Biochemical Characterization of the insertase Alb3.1 involved in the Assembly of Light-Harvesting Chlorophyll a/b complexes

集光性クロロフィル a/b 複合体の分子集合に関与するインセルターゼ Alb3.1 の生化学的解析

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Abbreviations

Chlamydomonas reinhardtii

Photosynthesis

Photosystem

Antenna

Light-harvesting complex

Chl a -Chlorophyll a

Chl b -Chlorophyll b

Chloroplasts

Thylakoid membranes

ATP: adenosine 5´-triphosphate

ADP: adenosine 5´-diphosphate

NADP: nicotinamide adenine dinucleotide phosphate

ALB3: Albino3.1, Albino3.2

Affinity purification

cpSRP: chloroplast signal recognition particle

Translocon

TAP-Tris Acetate phosphate

β-DM -n-dodecyl-β-maltoside

α-DM -n-dodecyl-α-maltoside

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General Introduction

Photosynthesis

Photosynthesis is the process that can utilize unicellular green alga, plants and other certain organisms convert solar energy or sunlight into chemical energy in biologically available form. This process make use of the reducing power derived from the splitting water molecule to drive the electron transport reactions in order to produce ATP and NADPH utilized for fixing the CO_2 in the form of carbohydrate.

The classical equation of photosynthesis is as follows:

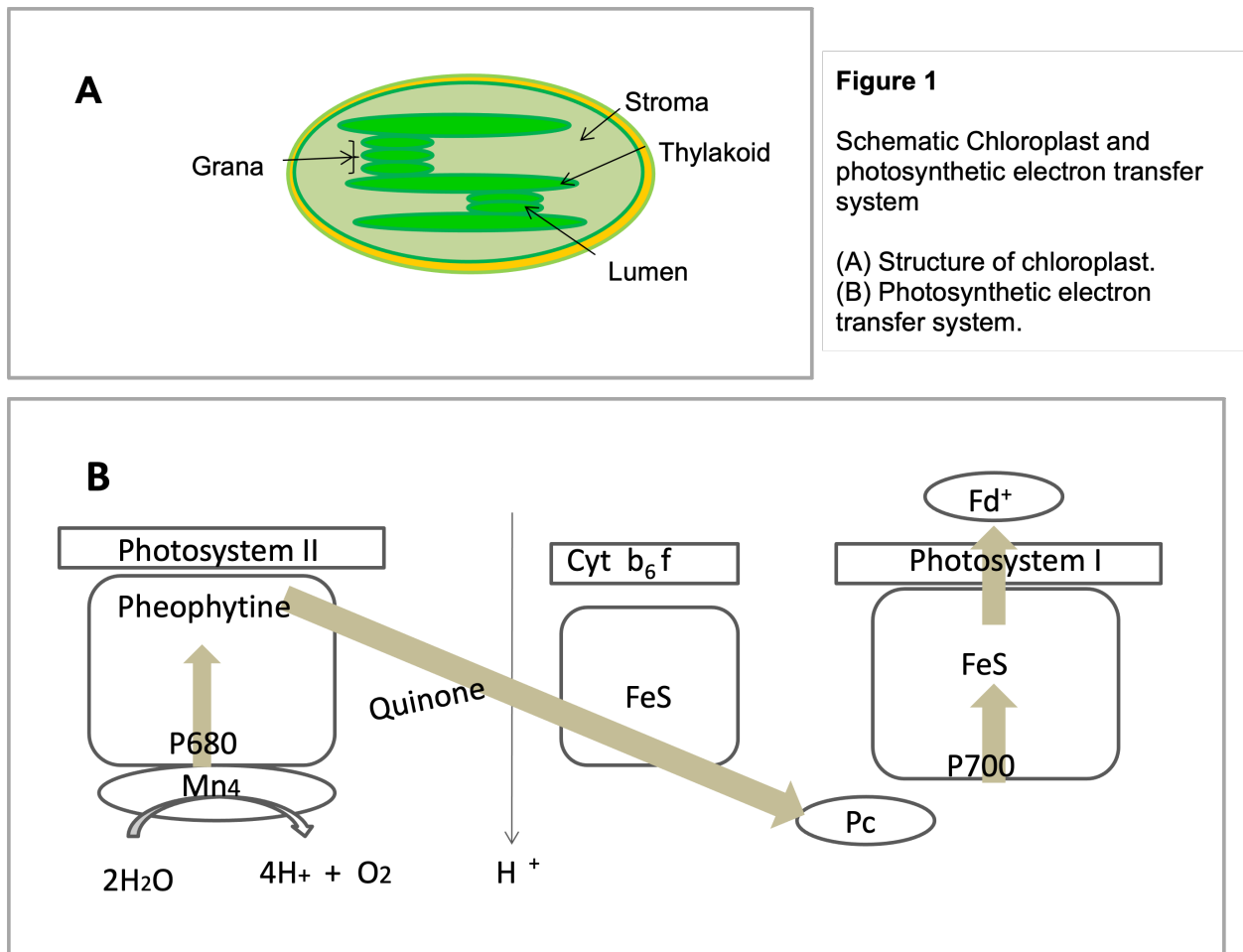$$6 \, CO_2 + 6 \, H_2O \xrightarrow{\;h\nu\;} C_6H12O_6 + 6 \, O_2$$
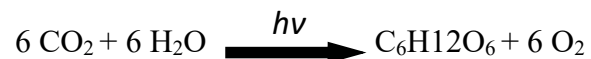
This work has paved the way for a better understanding of photosynthesis. To fix carbon, the process by green algae or plants and other organism convert solar technologies or sunlight into electrical energy which means the final product is Carbohydrate. This process can be classified into two types of reactions based on the utilization of light. Light reactions or a light-dependent reaction that takes place in thylakoid membranes and dark reactions or light-independent reactions that occurs in the four stromal matrix of chloroplast. Light reactions harness light energy to produce ATP and NADPH that is utilized by dark reactions to fix CO_2.

Chloroplast Structure

The structure of the chloroplast is shown in (Fig.1A). Chloroplasts are involves in the synthesis and storage of food. Large number of Chloroplast is present in an all green tissues. The internal structure of Chloroplast is highly organized within it there are closely packed thylakoid membranes. Chloroplasts are enclosed by two-layer envelope membranes, and inside the envelope membranes are stroma and thylakoid membranes. Chloroplast has two membranes which are an exterior membrane and an interior membrane. Exterior one forms the outer surface of it. Similarly, an interior membrane lies just a lower membrane. Outer membranes are freely permeable to molecules and inner membranes are selectively permeable for the molecules. In addition, there is a thin gap called Inter membrane which exists between the exterior and interior membranes. Jelly like matrix called as a Stroma surrounds thylakoids and grana. Stroma refers to the fluid filled inner space of chloroplasts bordering thylakoids and grana. It provides support for the pigmented thylakoids, however, stroma contains starch, chloroplast DNA and ribosomes, as well as all the enzymes required for light-independent reactions of photosynthesis. The thylakoids involve chlorophylls (a/b) and carotenoids, and these light harvest chlorophyll complexes are absorbed light by these pigments during photosynthesis. The assembling of light-absorbing pigments takes place with proteins which results in the formation of complexes. Photosystems has two type they are photosystem I (PSI) and photosystem II (PSII); also, these photosystems have roles in different parts of the light-dependent reactions.

The stroma contains its own DNA and ribosomes, and stroma enzymes produce complex organic molecules; they store energy in the form of carbohydrates. A brief electron transfer system of oxygenic photosynthesis is shown in (Fig.1B) Photosynthesis has two stages. Stage 1 called is light reaction, in the first stage, reactions that depend on the lights. Stage 2 called is light-independent and also called dark reaction. In the first stage, reactions form adenosine triphosphate (ATP) "the energy currency of the cell" and nicotinamide adenine dinucleotide phosphate (NADPH) and it happens by capturing sunlight through chlorophylls and carotenoids. The second stage called Calvin cycle (C3 cycle) and light-independent reactions three phases, reduction, carboxylation and ribulose 1,5-bisphosphate(RuBP). The chemical reactions that convert CO_2 and hydrogen (H_2) compounds into glucose molecules. In the Calvin cycle, the electrons which the NADPH carry, convert inorganic carbon dioxide to a carbohydrate organic molecule, for this process called as CO_2 fixation.

Figure 1

Figure 1

Schematic Chloroplast and photosynthetic electron transfer system

(A) Structure of chloroplast.
(B) Photosynthetic electron transfer system.

