HL (Richard *et al.*, 2000), low CO_2 (Miura *et al.*, 2004) or low iron (Naumann *et al.*, 2007) conditions. A study on the diatom ecotypes revealed that the level of LHCSR (LHCX) correlated with the amplitude of qE quenching (Bailleul *et al.*, 2010). Although PSBS cannot bind pigments, LHCSR3 is capable of binding Chl *a* and *b*, as well as xanthophylls (Bonente *et al.*, 2011). Furthermore, a recombinant LHCSR3 polypeptide reconstituted with Chls and xanthophylls dissipated excitation energy in a low pH buffer, suggesting that this protein is a quenching site in *C. reinhardtii* (Bonente *et al.*, 2011). In addition to this 'HL-specialized' antenna protein involved in quenching, genetic analysis in *C. reinhardtii* has led to identification of other LHCs related to quenching. Depletion of one of the major trimeric LHCII proteins, LHCBM1, in the *npq5* mutant decreased its capacity for thermal energy dissipation (Elrad *et al.*, 2002). It is possible that other LHC proteins are also involved in HL acclimation in *C. reinhardtii* (Ferrante *et al.*, 2012).

Current model for induction of qE quenching in *C. reinhardtii*

Signal transduction pathway(s) for LHCSR3 expression have not yet been clarified. Petroutsos *et al.* (2011) reported that a chloroplast-localized calcium sensor protein (CAS) affected HL-dependent LHCSR3 expression, whereby an artificial microRNA knockdown mutant of CAS did not express LHCSR3 under HL conditions unless a high concentration of calcium was present. They also found that the calmodulin antagonist W7 inhibited the HL-dependent accumulation of LHCSR3 (Petroutsos *et al.*, 2011), suggesting that HL-dependent *LHCSR3* gene expression or HL-dependent import of LHCSR3 protein into the chloroplast are mediated by calmodulin and/or calmodulin-affected calcium transients. More recently, gene expression of *LHCSR3.1* and *LHCSR3.2* as well as *LHCSR1* were studied by quantitative PCR, confirming that transcription of all three *LHCSR* genes was induced by HL (Maruyama *et al.*, 2014). However, their sensitivities to the inhibitors varied. Expression of *LHCSR3.1* and *LHCSR3.2* was almost completely inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or W7, whereas that of *LHCSR1* was only partially inhibited by W7 and was insensitive to DCMU (Maruyama *et al.*, 2014). These results led Maruyama *et al.* (2014) to propose that there are two kinds of light signals for expression of the *LHCSR* genes: one is dependent on the photosynthetic electron transport and the other is independent of photosynthesis. These studies on the *LHCSR* gene expression indicate a few hours elapse before the LHCSR proteins are expressed under HL conditions. The cells are thus exposed to HL without qE quenching in the first few hours. However, a recent report suggested that *C. reinhardtii* cells actually alleviate such initial HL stress by performing state transitions (Allorent *et al.*, 2013).

Although LHCSR3 is necessary to activate qE quenching in *C. reinhardtii*, where and how the LHCSR3 protein contributes has remained unclear. A recent study clarified where LHCSR3 is localized in the thylakoid membranes and how it dissipates excess energy (Tokutsu and Minagawa, 2013). By comparing the PSII–LHCII supercomplex isolated from wild-type (WT) *C. reinhardtii* cells with that from *npq4*, Tokutsu and Minagawa (2013) found that LHCSR3 was present only in the PSII supercomplex from HL-grown WT cultures and not in that from LL-grown WT or HL-grown *npq4*. The purified PSII–LHCII–LHCSR3 supercomplex was in a high-fluorescence (light-harvesting) state at neutral pH (7.5), as evaluated by time-correlated single-photon counting. However, the complex was in a low-fluo-

rescence (energy-dissipative) state at pH 5.5, similar to the effect of lumenal acidification after HL illumination of the thylakoid membranes (Tokutsu and Minagawa, 2013). The switch from a light-harvesting state to an energy-dissipative state observed in the PSII–LHCII–LHCSR3 supercomplex was sensitive to dicyclohexylcarbodiimide (DCCD), a protein-modifying agent that is specific for protonatable amino acid residues. The switch from a light-harvesting state to an energy-dissipative state was probably regulated by a conformational change in the C-terminus of LHCSR3 (Liguori *et al.*, 2013). We therefore propose that HL-induced expression, association of LHCSR3 with the PSII–LHCII supercomplex, and a conformational change in the C-terminus induced by lumenal acidification are the necessary steps for dissipating excess absorbed energy in *C. reinhardtii* (Figure 4).