Chlamydomonas reinhardtii

The genus Chlamydomonas is the name given to the unicellular flagellates named by C.G.Ehrenberg (1833, 1838). The C. reinhardtii species was described by Danreard in 1888 and named after the Ukrainian botanist Ludwig Reinhard who isolated this species in 1876. By 1960 it became a widely used strain in laboratory work. In the year 1945 this species was redefined based on the wild type strain isolated in Massachusetts. The redefined features are two anterior flagella with equal length, flagella points of emergence from the cell body that are not widely separated; contains a cell wall; and a single chloroplast or chromatophore having one or more pyrenoids (Fig.2A) and a cell observed by microscope is shown in (Fig.2B). C. reinhartii has model organism for the study of photosynthesis for the following reasons. Firstly, C. reinhartii can grow heterotrophycally in medium that contains acetic acid as a carbon source (TAP). This property provides a window for the isolation and maintenance of the mutants that are deficient in photosynthesis. Unlike higher plants, in C. reinhardtii, Chlorophyll biosynthesis can occur in dark condition. This helps in the study of light sensitive mutants and their photosynthetic apparatus assembly. Secondly, the C. reinhardtii genetic level establishment is strong in nuclear, chloroplast and mitochondrial genome which means manupulation of chloroplast, mitchondrial and nuclear genomes are feasible. Efficient transformation protocols for the nuclear and chloroplast compartments have been developed (Kindle, 1990) which allows one to isolate nuclear genes by genomic rescue of nuclear photosynthetic mutants with appropriate cosmid libraries. Thirdly, this organism chloroplast transformation occurs through homologous recombination, hence disruption or site-directed mutagenesis of plastid genes is possible.
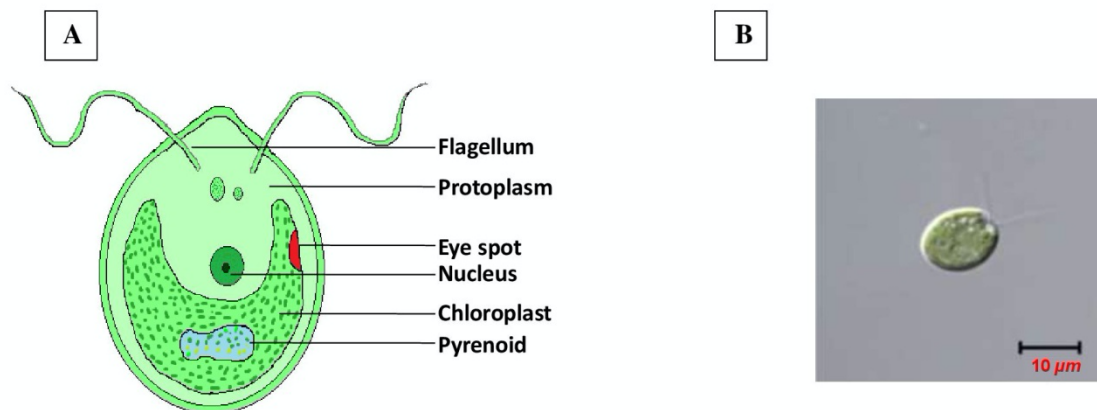
Figure 2

Figure 2 Chlamydomonas reinhardtii

A. Central architecture of *Chlamydomonas reinhardtii* is shown schematically
B. Microscopic image of a *Chlamydomonas reinhardtii* cell at 1000-folds magnification. Scale bar represents 10 µm (from Ozawa)

Powerful spectroscopic methods have been developed to study photosynthetic electron flow in this organism. With the combination of biochemistry, biophysics and genetic approaches, it is possible to study many aspects of photosynthesis. Finally, the 130 000 ESTs produced in Chlamydomonas genomic project have greatly helped in the identification of genes and gene families in Chlamydomonas (Rochaix, 2002).

Antenna systems

The energy density of sunlight is relatively low for efficient photochemical reactions. To utilize the low-density light energy efficiently, light-harvesting systems have been developed in photosynthetic organisms. One of the systems, light-harvesting chlorophyll complex (LHC) contains chlorophylls and carotenoids that are specialized to harvest photons and transmit excitation energy to photosystems. - 6 - Carotenoids in LHC also quench or dissipate excess energies as heats by xanthophyll cycle. LHCI stably binds to PSI and forms PSI-LHCI supercomplex whereas LHCII weakly associates with PSII to form PSII-LHCII supercomplex. Four distinct LHCIs bind PSI core in higher plants (Klimmek et al., 2005; Amunts et al., 2007), but under certain conditions two additional LHCIs may bind to PSI (Ganeteg et al., 2004; Lucinski et al., 2006). In contrast to higher plants, C. reinhardtii contains nine distinct LHCI subunits and all of which bind to PSI core complex (Stauber et al., 2003; Takahashi et al., 2004; Tokutsu et al., 2004).
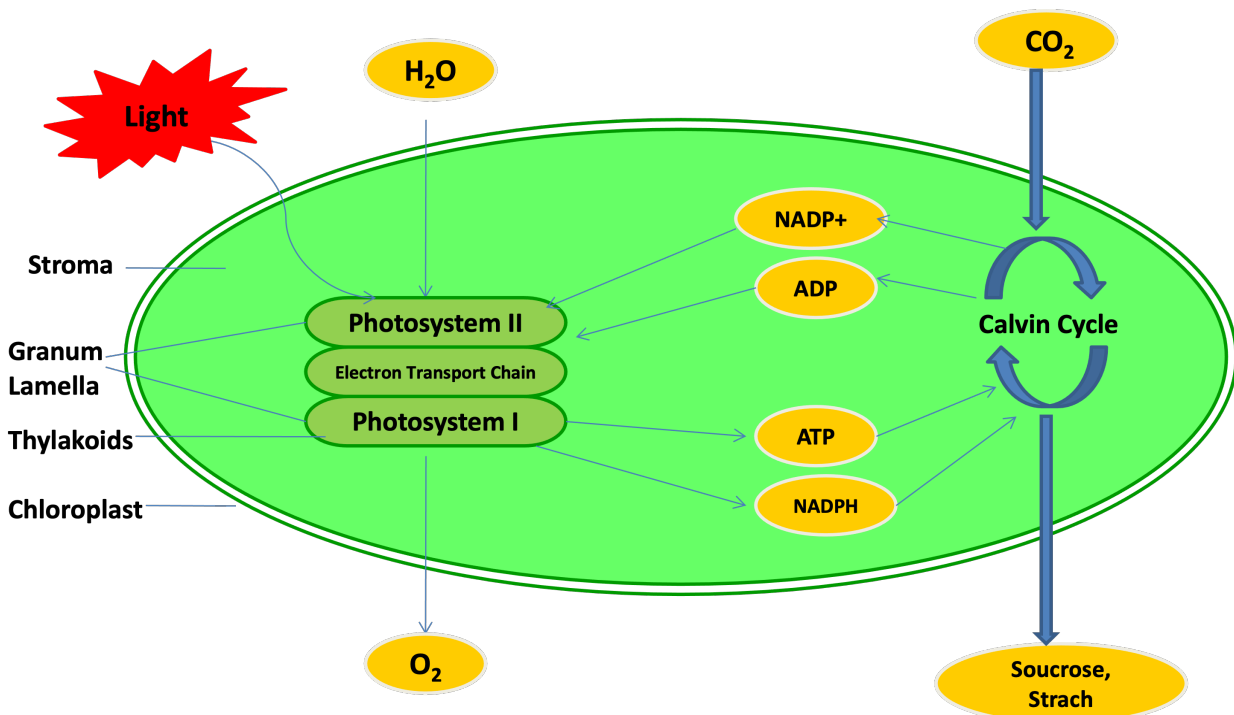
Figure 3

Figure 3 Schemes of noncyclic and cyclic pathways of electron transport in chloroplasts

Photosystem II

Photosystem II is a multiprotein membrane complex that oxidizes water molecule to generate reducing equivalents to run photosynthesis. This complex is made up of not less than 30 proteins Within the photosystem, enzymes capture photons of light to energize electrons that are then transferred through a variety of coenzymes and cofactors to reduce plastoquinone to plastoquinol. PS II uses light energy replaced by oxidizing water to form hydrogen ions and molecular oxygen.(Umena et al., 2011).

Light harvesting complexes II associated with PSII

Chlamydomonas genome consists of twelve nuclear coded LHCB genes that encodes polypeptides associated with PSII. They can be classified into major LHCII: LHCBM1-9 (M Major) and minor LHCs LHCB4, LHCB5 and LHCB7. Based on phylogenetic relationships major LHC is subdivided into four groups (Minagawa and Takahashi, 2004).

Type I: LHCBM3, LHCBM4, LHCBM6, LHCBM8, and LHCBM9

Type II: LHCBM5

Type III: LHCBM, LHCBM2

Type IV: LHCBM1

LHCII monomer is made of three transmembrane helices with N-terminus exposed to stromal side and luminally exposed C-terminus. It binds to 8 Chlorophyll a, 6 Chlorophyll b, 2 leutins and 19`cisneoxanthin (Liu et al., 2004). PS II is surrounded by three layers of light harvesting complexes: 1) Inner layer close to reaction center called as core antenna CP43 and CP 47 containing Chlorophyll a. 2) Between core antenna and major LHC the less abundant minor monomeric antenna Chl a and Chl b containing CP26 and CP29 in C. reinhardtii and there is additional CP24 in higher plants (Yakushevska et al., 2003). 3) Outer peripheral more abundant major LHC trimetric antenna. All the major LHCII proteins have a conserved WYGPDR domain that is responsible for trimerization. CP26 in (Minagawa et al., 2013). T-strain that lacks one of the major LHCII polypeptides has been found to undergo trimerization. This is attributed to the presence of WYGPDR domain in CP26. Therefore WYGPDR is important for trimerization (Minagawa and Takahashi, 2004). LHCII trimers of C. reinhardtii are less stable compared to that of plants which dissociate into monomers upon treatment with detergent. Trimers containing LhcbM5 are less stable. CP26 may form homo/heterotrimers in C. reinhardtii and is associated with LHCII trimer. 10 LhcbM2/7, LhcbM1, LhcbM3 are the most abundant proteins in C. reinhardtii. Trimetric LHCII bind 4 carotenoids per 14 Chls. The ratio of Chl a/b of monomers is 1.29, while that of trimers is Chl a/b. The structure of minor monomeric LHCs and LHCI has not been elucidated but they are thought to have a topology similar to LHCII. But there is a difference in pigment composition, CP26 binds to 6 Chl a and 9 Chl b (Croce et al., 2002a), CP29 binds with 6 Chl a and 2 Chl b (Sandona et al., 1998). LHCI polypeptides binds with 8 Chl a and 2 Chl b (Bassi et al., 1992).

Supramolecular organization of PSII

Because of its structural complexity and fragility, it is not possible to study the structure of PSII-LHCII complexes in X-ray crystallography. With less resolution it is, however, possible to deduce the structure of these complexes by single particle analysis (Tokutsu et al., 2012). With recent 11 evidences of atomic structures of PSII dimer from cyanobacteria (Guskov et al., 2009, Umena et al., 2011) spinach and pea, LHCII trimers (Liu et al., 2004, Standfuss et al., 2005) and monomers (Pan et al., 2011) are available that can be useful to predict the structure by single particle analysis. PSII core (C) as dimer is flanked by LHCII trimers on both side that are bordered by monomeric minor antenna. LHCII trimers are classified into three types based on their binding strength to the PSII dimer: Strongly bound (S- Trimer), moderately bound (M-Trimer) and loosely bound (L-Trimer).

Earlier studies in spinach reported presence of C2S2M1L1, C2S2M2L1, C2S2L1, or C2S2L2 supercomplexes. Whereas in A. thaliana, C2S2M2 is the largest but no L trimer in this PSII- 12 LHCII supercomplex observed (Caffarri et al., 2009). In C. reinhartii, the largest PSII-LHCII complex is isolated having C2S2M2L2 (Umena et al., 2011). It was proposed that due to absence in CP24, this L-LHC II trimer directly bound to the PSII core that could directly deliver the excitation energy to PSII core (Tokutsu et al., 2012).
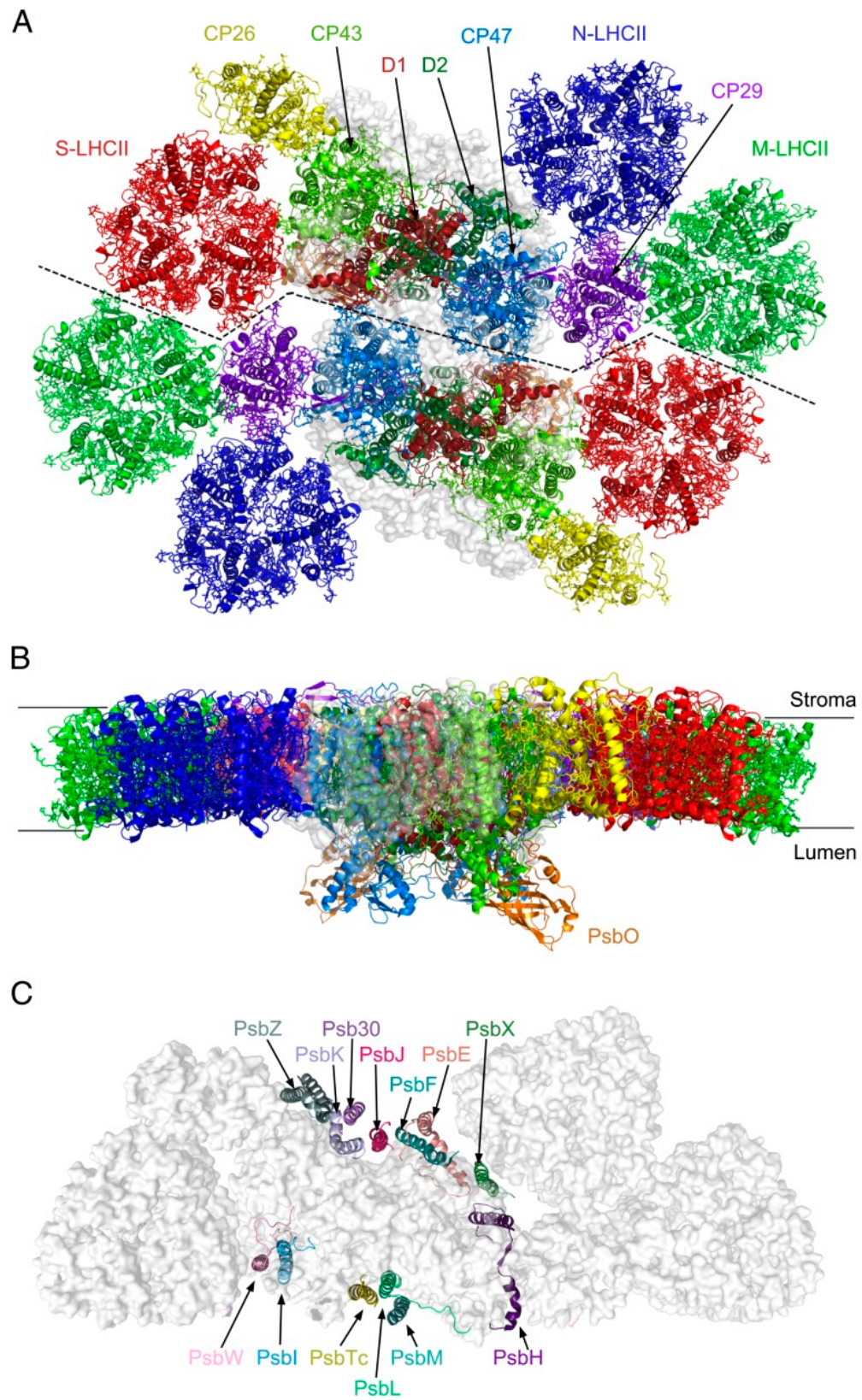
Figure 4

Figure 4 Overall architecture of the C2S2M2N2-type PSII–LHCII supercomplex from C. reinhardtii. (A) Cartoon representation of the overall structure with a top view from the stromal side. Four core subunits (D1, D2, CP43, and CP47), 1 extrinsic subunit PsbO, and the peripheral antennae are colored differently, whereas the 13 small intrinsic subunits are shown as a gray transparent surface. (B) Side view along the membrane plane. (C) The arrangement of 13 small membrane-intrinsic proteins in each monomer of the C2S2M2N2-type PSII–LHCII supercomplex viewed from the stromal side. (Shen, L., et al. PNAS 2019)

Photosystem I

Photosystem I is an integral membrane protein complex that uses light energy to catalyze the transfer of electrons across the thylakoid membrane and using light energy by the membrane energy barrier.

Light Harvesting Complexes I associated with PSI

LHCs are meant to monitor the time scales of energy transfer reactions in photosynthesis. They harvest the energy from light source. RCs could have been evolved without the presence of LHCs, but they have to become integral part of photosystems. Without LHCs, the RCs have to 15 wait for a relatively longer time between successive hits from incoming photons. This is because the redox reactions at RCs occur at a faster rate compared to that of successive photon hits. Moreover, the redox reactions at RCs require more than 1 electron. The time delay between successive hits pave way for back reactions, thereby decreasing quantum efficiency. The presence of LHCs clear all these hurdles by increasing cross sectional area for photon capturing and hence continuously supply photons to RC (Cogdell et al., 2008).

(Table 2) Genes encoding LHCI proteins in C. reinhardtii

Gene	Molecular weight(Da)	Protein	Accession number
LHCA1	23900.28	p22/p22.1	BAD06923
LHCA2	26922.26	p19	EDP05477
LHCA3	28886.33	p14.1	BAD06919
LHCA4	28701.92	p14	BAD06918
LHCA5	28228.62	p15.1	BAD06920
LHCA6	27781.42	p18.1	BAD06922
LHCA7	26222.88	p15	BAD06924
LHCA8	25920.87	p18	BAD06921
LHCA9	22844.34	P22.2	EDP04026

Photosystem I binds to the antenna known as LHC I. In green plants they are of four types Lhca11 to Lhcal4 (Jansson, 1999). Lhca1 and Lhca4 forms heterodimer which is called

LHCI700 and Lhca2 and Lhca3 forms another dimer called LHC680 (Ganeteg et al., 2001, Croce et al., 2002b). These LHCs binds to Chls and xanthophylls. Lhca1, Lhca2 and Lhca4 binds to 12 Chls and Lhca3 binds to 11 Chls. Additional pigments called gap Chls are present in LHCI binding interface. Green algae C. reinhardtii genome has nine types of LHCI polypeptides peptides. LHCA1-9 forms two crescent rings on one side of PSI core. Thus they are bound to core as two layers towards PsaJ PsaF and PsaG components of PSI. Whereas during state transition PSI binds to LHCII trimers and CP29 at PsaI Psa H and Psa L, the opposite side of the LHCI binding site.