CYCLIC ELECTRON FLOW

Regulation of cyclic electron flow

Electrons that are generated in the photosystems flow into two pathways in the thylakoid membranes: the linear electron flow (LEF) pathway from water to $NADP^+$ via PSII and PSI in series, and the cyclic electron flow (CEF) pathway around PSI. The correct balance of ATP and NADPH in the stroma (3:2) is crucial for assimilating CO_2 in the Calvin–Benson cycle; however, this balance cannot be achieved by LEF alone (Allen, 2003). As illustrated in Figure 5, assuming the quantum yield of the photochemistry in PSII is approximately 0.8, four protons are released into the lumen and two molecules of reduced PQ are released to the inter-system pool during four turnovers of PSII upon capturing five photons (Björkman and Demmig, 1987). At the Qo site of Cyt *bf*, two molecules of reduced PQ release four protons into the lumen and transfer two electrons toward Pc. This yield of protons and electrons is doubled through the Q-cycle mechanism (Mitchell, 1976; Sacksteder *et al.*, 2000). Overall, eight protons are released from Cyt *bf* into the lumen, and four Pc are reduced, and ultimately two NADPH accumulate on the stroma side of the membrane. Protons that are released into the lumen are used to rotate the CFo sub-complex of ATP synthase, in which three ATP molecules are synthesized per single rotation. As there are 14 proton-binding c-subunits in the CFo sub-complex (Seelert *et al.*, 2000), the 12 protons generated by LEF may only be used to rotate the CFo sub-complex by 0.86 rounds, which is coupled to synthesis of 2.6 molecules of ATP. To synthesize three molecules of ATP, plants and algae need to re-route one electron from PSI to Cyt *bf* to pump two extra protons into the lumen. CEF enables such electron re-routing from PSI to Cyt *bf*, and is therefore essential to achieve the correct balance (3:2) of ATP and NADPH in the stroma for CO_2 assimilation (Munekage *et al.*, 2004). The scheme shown in Figure 5 indicates that the quantum yield of PSII, in which five photons are required for four turnovers, is lower than that of PSI, in