PSI-LHCI complex

The crystal structure of PSI-LHCI supercomplex solved from Pisum sativum reveals that one side of PSI is occupied by LHCI, while the other is left unoccupied. The possible explanation for this one-sided vacancy is that it provides room for shuttling LHCII that migrate to PSI during state transitions. The recent structure reveals the detailed arrangement of pigments and other cofactors especially within LHCI as well as various interactions between the PSI core and LHCI (Qin et al., 2015, Mazor et al., 2017). PSI of C. reinhardtii contains nine LHCI proteins arranged in the form of double half-ring shape as revealed by single particle analysis. PSI-LHCI of C. reinhardtii is less stable compared to that of higher plants. The outer belt of LHCI consists of Lhca2, Lhca4, Lhca5, Lhca6, and Lhca9 while the inner belt has Lhca1, Lhca3, Lhca7, and Lhca8. Lhca2 occupies the site near Lhca1 in the crystal structure of higher plants as revealed by the superposition of EM images and the crystal structure. PsaL, PsaK, Lhca2 and Lhca9 are loosely bound to the supercomplex (Drop et al., 2011) as suggested by their loss from supercomplex even at low concentrations of mild detergent. PsaF, PsaG and PsaK participate in stabilizing the PSI-LHCI complex. PsaH is oriented away from Lhcas in the PSILHCI supercomplex (Minagawa et al., 2013). And Lhca(s) associated with PSI are classified into 3 subclasses depending on their fluorescence emission maxima. The PSI-LHCI supercomplex is heterogeneous in C. reinhardtii due to the differences in stoichiometry of PSI core and LHCI. Lhca2, Lhca5, Lhca6, Lhca8, Lhca9 are in sub stoichiometric ratio with the PSI core. Lhca1, Lhca4, Lhca7 in 1:1 ratio with the PSI core. Lhca3 in 2:1 ratio with the PSI core. Altogether PSI core is associated with 14 Lhca subunits, where most of Lhca are situated at PsaF/PsaJ site and 2-3 Lhca are situated at PsaH (Drop et al., 2011).
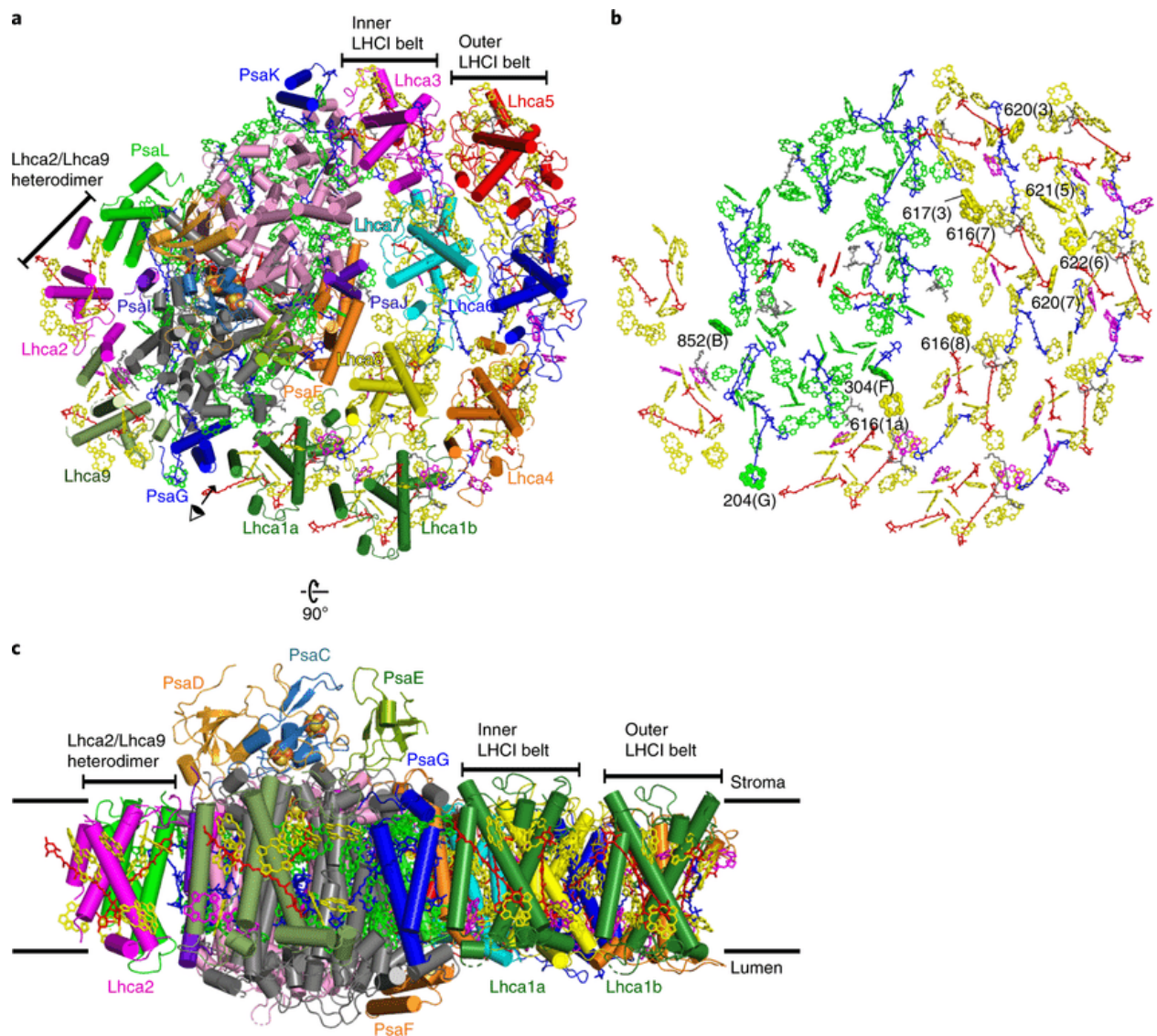
Figure 5

Figure 5 Cryo-EM structure of the PSI–LHCI supercomplex from C. reinhardtii a, Overall structure viewed along the membrane normal (a direction vertical to the membrane plane) from the stromal side. b, Arrangement of pigments and other co-factors, with the same viewing direction as in a. Chlorophylls arranged in thick sticks are largely changed in structure or newly found in comparison with the plant PSI–LHCI (PDB ID: 5ZJI). c, Side view of the PSI–LHCI supercomplex from the PsaG aspect (viewing direction indicated by arrow in a). Colour coding for co-factors: green, Chls a of the PSI core; yellow, Chls a of LHCI; magenta, Chls b; red, P700, naphthoquinones and luteins; blue, β-carotenes; grey, lipids. The colour coding shown here is used for all Figures unless otherwise noted (*Suga, M. et al Nature Plants 2019*).

The BF4 mutant

A pale green mutant BF4 of the unicellular green alga Chlamydomonas reinhardtii is defective in the insertase Alb3.1, which showed a marked decrease in chlorophyll content for cell and high chl a/b ratio due to their deficiency in peripheral antenna proteins. Most of the mutants fall in either of two categories, peripheral antenna mutants or chlorophyll biosynthesis mutants. Mutants of chlorophyll biosynthesis may also be photosensitive because of the accumulation of chlorophyll biosynthesis intermediates. This mutant is deficiencies in most of the peripheral antenna proteins associated with either photosystem I or photosystem II. The BF4 mutant rather belonged to the first category since they were not photosensitive and their pale green phenotype was observed in darkness as well as under dim or moderate light for growth, independent of the mixotrophic or photoautotrophic light growth condition (Sandrine Bujaldon et al., 2020).

Transport into the thylakoid membrane

Light-harvesting chlorophyll-binding proteins (LHCPs) are integral proteins with three transmembrane helices (TM1-3) and are the most abundant proteins in the thylakoid membranes. LHCPs are translated in the cytosol as precursor proteins with an N-terminal chloroplast targeting peptide (TP) and are translocated to and integrated into the thylakoid membranes (Dall'Osto et al. 2015; Richter et al. 2010; Ziehe et al. 2017, 2018). The TP is used to direct the precursor proteins into the chloroplast by translocating across the envelope membranes via TOC/TIC translocon. Upon translocation, the TP is cleaved off by a stromal processing peptidase and the resulting mature proteins form a transit complex with the chloroplast signal recognition particle (cpSRP). The translocation of LHCPs from the TIC/TOC translocon to cpSRP is mediated by the LTD (LHCP translocation defect) protein (Jeong et al. 2018; Ouyang et al. 2011). The formation of the cpSRP-LHCP complex allows the hydrophobic LHCPs to remain soluble in the stroma and prevents their aggregation. The cpSRP-LHCP complexes are directed to the thylakoid membranes via the cpSRP receptor cpFtsY peripherally bound to the thylakoid membranes. Both cpFtsY and cpSRP54 contains a GTP binding and hydrolysis domain (Ziehe et al. 2018). Finally, the LHCPs are inserted into the thylakoid membranes by Albino 3 (Alb3) (Sundberg et al. 1997). Alb3 is a chloroplast homolog of the mitochondrial Oxa1p and *Escherichia coli* YidC, which are essential for integrating membrane proteins in membranes. Alb3 has five putative transmembrane helices and is present in the thylakoid membranes. The green algae also contain LTD, cpSRP43, cpSRP54, CpFtsY, and Alb3 (Ziehe et al. 2017). *C. reinhardtii* contains two Alb3 proteins, Alb3.1 and Alb3.2. The Alb3.1 is required for the accumulation of LHCPs (Bellafiore et al. 2002; Bujaldon et al. 2020), whereas the Alb3.2 appears to have a wider role than Alb3.1 and is also required for the accumulation of PSI and PSII complexes and is essential for cell survival (Gohre et al. 2006). Alb3.1 and Alb3.2 are located in the thylakoid membranes and are associated with a large complex. In addition, co-immunoprecipitation experiments suggested that these two proteins interact directly or indirectly with each other (Gohre et al. 2006).

In a previous study, Alb3.1 is essential for proper LHCP assembly in the thylakoid membranes (Bujaldon et al. 2020). In the present study, complemented BF4 with vectors that express Alb3.1, Alb3.1-HA (Alb3.1 fused with a single HA tag at the C-terminus), or Alb3.1-3HA (Alb3.1 fused with a triple HA tag at the C-terminus) and confirmed that these complemented strains accumulate LHCPs at nearly wild type levels. The purification and characterization of HA-tag fused Alb3.1 reveal an intimate interaction of cpSRP43, cpSRP54, and many LHCI and LHCII proteins with Alb3.1. From our data suggests that the Alb3.1-cpSRP-LHCP complexes transiently associate an assembly intermediate of PSI-LHCI supercomplex.