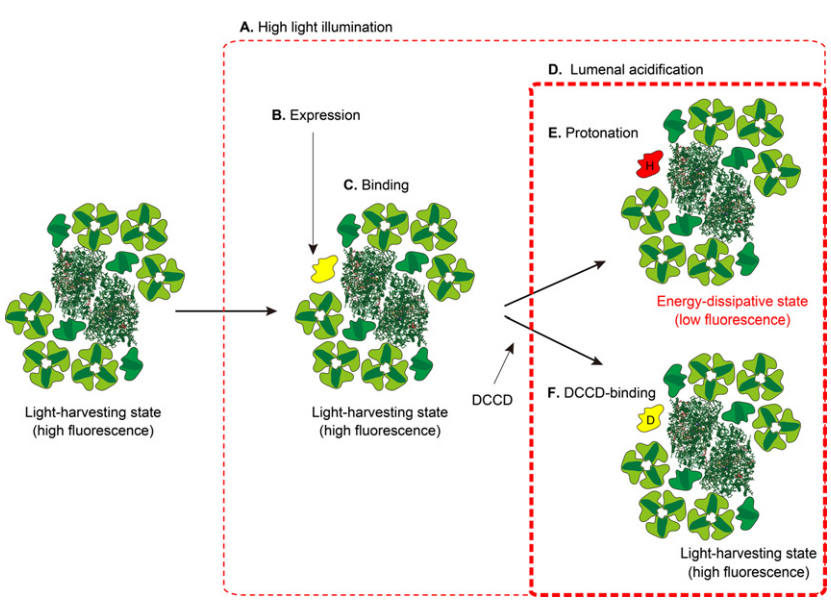

Figure 4. Current model for the induction of qE quenching in *C. reinhardtii*.
When *C. reinhardtii* cells are exposed to HL for a few hours (A), expression of LHCSR3 is induced (B). LHCSR3 (yellow) then associates with the PSII–LHCII supercomplex to form the PSII–LHCII–LHCSR3 supercomplex (C). Although the PSII–LHCII–LHCSR3 supercomplex is still in a light-harvesting state under dark or LL conditions, it becomes energy-dissipative upon protonation of LHCSR3 when the lumen of the thylakoid membranes is acidified under HL conditions (D), which causes a conformational change in the C-terminal region of LHCSR3 (red; 'H' indicates protonation) (E). When DCCD binds to LHCSR3 (yellow; 'D' indicates DCCD), the PSII–LHCII–LHCSR3 supercomplex cannot become energy-dissipative (F). The crystal coordinates were obtained from the Protein Data Bank: PSII core, PDB ID 3ARC; LHCII, PDB ID 2NHW; PSI–LHCI supercomplex, PDB ID 2WSC.

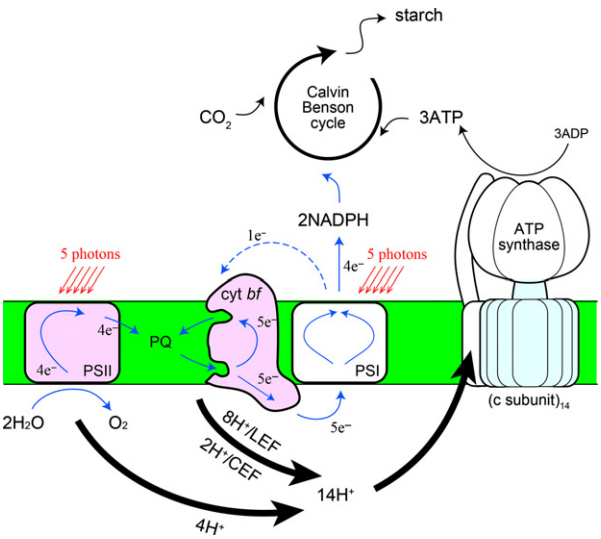

Figure 5. Schematic representation of the movement of protons and electrons by the photosynthetic electron transport chain in the chloroplast.
The pathways and stoichiometry of light-driven electron transport, proton translocation and ATP synthesis in the thylakoid membranes are shown. Thick arrows represent the pathways of protons; thin solid arrows and thin dashed arrows represent LEF and CEF, respectively.

which five photons are required for five turnovers. In other words, because 20% of the electrons in PSI are circulated along the CEF pathway, the quantum yield for PSII is reduced by 20%. Under natural conditions, ATP and NADPH are consumed in various cellular reactions, and the demand for each fluctuates from time to time. Therefore, photosynthetic organisms must constantly adjust the ratio between the two modes of electron flow. Another important role of CEF is to establish a high proton motive force across the thylakoid membrane, which is required to activate qE quenching. Moreover, Kukuczka *et al.* (2014) recently demonstrated that the *npq4* mutant deficient in LHCSR3 displayed an increased expression of PROTON GRADIENT REGULATION 5 LIKE 1 (PGRL1) protein, a critical factor for CEF as mentioned below, suggesting that CEF may even compensate for loss of the qE effector. CEF is therefore crucial for CO_2 assimilation and for protection of the photosynthetic machinery from HL. Chloroplasts must thus regulate the rate of CEF in response to the energy status and stress conditions.

In *C. reinhardtii* WT cells, modulation of CEF normally occurs in parallel with state transitions (Finazzi *et al.*, 2002). When the light-induced reduction of Cyt *bf* was probed in state 1- or state 2-adapted cells, a differential sensitivity to addition of the PSII inhibitor DCMU was observed. In the presence of DCMU, reduction of Cyt *bf* was suppressed in state 1 but not in state 2. An identical sensitivity to an inhibitor of Cyt *bf*, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone (DBMIB), was observed in both state 1 and state 2, suggesting that PSII-independent Cyt *bf* reduction occurs only in state 2 (Finazzi *et al.*, 1999). However, recent reports provided evidence that state transitions and the CEF/LEF switch are not mechanically linked in *C. reinhardtii* but the two phenomena are instead coincidental (Terashima *et al.*, 2012; Takahashi *et al.*, 2013). Under anaerobic conditions, independent knockdown of three thylakoid membrane proteins, PGRL1, CAS and ANAEROBIC RESPONSE 1 (ANR1), resulted in decreased CEF activity, but the ability to undergo state transitions was unaffected (Tolleter *et al.*, 2011; Terashima *et al.*, 2012). This was further supported by the low CEF activity in a state 2-locked mutant of *C. reinhardtii* (Takahashi *et al.*, 2013). Although lateral migration of mobile LHCIIs occurred in the *ptox2* mutant, which was locked in state 2 due to a lack of plastid terminal oxidase 2 (Houille-Vernes *et al.*, 2011), the effects on $P700^+$ re-reduction were negligible (Takahashi *et al.*, 2013). CEF was thus proposed to be correlated with a reduced state of the stroma (Figure 2). However, the mechanism by which increased reducing power in the stroma promotes CEF remains to be unraveled (Takahashi *et al.*, 2013).

Molecular models for cyclic electron flow

Two possible electron transfer pathways have been proposed for CEF: an Fd-dependent pathway (Fd-CEF) and an NAD(P)H dehydrogenase-dependent pathway (Shikanai, 2007). In the NAD(P)H dehydrogenase-dependent pathway, NAD(P)H dehydrogenase mediates NADPH oxidation and PQ pool reduction in much the same way that complex I does in mitochondria (Ogawa, 1991). However, how electrons on Fd are transferred to the PQ pool in the Fd-dependent pathway had been unclear until recently. The molecular machinery for Fd-CEF was first identified in *C. reinhardtii* cells treated with carbonyl cyanide 4- (trifluoromethoxy)phenylhydrazone (FCCP) (Iwai *et al.*, 2010a). FCCP depletes intracellular ATP pools by uncoupling the energized membranes, and stimulates glycolysis according to the Pasteur effect (Cardol *et al.*, 2009), reducing the stroma of the chloroplast. Probably because of this process, CEF becomes activated and the PQ pool in the thylakoid membrane is reduced, inducing the cells to shift to state 2 (Bulté *et al.*, 1990). By loading the solubilized thylakoid membranes onto a sucrose density gradient, Iwai *et al.* (2010a) obtained four discreet green bands, including the heaviest fraction (the A4 fraction). The A4 fraction contained PSI with its own antenna system (the PSI–LHCI supercomplex), major LHCIIs, minor LHCIIs, Cyt *bf*, PETC, PetD, PETO, Fd NADPH oxidoreductase (FNR) and PGRL1, with equimolar amounts of Cyt *bf* and PSI. Use of four different methods suggested that the proteins in the A4 fraction formed a single supercomplex: (i) a single peak with a molecular mass corresponding to 1400–1600 kDa was observed on a gel-filtration chromatogram, (ii) the constituents of the A4 band were disassembled by salt treatment

into complexes of lower molecular mass, indicating they were originally confined in a super-supercomplex, (iii) the A4 fraction was absent in mutants for PSI or Cyt *bf*, and (iv) the supercomplex, after being affinity-purified using a His-tagged PsaA subunit of PSI, displayed the same protein library as the original A4 fraction (Iwai *et al.*, 2010a).

To investigate whether the supercomplex in the A4 fraction performs CEF, the electron transfer activities of the supercomplex on its stromal and lumenal sides were examined by a series of spectroscopic analyses (Iwai *et al.*, 2010a). The stromal-side electron transfer was first probed by measuring the reduction of Cyt *b*. When Cyt *b* in the supercomplex was reduced after incubation with both NADPH and Fd, the resulting signal was approximately half as large as that produced upon reduction of both *b*-type hemes using dithionite, suggesting that only one of the *b*-type hemes in Cyt *bf*, most likely the high-potential heme b_H, was reduced. When the supercomplex was illuminated in the presence of Fd, Cyt *b* was reduced. This indicated that one of the *b*-type hemes in Cyt *bf*, probably heme b_H, was photoreduced by electrons from PSI. The lumenal-side electron transfer was evaluated by studying the flash-induced kinetics of $P700^+$ re-reduction and Cyt *f* oxidation within the supercomplex. After P700 was oxidized by a single-turnover actinic flash, Cyt *f* was rapidly oxidized in the presence of Pc, and most of the P700 accepted electrons from Pc (Iwai *et al.*, 2010a).