Figure 6

"A Hypothesis model" Overview of LHCP transport into the thylakoid membrane
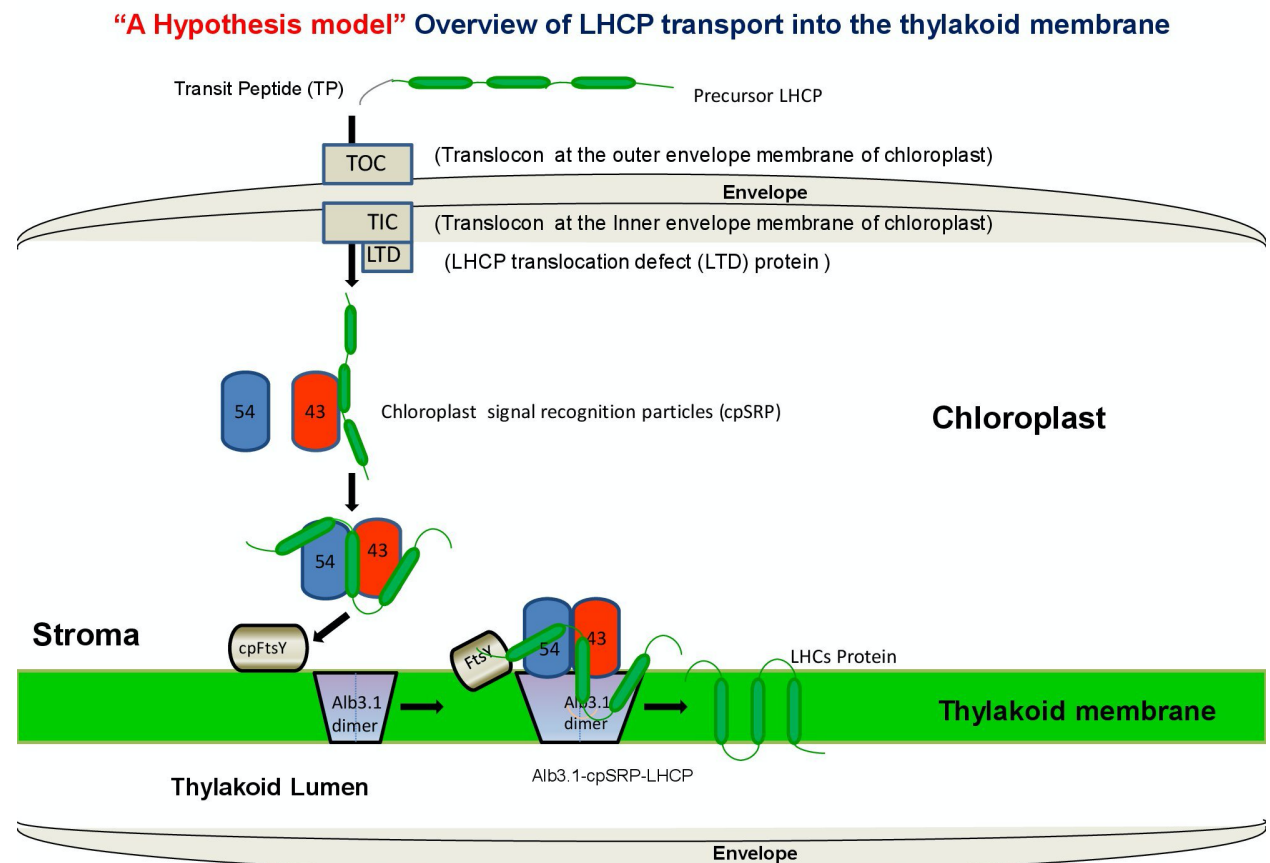
This model modified from *Dominik Ziehe, 2018* and *Beatrix Dunschede, 2011*

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Assembly apparatus of light-harvesting complexes; Identification of Alb3.1-cpSRP-LHCP complexes in the green alga *Chlamydomonas reinhardtii*

ABSTRACT

The unicellular green alga, *Chlamydomonas reinhardtii*, contains many light-harvesting complexes (LHCs) associating chlorophylls *a*/*b* and carotenoids; the major light-harvesting complexes, LHCIIs (types I, II, III, and IV), and minor light-harvesting complexes, CP26 and CP29, for photosystem II, as well as nine light-harvesting complexes, LHCIs (LHCA1-9), for photosystem I. A pale green mutant BF4 exhibited impaired accumulation of LHCs due to deficiency in *Alb3.1* gene which encodes the insertase involved in insertion, folding and assembly of LHC proteins in the thylakoid membranes. To elucidate the molecular mechanism by which ALB3.1 assists LHC assembly, we complemented BF4 to express ALB3.1 fused with no, single, or triple Human influenza hemagglutinin (HA) tag at its C-terminus (cAlb3.1, cAlb3.1-HA, or cAlb3.1-3HA). The resulting complemented strains accumulated most LHC proteins comparable to wild-type levels. The affinity purification of Alb3.1-HA and Alb3.1-3HA preparations showed that ALB3.1 interacts with cpSRP43 and cpSRP54 proteins of chloroplast signal recognition particle cpSRP and several LHC proteins; two major LHCII proteins (types I and III), two minor LHCII proteins (CP26 and CP29), and eight LHCI proteins (LHCA1, 2, 3, 4, 5, 6, 8, and 9). Pulse-chase labeling experiments revealed that the newly synthesized major LHCII proteins were transiently bound to the Alb3.1 complex. We propose that Alb3.1 interacts with cpSRP43 and cpSRP54 to form an assembly apparatus for most LHCs in the thylakoid membranes. Interestingly, PSI proteins were also detected in the Alb3.1 preparations, suggesting that the integration of LHCIs to a PSI core complex to form a PSI-LHCI subcomplex occurs before assembled LHCIs dissociate from the Alb3.1-cpSRP complex.

INTRODUCTION

Capture of light energy by photosynthetic pigments is the initial step of photosynthesis reactions. In plants and green algae, there are two types of light-harvesting apparatus: core and peripheral antenna complexes. The core antenna complexes associate with photosystem I (PSI) and photosystem II (PSII) core complexes that are mainly composed of intrinsic proteins encoded by chloroplast genes. The peripheral antenna complexes consist of light-harvesting chlorophyll-binding proteins (LHCPs), which bind chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid molecules. The LHCPs are encoded by two multigenic gene families in the nucleus and form light-harvesting complexes (LHCs) in the thylakoid membranes. The *lhca* genes encode LHCI proteins which associate with PSI core complex to form PSI-LHCI supercomplex(Ben-Shem et al. 2003; Ozawa et al. 2018), whereas the *lhcb* genes code for LHCII proteins which mainly bind to PSII core complex to form PSII-LHCII supercomplex (Sheng et al. 2021). Excitation energy trapped by LHCs is transferred to the core antenna complexes and is finally used for photochemical reactions in PSI and PSII reaction centers (RCs).

LHCPs are integral proteins with three transmembrane helices (TM1-3) and are the most abundant proteins in the thylakoid membranes. LHCPs are translated in the cytosol as precursor proteins with an N-terminal chloroplast targeting peptide (TP) and are translocated to and integrated into the thylakoid membranes (Dall'Osto et al. 2015; Richter et al. 2010; Ziehe et al. 2017, 2018). The TP is used to direct the precursor proteins into the chloroplast by translocating across the envelope membranes via TOC/TIC translocon. Upon translocation, the TP is cleaved off by a stromal processing peptidase and the resulting mature proteins form a transit complex with the chloroplast signal recognition particle (cpSRP). The translocation of LHCPs from the TIC/TOC translocon to cpSRP is mediated by the LTD (LHCP translocation defect) protein (Jeong et al. 2018; Ouyang et al. 2011). The formation of the cpSRP-LHCP complex allows the hydrophobic LHCPs to remain soluble in the stroma and prevents their aggregation.

In vascular plants, cpSRP consists of a heterodimer of cpSRP43 and cpSRP54. The cpSRP54 is an ortholog of the cytosolic and prokaryotic SRP54 (Dunschede et al. 2015). Although SRP54 contains a conserved RNA, cpSRP54 lacks the RNA and instead recruited cpSRP43 (Ziehe et al. 2017). In vitro assay using synthetic peptides suggested that cpSRP43 binds LHCPs through the L18 region located between the two transmembrane helices, TM2 and TM3, of LHCPs, whereas no significant interaction between cpSRP54 and LHCPs is observed (DeLille et al. 2000; Stengel et al. 2008; Tu et al. 2000). In Arabidopsis, cpSRP43 is sufficient for forming a soluble cpSRP43-LHCP complex in the stroma (Dunschede et al. 2015; Tu et al. 2000), while the integration of LHCPs into the thylakoid membranes requires cpSRP54, cpFtsY, Alb3, and GTP.

The cpSRP-LHCP complexes are directed to the thylakoid membranes via the cpSRP receptor cpFtsY peripherally bound to the thylakoid membranes. Both cpFtsY and cpSRP54 contains a GTP binding and hydrolysis domain (Ziehe et al. 2018). Finally, the LHCPs are inserted into the thylakoid membranes by Albino 3 (Alb3) (Sundberg et al. 1997). Alb3 is a chloroplast homolog of the mitochondrial Oxa1p and *Escherichia coli* YidC, which are essential

for integrating membrane proteins in membranes. Alb3 has five putative transmembrane helices and is present in the thylakoid membranes.