The physical association of PSI, Cyt *bf* and FNR had long been suggested as a platform for Fd-CEF (Joliot and Joliot, 2002; Eberhard *et al.*, 2008). Mathematical modeling of electron transfer (Laisk, 1993) and *in vivo* observation of its high efficiency (Joliot and Joliot, 2002) suggested that Fd-CEF operates in a supercomplex. However, the enigmatic 'super-supercomplex' comprising PSI, Cyt *bf* and FNR had never been detected. The above-mentioned study in *C. reinhardtii* (Iwai *et al.*, 2010a) demonstrated that the CEF supercomplex exists. Notably, electron transfer activity was detected in this supercomplex on both the stromal and lumenal sides. Because CEF and LEF share many redox carriers (PQ, Cyt *bf*, Pc, PSI, Fd and FNR), they are potentially in competition with one another. Furthermore, the redox poise of the CEF components may be disturbed if reduced LEF components co-exist. The formation of a super-supercomplex specific for Fd-CEF (a PSI–LHCI–LHCII–FNR–Cyt *bf*–PGRL1 supercomplex) appears to compartmentalize CEF by localizing the mobile electron carriers, leading to formation of a functional pool of CEF components to sustain CEF activity as discussed previously (Breyton *et al.*, 2006; Peng *et al.*, 2009; Johnson, 2011).

As described above, *C. reinhardtii* cells carrying mutations in either CAS or ANR1 showed decreased CEF activity under anaerobic, which was partially restored by addition of calcium under anaerobic conditions, whereas the activity of the *pgrL1* mutant was only marginally restored (Terashima *et al.*, 2012). CAS and ANR1 were thus proposed as additional components of the CEF supercomplex and regulate CEF activity via calcium signaling. Furthermore, the CEF supercomplex was present under anaerobic but not aerobic conditions in WT, *ptox2* (state 2-locked) or *stt7* (state 1-locked) cells (Takahashi *et al.*, 2013). This observation suggests that a reduced stromal environment, which was technically achieved by anaerobic treatment, was responsible for the LEF/CEF switch but not LHCII phosphorylation. Using a mutant defective in either Rubisco or ATPase activity in chloroplast, it was proposed that CEF was activated when the stromal carriers were reduced in *C. reinhardtii* (Johnson *et al.*, 2014).

Finally, we discuss the possible CEF inhibitor antimycin A (AA) and the intriguing CEF components PGR5 and PGRL1. Among NPQ mutant lines, Munekage *et al.* (2002) isolated several *pgr* mutants in *A. thaliana* that have a limited capacity for proton gradient formation across the thylakoid membranes. Because reduction of the PQ pool by way of CEF was previously described as AA-sensitive (Tagawa *et al.*, 1963), the elusive AA-binding enzyme Fd-PQ oxidoreductase (FQR) had been proposed to bypass Cyt *bf*. Unlike its sister enzyme, the cytochrome bc_1 complex in mitochondria (Huang *et al.*, 2005), Cyt *bf* is not sensitive to AA with respect to reduction of the PQ pool (Moss and Bendall, 1984). Because the *A. thaliana* mutant *pgr5* affected the AA-sensitive PSII fluorescence increase from ruptured chloroplasts, PGR5 was postulated to be part of FQR (Shikanai, 2007). PGR5 is only an 8 kDa polypeptide, and probably forms a complex with other membrane-spanning subunit(s) to constitute possible FQR. A mutant deficient in PGRL1 was subsequently reported to display a limitation of the increase in PSII fluorescence in much the same way that *pgr5* did (DalCorso *et al.*, 2008). Subsequent *in vitro* studies led Hertle *et al.* (2013) to propose a molecular mechanism for Fd-CEF in *A. thaliana* dependent on the PGR5–PGRL1 heterocomplex. Recombinant PGRL1 was reduced by Fd when it formed a heterocomplex with PGR5, although such a complex was not detected *in vivo* (Hertle *et al.*, 2013). The reduced and monomerized PGRL1 in turn reduced a PQ analog 2,6-dimethyl-*p*-benzoquinone (DMBQ) *in vitro* in an AA-sensitive manner, although the AA concentration used here was 500 times higher than that in studies using ruptured chloroplasts (Hertle *et al.*, 2013).

Although genes for both PGR5 and PGRL1 are present in the genome of *C. reinhardtii* (Merchant *et al.*, 2007) and transcription of the two genes was observed (Petroutsos *et al.*, 2009), only PGRL1 was detected in the active CEF supercomplex in *C. reinhardtii* (Iwai *et al.*, 2010a), which may suggest that PGR5 is not an essential component for CEF activity. Indeed, a significant amount of the reduced form of PGRL1 was detected in *pgr5* leaves upon illumination, implying that PGRL1 may relay CEF without PGR5 (Hertle *et al.*, 2013). In addition, multiple laboratories reported that

CEF may occur in the absence of PGR5. Avenson *et al.* (2005) estimated that the PGR5 pathway in *A. thaliana* contributed up to 13% of proton flux *in vivo*, and many of the *pgr5* phenotypes could be attributed to the effects on ATP synthase. The leaves of *pgr5* in *A. thaliana* were able to perform CEF at a rate similar to the WT (Nandha *et al.*, 2007). Livingston *et al.* (2010) detected a high CEF activity in the *hcef1* mutant of *A. thaliana*, although it expressed PGR5 at only half the level of the WT, and such activity was not compromised by crossing with *pgr5*. Moreover, the main role of PGR5 was proposed not to be to regulate CEF, but to regulate LEF to prevent damage of PSI under fluctuating light conditions (Suorsa *et al.*, 2012). Similarly, a recent report also indicated that the electrochromic shift (ECS) signal, which is solely due to CEF when PSII activity is blocked by DCMU, decreased by half in the *pgr5* mutant in *C. reinhardtii* (Johnson *et al.*, 2014). All these studies observed effects of the *pgr5* mutation to some extent, but none of them reported that CEF activity was abolished by loss of PGR5. Given the results of studies of the CEF supercomplex (Iwai *et al.* 2010a), the mutant studies suggested that PGR5 may have been present and contributed to the activity in the CEF supercomplex but escaped scrutiny because it was expressed at a level that was seven times lower than that of its binding partner PGRL1 (Hertle *et al.*, 2013). Alternatively, PGR5 may indeed be absent from the isolated CEF supercomplex, such that the complex cannot exert maximal CEF activity. The most likely scenario is that the CEF supercomplex harboring only PGRL1 exhibits CEF activity (Iwai *et al.*, 2010a), but such activity may be switched on more rapidly (Joliot and Johnson, 2011) or may be enhanced up to 200% (Hertle *et al.*, 2013) if a minor amount of PGR5 is present (Figure 2).

The above hypothesis regarding the role of PGR5 on CEF, which is thought to be AA-sensitive (Leister and Shikanai, 2013), cannot account for the finding that $P700^+$ reduction in *C. reinhardtii* cells was not sensitive to AA (Iwai *et al.*, 2010a). A recent study comparing the sensitivity to AA and the sequence variations of PGR5 in two plant species may provide a clue to solve this problem (Sugimoto *et al.*, 2013). The authors found that the third residue of PGR5 in AA-sensitive *A. thaliana* was Val, whereas that in AA-insensitive *Pinus taeda* was Lys. Because strain 137c, a common WT strain of *C. reinhardtii* used in isolation of the CEF supercomplex harbors Lys at this position, any PGR5–PGRL1-dependent CEF supercomplex was not supposed to be sensitive to AA.