The green algae also contain LTD, cpSRP43, cpSRP54, CpFtsY, and Alb3 (Ziehe et al. 2017). The green alga *Chlamydomonas reinhardtii* is an excellent model organism for studying the assembly of the LHCs because all the lhc genes and LHCPs have been identified and a variety of LHC-deficient mutants is available. In particular, mutants deficient in LTD (Jeong et al. 2018), cpSRP43 (Bujaldon et al. 2020; Kirst et al. 2012b), cpSRP54 (Jeong et al. 2017), and CpFtsY (Kirst et al. 2012a) have been isolated and characterized. These mutants except for cpSRP54 mutant are pale green and contain significantly reduced levels of LHCPs. The cpSRP54 mutant shows a milder pale green phenotype and accumulates more LHCPs than the other mutants, suggesting that the cpSRP54 is less important for the assembly of LHCs (Jeong et al. 2017).*C. reinhardtii* contains two Alb3 proteins, Alb3.1 and Alb3.2. The Alb3.1 is required for the accumulation of LHCPs (Bellafiore et al. 2002; Bujaldon et al. 2020), whereas the Alb3.2 appears to have a wider role than Alb3.1 and is also required for the accumulation of PSI and PSII complexes and is essential for cell survival (Gohre et al. 2006). Alb3.1 and Alb3.2 are located in the thylakoid membranes and are associated with a large complex. In addition, co-immunoprecipitation experiments suggested that these two proteins interact directly or indirectly with each other (Gohre et al. 2006).

In a previous study, we reported that the pale green mutant BF4 is defective in *ALB3.1* gene and accumulates the LHCPs at <25% of wild type levels and concluded that Alb3.1 is essential for proper LHCP assembly in the thylakoid membranes (Bujaldon et al. 2020). In the present study, we complemented BF4 with vectors that express Alb3.1, Alb3.1-HA (Alb3.1 fused with a single HA tag at the C-terminus), or Alb3.1-3HA (Alb3.1 fused with a triple HA tag at the C-terminus) and confirmed that these complemented strains accumulate LHCPs at nearly wild type levels. To further elucidate how Alb3.1 assists the assembly of LHCs in the thylakoid membranes, we purified Alb3.1-HA and Alb3.1-3HA from the thylakoid membrane extracts by affinity spin column to identify interacting proteins. The purification and characterization of HA-tag fused Alb3.1 reveal an intimate interaction of cpSRP43, cpSRP54, and many LHCI and LHCII proteins with Alb3.1. We conclude that LHCs are assembled in the thylakoid membranes by formation of a transient assembly apparatus of Alb3.1-cpSRP-LHCP complexes. We also suggest that the Alb3.1-cpSRP-LHCP complexes transiently associate an assembly intermediate of PSI-LHCI supercomplex.

RESULTS

Complementation of BF4 with Alb3.1 cDNA

The pale green BF4 mutant cells grow photoautotrophically but accumulate light-harvesting proteins (LHCPs) at about 20% of wild type level (Bujaldon et al. 2020). This mutant is defective in the insertase Alb3.1. To address the molecular mechanism by which light-harvesting complexes (LHCs) are assembled in the thylakoid membranes, we complemented BF4 mutant with three different complementation vectors to express Alb3.1, Alb3.1-HA, or Alb3.1-3HA from the *ALB3.1* cDNA driven by *PSAD* promoter/terminator (Figure 1A). After introduction of one of these vectors into BF4 cells by electroporation as described in Materials and Methods, putative transformants were selected on agar plates containing paromomycin as shown in Supplementary Figure. When transformed with the vector expressing Alb3.1-3HA, the color of the cells on agar plates is variable as shown in Supplementary Figure; some are as green as wild type, some are pale green like BF4, and the others are intermediates. The clones as green as wild type were selected for further analysis. The total cellular DNA was isolated and used as a template to amplify the insertion of the transforming vector and the resulting DNA fragments were analyzed by agarose gel electrophoresis (Figure 1B). No band is detected in wild type and BF4 as expected, whereas DNA fragments of 135 bp, 162 bp, and 216 bp are amplified in the three complemented strains, cAlb3.1, cAlb3.1-HA, and cAlb3.1-3HA, respectively, indicating that the introduction of the complementation vectors to the nuclear genome is successful. Since antibody against Alb3.1 is not available, Alb3.1-HA and Alb3.1-3HA were detected using anti-HA antibody. A weak signal of Alb3.1-HA is detected around 50 kDa in Alb3.1-HA cells, while a strong signal of a slightly larger size is detected in Alb3.1-3HA cells (Figure 1C). The strong signal of Alb3.1-3HA may be ascribed to the fact that the anti-HA antibody reacted more intensively with the triple HA tag than with the single HA tag of Alb3.1-HA. Although BF4 cells accumulate LHCI and LHCII proteins at significantly reduced levels as reported previously (Bujaldon et al. 2020), the three complemented strains have restored the accumulation of LHCI and LHCII proteins; LHCI proteins accumulate to substantial levels but to slightly decreased levels compared with those in wild type cells, while LHCII proteins to nearly wild type levels (Figure 1C). The similar amount of LHCI and LHCII proteins in the three complemented strains indicates that the fusion of the single or triple HA tag at C-terminus of Alb3.1 does not significantly affect its ability to contribute to the membrane insertion and accumulation of LHC proteins.

Alb3.1 associates with cpSRP43 and cpSRP54

To identify proteins interacting with Alb3.1, HA-tagged Alb3.1 was used as bait, and proteins interacting with Alb3.1 were isolated by affinity spin columns. Since Alb3.1 is localized in the thylakoid membranes, we isolated the thylakoid membranes from cAlb3.1-HA and cAlb3.1-3HA strains, solubilized them with n-dodecyl-α-D-maltoside (α-DDM), and applied the resulting extracts to affinity spin columns. As a negative control, the thylakoid extracts from

wild-type cells were used. The purified preparations were incubated with SDS and dithiothreitol, and the solubilized polypeptides were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver (Figure 2A). The preparation from wild type (WT) as a negative control shows several polypeptides in high molecular region (>60 kDa), which are non-specific contaminations. The strong band below 66 kDa marker protein was identified as AtpA (the α-subunit of chloroplast ATP synthase) by mass spectrometry analysis, the intensity of which differs from preparation to preparation. A more complex and quite similar polypeptide pattern was observed in the Alb3.1-HA and Alb3.1-3HA preparations. The most significant difference between the two Alb3.1preparations was found in the 45-66 kDa region (marked with asterisks in Figure 2A). To further characterize the Alb3.1 preparations, we performed large-scale purifications and separated their polypeptides by two different SDS-PAGE systems, a Tris-HCl resolving gel buffer system (Laemmli 1970) (Figures 2B) and a urea-MES-Tris resolving gel buffer system (Kashino et al. 2001) which better separates LHCI polypeptides from LHCII polypeptides (Figures 2C). The polypeptides of these preparations were also compared with the polypeptides of LHCII and PSI-LHCI preparations from wild type cells, shown on the left side of Figure 2A. The comparison of the polypeptides from LHCII and PSI-LHCI complexes with those from the Alb3.1 preparations suggests that the Alb3.1 preparations contain several LHCI and LHCII polypeptides.

Next, the separated polypeptides (bands 1-19) were digested with trypsin and were subjected to LC-MS/MS for sequence identification of their peptide profile. Band2/3 in the Alb3.1-HA preparation and the band 2 in the Alb3.1-3HA preparation in Figure 2B, which correspond to the bands marked by asterisk in Figure 2A, contain Alb3.1-HA and Alb3.1-3HA, respectively, as well as AtpA (Table 1). The relative migration of the two Alb3.1 bands well corresponds to signals detected with anti-HA antibody as shown in Figure 1C. The difference in size is ascribed to the number of HA tag fused at the C-terminus of Alb3.1. Band 3 of the Alb3.1-3HA preparation and band 2/3 of the Alb3.1-HA preparation contain cpSRP54 (Figure 2B and Table 1). Band 4 detected in both Alb3.1-HA and an Alb3.1-3HA preparation is assigned cpSRP43. To confirm that cpSRP54 and cpSRP43 are exclusively present in Alb3.1 preparations, we analyzed the presence of Alb3.1, cpSRP54, and cpSRP43 in the negative control lane. Six gel slices from the region between 45 kDa and 66 kDa marker proteins were cut and analyzed with an LC-MS/MS. This region of the gel may possibly contain Alb3.1, cpSRP54, and cpSRP43, but no peptides of Alb3.1, cpSRP54, and cpSRP43 were detected except for peptides of AtpA and AtpB which were also detected in the Alb3.1 preparations (see Table 1). Thus, cpSRP54 and cpSRP43 are exclusively associated with Alb3.1. CpSRP54 and cpSRP43 form the cpSRP complex and are mainly present in the stroma as a soluble complex (Ziehe et al. 2018). However, the present finding reveals that part of cpSRP is associated with Alb3.1 and is thus bound to the thylakoid membranes. It is of note that the amount of cpSRP54 is much lower than that of cpSRP43 in the Alb3.1 preparations, based on the staining intensities on the gels. Although it has already been reported by in vitro experiments that cpSRP has an affinity with Alb3.1 (Dunschede et al. 2011; Lewis et al. 2010), the present study indicates that Alb3.1 and cpSRP interact in the thylakoid membranes strongly enough to resist solubilization of the thylakoid membranes with α-DDM.

It is of interest that magnesium-protoporphyrin IX monomethyl ester cyclase, which is involved in chlorophyll synthesis, also is also detected in the band 6. However, the thylakoid membrane-associated SRP receptor, cpFtsY, and Ab3.2 are not detected in these experiments.

Alb3.1associates with LHCPs

The Alb3.1-HA and Alb3.1-3HA preparations contain several polypeptides the relative electrophoretic migrations of which correspond to those of LHCI and LHCII polypeptides (Figure 2B and 2C). LC-MS/MS analysis reveals that, apart from band 10, bands 8-16 contain peptides from LHC proteins (Table 1). Band 8 is assigned to be minor LHCIIs, CP26 and CP29, which are not separated as discrete bands under the present electrophoresis conditions. Bands 9 and 13 are assigned to LHCII types I and III, respectively, but LHCII types II and IV were not detected. In addition, six LHCI proteins, LHCA4, LHCA3/LHCA6, LHCA8, and LHCA1/LHCA9 are assigned to bands 11, 12, 14, and 16, respectively. In addition to these LHCPs, some proteins localized in the thylakoid membranes are detected; PSAF of PSI (band 17), PsbC (CP43) and PSBP1 of PSII (bands 5 and 15), PetA (Cyt f) and PETO of Cyt. b_6f (bands 7 and 18), and AtpA of ATP synthase (band 2) (Table 1). It remains to be determined whether these are specifically associated with Alb3.1, but it is highly likely that AtpA is a non-specific contamination because this protein is detected in the negative control sample as well.

Immunological identification of proteins in the Alb3.1-3HA preparation

The polypeptides from the Alb3.1-3HA were also analyzed by immunoblotting. When probed with anti-HA antibody, a very strong signal is detected in the Alb3.1-3HA preparation, indicating that the Alb3.1-3HA is significantly enriched in the preparation, as expected (Figure 3). The anti-LHCII antibody which reacts with major LHCII proteins (LHCII types I-IV) detected signals corresponding to LHCII types I and III but did not detect LHCII types II and IV, which is consistent with the results by LC-MS/MS analyses. Probing with an anti-LHCII type I antibody, which reacts with both LHCII type I and CP29, reveals that these two proteins are present. In addition, the presence of CP26 is confirmed immunologically. Although faint bands of CP26 and LHCII type I were detected in the preparation from the wild-type thylakoid extracts, the intensity of the signals was significantly weaker than those of the corresponding signals in the Alb3.1 preparation. Thus, LC-MS/MS and immunoblot analyses confirmed the presence of LHCII proteins except for LHCII types I and IV in the Alb3.1-3HA preparation. Immunoblot analyses also detected several LHCI proteins; LHCA1, 2, 5, 8, and 9 but not LHCA7. Since the activity of the antibodies against LHCA3, LHCA4 and LHCA6 is rather weak, it proved difficult to confirm the presence of either of these three LHCI proteins although LC-MS/MS detected peptides from their sequence (Table 1). We noted that the immunoblotsrevealed the presence of the three photosystem I (PSI) proteins, PsaA, PSAD, and PsaF, but not the presence of PSII proteins, PsbA (D1) and PsbC (CP43). It is unlikely that Alb3.1 is required for the insertion of PsaA, which has eleven transmembrane helices, into the thylakoid membranes, because PsaA is

encoded by the chloroplast *psaA* gene, and the PSI complex is properly assembled in BF4 mutant cells as we reported previously (Bujaldon et al. 2020) and as documented in Figure 1. Although PSAD, which is peripherally located on the stromal side of PSI reaction center complex, and PSAF, which has one transmembrane helix with which most hydrophilic region located on the lumenal side of PSI reaction center complex, are encoded by nuclear genes, the BF4 mutant cells also accumulated these two proteins. Thus, we suspect that their interaction of the PSI proteins with Alb3.1 is indirect as will be discussed below.

Alb3.1 transiently associates newly synthesized LHCII proteins

The cpSRP pathway suggests that the cpSRP-Alb3.1 complex transiently associates with newly synthesized LHCPs. To address this question, we isolated an Alb3.1 preparation from cells that were radiolabeled with ^{35}S. Figure 4 shows the fluorography of the labeled polypeptides of the thylakoid membranes and the purified Alb3.1 preparation separated by a longer gel for a better resolution. The pulse-labeled polypeptides of the thylakoid membranes showed heavily labeled bands corresponding to PSII proteins (CP47/CP43 and D1/D2) and sharpbands of LHCII types I/II and III/V. CP26 and CP29 migrated in the same region as D1 under the present electrophoresis condition. The labeling of LHCI proteins was much weaker, as expected from their much lower content in the thylakoid membranes when compared with LHCII proteins (Ozawa et al. 2010). During the 4-h chase period, the intensity of PSII proteins becomes weaker because they turnover rapidly, whereas the intensity of the signal of LHCPs remains almost constant.

Many pulse-labeled polypeptides were also detected in the Alb3.1 preparation. Pulse-labeled bands in 45-60 kDa region appear to correspond to Alb3.1-HA, cpSRP54, and cpSRP43, and these signals are stable during the chase experiment. The sharp band due to AtpA is also detected above the Alb3.1-HA under the present electrophoresis condition. In addition, we detected several labeled LHCII proteins; LHCII type I (LHCII-I) and type III (LHCII-III) as well as CP26/CP29. The intensity of these signals significantly decreases during chase experiments. We hardly detected any labeled LHCI proteins in line with their lower labeling in the thylakoid membranes. The decreased labeling of LHCII proteins upon 4-h chase experiments is consistent with the fact that the newly synthesized LHCII proteins transiently associate with the cpSRP-Alb3.1 complex, while they insert into the thylakoid membranes.

DISCUSSION

Alb3.1 has a stable interaction with cpSRP

In vascular plants, cpSRP43 and cpSRP54 forms a heterodimer complex, and the cpSRP binds LHC apoproteins in the stroma (Aldridge et al. 2009; Horn et al. 2015; Ziehe et al. 2018). The L18 motif located between second and third transmembrane helices of LHCP binds to the ankyrin repeats of cpSRP43 (Horn et al. 2015; Stengel et al. 2008; Tu et al. 2000). The C-terminal ARRKR motif of the cpSRP54 binds to the C-terminal chromodomain 2 (CD2) of the cpSRP43 (Horn et al. 2015; Richter et al. 2010). The cpSRP-LHC complex is transported as a stroma-soluble transient complex to the thylakoid membranes where LHC apoproteins are membrane-integrated with the assistance of Alb3. Thus, it is inferred that cpSRP is mainly present in the stroma and only transiently binds to Alb3 in the thylakoid membranes.Crystal structure of cpSRP43-A3CT (Alb3 C-terminal tail) complex revealed that two positively charged motifs in the A3CT binds to the C-terminal chromodomain 3 (CD3) of cpSRP43(Horn et al. 2015). In green algae, the twin arginine of the ARRKR motif is conserved in cpSRP54 but the alanine is replaced by valine which has a bulkier side chain that may sterically prevent the interaction with cpSRP43 (Dunschede et al. 2015). Also, some critical amino acid residues in the CD2 domain are not conserved in cpSRP43, which may alter the cpSRP43-cpSRP54 interaction (Dunschede et al. 2015). Actually, yeast-two-hybrid, in vitro pulldown experiments, and size-exclusion chromatography separation of total protein extracts from *Chlamydomonas* cells revealed that cpSRP54 and cpSRP43 do not form a complex (Dunschede et al. 2015). However, we found in the present study that the purified Alb3.1 preparations contain both cpSRP43 and cpSRP54 although the amount of cpSRP54 appears lower than that of Alb3.1 and cpSRP43 (Figure 2 and Table).

Thus, our finding indicates that at least part of cpSRP43 and cpSRP54 associates with Alb3.1 in the thylakoid membranes. It is inferred that the interaction between Alb3.1 and cpSRP43/cpSRP54 may be transient but that it is rather stable since it resisted solubilization with the detergent. The presence of both cpSRP43 and cpSRP54 in the Alb3.1preparations suggests that either these two factors interact as a cpSRP in the Alb3.1 preparations or that they bind to Alb3.1 individually.The Chlamydomonas truncated light-harvesting antenna 4 (tla4) mutant, which has a lesion of *cpSRP54* gene, showed a pale green phenotype and a reduced accumulation of LHCPs (Jeong et al. 2017). However, it accumulated more LHCPs than the *tla3* and *p71* mutants deficient in cpSRP43 (Bujaldon et al. 2020; Kirst et al. 2012b), the *tla2* mutant deficient in cpFtsY(Kirst et al. 2012a), and the *ac29-3* and BF4 mutants deficient in Alb3.1 (Bellafiore et al. 2002; Bujaldon et al. 2020). These results argue for a more limited role of cpSRP54 in the accumulation of LHCPs as compared to that of the other biogenesis factors in *C. reinhardtii*. Our present finding that a smaller amount of cpSRP54 than of cpSRP43 is present in the Alb3.1 preparations further supports this view.