Remaining problems

The isolated CEF supercomplex was shown to perform Fd-CEF in *C. reinhardtii* (Iwai *et al.*, 2010a). An alternative PGR5–PGRL1 heterocomplex model, as originally shown in *A. thaliana* using recombinant polypeptides (Hertle *et al.*, 2013), was also proposed in *C. reinhardtii* (Johnson *et al.*, 2014). These reports established a framework for studying Fd-CEF at the molecular level, and related findings are rapidly accumulating. However, as discussed above, there are currently several differences and discrepancies among those studies. These discrepancies may have arisen in part due to differences in the methodologies used for measurement of CEF activity, such as increased PSII fluorescence as a marker of PQ pool reduction in osmotically ruptured chloroplasts (Munekage *et al.*, 2002; DalCorso *et al.*, 2008; Hertle *et al.*, 2013), re-reduction of $P700^+$ in the absence of PSII activity in the cells (Munekage *et al.*, 2002; DalCorso *et al.*, 2008; Petroutsos *et al.*, 2009), dark-interval relaxation of ECS in the absence of PSII activity in the cells (Johnson *et al.*, 2014), reduction of the PQ analog DMBQ by a recombinant polypeptide (Hertle *et al.*, 2013), and photoreduction of Cyt b_H in the isolated complex (Iwai *et al.*, 2010a). A recent evaluation by Fisher and Kramer (2014) provided important progress toward clarifying this problem. The authors re-evaluated the induction kinetics and inhibitor sensitivity of the PSII fluorescence from ruptured spinach chloroplasts, and noted that the AA-sensitive fluorescence increase was not inhibited by the PSII inhibitor DCMU, suggesting that the observed fluorescence increase did not reflect the reduction of the PQ pool, but reduction of the low-potential variants of the primary acceptor (Q_A) in PSII, and that the observed effects of AA on the fluorescence increase were due to effects on the O_2-evolving complex in PSII (Fisher and Kramer, 2014). One should thus bear in mind that 'AA has multiple effects on photosynthesis, and ascribing functional significance to any one process may be misleading' (Fisher and Kramer, 2014). Takahashi *et al.* (2013) raised the issue of acceptor-side limitation on the measurements of the P700 redox state under anaerobic conditions in *C. reinhardtii*. They found that the yield of PSI charge recombination increased when the electron acceptors of PSI had been reduced before light-induced oxidation of P700. To overcome this problem, they proposed a method with a prolonged incubation under anaerobic conditions prior to measurements. Kirchhoff *et al.* (2004) also reported that the change in P700 absorbance was affected by diffusion of Pc on the lumenal side of the thylakoid membranes, suggesting the possibility of errors when estimating CEF activity from the redox state of P700. Because of these problems, the ECS signal of carotenoids in the thylakoid membranes due to proton pumping into the lumen is becoming more popular as an indicator of CEF. Lucker and Kramer (2013) reported that absorbance changes at 520 nm (for ECS of carotenoid absorption) and at 705 nm (for P700 redox) were linearly correlated when PSII activity was inhibited.

CHLAMYDOMONAS REINHARDTII IN THE STUDY OF THE DYNAMIC ASPECTS OF PHOTOSYNTHESIS

The availability of a crystal structure of the PSII core complex at 1.9 Å (Umena *et al.*, 2011) demonstrates that the static world of the photosynthesis research is becoming crystal clear and exploring its dynamic world represents one of the next challenges for biologists. Photosynthesis is dynamically regulated in nature, as required by environmental and developmental cues. However, such phenomena are generally complex, and exploring them has inevitably been complicated, as discussed above. Due to the large variety of historical mutant banks and the less complicated cell biology and physiology of unicellular life, *C. reinhardtii* has become one of the most advantageous experimental systems for studying the molecular cell biology of photosynthesis. Such advantages have also been realized in the study of dynamic aspects of photosynthesis. Together with advances in techniques for purifying large membrane protein supercomplexes, the existence of powerful proteomic approaches aided by genomic information, and the availability of computer-aided electron microscopy, these complex research topics in the dynamic aspects of photosynthesis are now within our reach.

The idea of acclimation via supramolecular reorganization of protein complexes is not particularly new, nor is it unique in the field of photosynthesis. However, what occurs in thylakoids is a large-scale and dynamic reorganization of the supercomplexes. Because these phenomena are in most cases triggered simply by specific light cues, the events in their entirety are truly amazing. In this review, we have summarized the various approaches that have been utilized during the past 10 years. State transitions, excess energy dissipation and CEF have received particular attention from researchers, and numerous new findings have been reported. The green alga *C. reinhardtii* and the vascular plant *A. thaliana* have received almost exclusive attention in this area. The accumulated findings indicate several differences in the results from these two species, such as in the role of PGR5 in CEF, the identity of the qE effector, and the significance and regulation of state transitions. Whether the differences may be explained in evolutionary terms or are instead merely superficial differences and represent small parts within some unified mechanisms is likely to be clarified in the next decade.

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