It was reported that the solubilized Alb3.1 is found in two complexes of 100-150 kDa and 600-700 kDa (Bellafiore et al. 2002). The apparent molecular mass of the smaller complex may be a dimer of Alb3.1, but the large complex is much larger than what would be expected for

Alb3.1-cpSRP. This observation suggests that the cpSRP43-Alb3.1 complex may associate with additional proteins. However, cpFtsY, the receptor protein of cpSRP, which is peripherally bound to the thylakoid membranes, is not detected in the Alb3.1 preparation. An reciprocal immunoprecipitation experiments suggested that Alb3.1 forms a complex with Alb3.2, which is the homolog of Alb3.1 and is also present in the thylakoid membranes (Gohre et al. 2006). However, we did not find Alb3.2 in the Alb3.1 preparations. Instead, we detected the magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase, which catalyzes the reaction to converts porphyrin to chlorine in chlorophyll biosynthesis pathway. That the enzyme involved in chlorophyll biosynthesis is found together with antenna proteins in association with their biogenesis complex suggests that the folding of LHCPs upon insertion in the thylakoid membranes must be coupled with the integration of chlorophylls. However, it remains to be addressed whether this enzyme is functionally bound to Alb3.1 since other enzymes involved in the chlorophyll biosynthesis pathway were not detected in the Alb3.1 preparations.

cpSRP-Alb3.1 acts as an assembly apparatus of LHCPs

LC-MS/MS and immunoblot analyses indicate that eight LHCI proteins (LHCA1, 2, 3, 4, 5, 6, 8, and 9) and four LHCII proteins (LHCII types I and III, CP26, and CP29) are present in the purified Alb3.1-3HA preparation. The hydrophobic apoproteins of LHCs must associate with cpSRP complex to remain soluble in the stroma and are targeted to the receptor cpFtsY and the insertase Alb3.1 to be integrated into the thylakoid membranes. Pulse-chase labeling experiments show that the LHCII types I and III proteins and probably CP26/29 bound to Alb3.1 are newly synthesized polypeptides (Figure 4). The limited pulse-labeled signals of most LHCI proteins contrasts with the significant labeling of LHCII proteins. This reflects the huge difference in abundance of these two types of antenna, LHCII being synthesized rather actively, whereas the synthesis of LHCI proteins is relatively slow (Ozawa et al. 2010). Although, LHCA7, and LHCII types II and IV are not detected in the Alb3.1-3HA preparation, it is highly likely that they also interact with the Alb3.1-cpSRP complex because the accumulation of LHCA7, and LHCII types II and IV is reduced to similar levels as the other LHC proteins in BF4 mutant cells. These proteins may be dissociated from the complex during the solubilization and/or purification proceduresbut the reason why the interaction of these proteins with cpSRP-Alb3.1 would be looser remains to be investigated.

It has been reported that the conserved L18 domain of LHC proteins, which consists of 18 amino acid residues and contains the crucial DPLG motif, binds to cpSRP43 proteins (Tu et al. 2000)(Horn et al. 2015). The L18 domain is well conserved even in LHCIIs types II and IV. Although the L18 domain is less conserved in most LHCI proteins than in LHCII proteins, those of LHCA2, LHCA8, and LHCA9 are well conserved among nine LHCI proteins (Bujaldon et al. 2020). This suggests that the affinity between LHC proteins and the Alb3.1-cpSRP complex is not determined primarily by the extent of the conservation of the amino acid sequence of the L18 domain. Other regions of the LHC proteins may also be involved in the stable interaction with Alb3.1-cpSRP complex.

Coupling between LHCI and PSI-LHCI assemblies

It is intriguing that PSI proteins (PsaA, PSAD, and PSAF) were detected in the Alb3.1-3HA preparation, as shown in Figure 3. This observation suggests that Alb3.1 is not only required for LHCI assembly but is also involved in the integration of LHCI complexes to a PSI core complex. It is also possible that Alb3.1 plays a role in a co-translational insertion of the chloroplast-encoded PSI reaction center (RC) subunits (PsaA and PsaB) with eleven transmembrane helices. Although BF4 mutant accumulates PSI normally, there may be a redundant pathway assisted by Alb3.2 or other factors(Gohre et al. 2006). However, it remains to be addressed whether Alb3.1 and Alb3.2 are involved in the PSI RC assembly. Figure 5 shows a working hypothesis of how Alb3.1 is engaged in assembling LHCI complexes and PSI-LHCI supercomplex. LHCI proteins translocated onto the stromal surface of the thylakoid membranes by the cpSRP complex would insert into the thylakoid membranes via Alb3.1. It remains unclear whether ten LHCI complexes bind to a PSI core one by one. Alternatively, LHCI oligomers are first assembled, and the resulting oligomers are integrated into a PSI core to form a PSI-LHCI subcomplex. It is of interest that a PSI-deficient mutant accumulates oligomeric LHCI complexes consisting of inner and outer tetramers , suggesting that the oligomerization of LHCI complexes proceeds before the association to a PSI core. The Alb3.1-cpSRP complex would subsequently detach after the assembly of the PSI-LHCI subcomplex is completed. The association of the Alb3.1-cpSRP-LHCP complex with PSI core may correspond to the large Alb3.1 complex of 600-700 kDa that we discussed above (Bellafiore et al. 2002).

In contrast, PSII proteins are not detected by immunoblotting although PsbC (CP43) was detected by mass spectrometry (Figure 3 and Table 1). This inconsistency may result from the fact that mass spectrometry is generally more sensitive than immunoblotting. It is known that a dimer of PSII core complex associates with LHCII trimers and monomers (CP26 and CP29) to form a PSII-LHCII supercomplex (Shen et al. 2019; Sheng et al. 2021), but this supercomplex is much more unstable than PSI-LHCI supercomplexes. Thus, it is possible that the interaction between Alb3.1-cpSRP-LHCPs and a PSII core might be dissociated during the solubilization and purification procedures. It is of interest that the assembly of one of the PSII reaction center proteins, D1, is retarded in the *Alb3.1* deletion mutant ac29, and that reciprocal coimmunoprecipitation experiments suggested a direct or indirect interaction between Alb3.1 and D1 (Ossenbühl et al. 2004). Thus, it remains to be addressed if a transient complex between a PSII core complex and Alb3.1-cpSRP-LHCP complex also is formed.

MATERIALS and METHODS

Strains and culture conditions; WT and mutant strains of *Chlamydomonas reinhardtii* were grown in tris-acetate phosphate (TAP) medium at 25 °C under continuous light of 50 μmol photons m^{-2} s^{-1} to the cell density of 2-5 x 10^6 cells ml^{-1}.

Plasmid construction and nuclear transformation;

DNA sequence of the *ChlamydomonasALB3.1* gene was retrieved at GenBank (accession number: AF492768). Part of the *ALB3.1* coding region was amplified by nested PCR (primers #1 and #2 for the 1st PCR, and primers #3 and #4 for the 2nd PCR) using *Chlamydomonas* cDNA library. The resulting PCR product was purified with illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Science, Backinghamshire), digested with *Nde*I and *Xba*I, and cloned into an *Nde*I/*Xba*I-digested pSL18 vector to generate plasmid pNK1. The pSL18 vector carries paromomycin resistant cassette and promoter/5'-UTR/terminator of PSAD gene to drive foreign gene expression. The N-terminal coding region of the *ALB3.1* was amplified by PCR with primers #5 and #6 using the *Chlamydomonas* cDNA library and the resulting DNA fragment was inserted into an *Nde*I/*Pml*I-digested pNK1 with In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga) to generate pNK2. Annealed oligo DNAs from primers #7 and #8 were inserted into an *Eco*RI/*Xba*I-digested pUC18 vector to generate pSXY2002, which carries an HA tag coding region. Two sets of annealed oligo DNAs (primers #9 and #10 and primers #11 and #12) were simultaneously inserted into an *Eco*RI/*Xba*I-digested pUC18 vector to generate pSXY2004, which carries the triple HA tag coding region. The C-terminal coding region of the *ALB3.1* was amplified by PCR using primers #13 and #14, and was then inserted into BstZ17I-digested pSXY2002 and pSXY2004 with In-Fusion HD Cloning Kit to generate pMKR2 and pMKR3, respectively. Plasmids pMKR2 and pMKR3 were digested with *Mlu*I and *Xba*I, and then cloned into an *Mlu*I/*Xba*I-digested pNK2 to generate pMKR4 and pMKR5, respectively. DNA segments amplified by PCR were confirmed by DNA sequencing.

Nuclear transformation was carried out by electroporation with an electroporator (Super Electroporator, NEPA21, typeII, Nepagene, Japan) basically as previously described (Nellaepalli et al. 2018) except that 10 μg/mL paromomycin instead of hygromycin was used for selection of the transformants. BF4 cells grown in TAP medium were harvested at 600 x g and resuspended in TAP-sucrose solution to the density of 1 x 10^8 cells mL^{-1}. Plasmids pMKR4 and pMKR5 were digested by *Sca*I and introduced into BF4 cells. Insertion of the HA tag was confirmed by using PCR KOD FX Neo DNA polymerase (Toyobo, Tokyo) by using primers #15 and #16. The resulting PCR products were separated by 2.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with a GelDoc Go Imaging System (BIO-RAD, Hercules, CA).

Oligo DNAs used in the present study are listed below.

#1: 5'-AAGTCTTGGGTGATGCCCTGCAC

#2: 5'-AACACGCAAACCACACGCATGCGCTG

#3: 5'-GGGCATATGTACGTGCTGGCCGACGCCTCG

#4: 5'-CCTCTAGAATCAAGCCGACGCACCGG

#5: 5'-CACAACAAGCCCATATGTCGAGCTCCATGTGCCTGGCG

#6: 5'-GTAGGAGTAGGGCACGTGCAGCTTGTCC

#7: 5'-AATTCGTATACCCCTACGACGTGCCCGACTACGCC<u>TAA</u>T

#8: 5'-CTAGA<u>TTA</u>GGCGTAGTCGGGCACGTCGTAGGGGTATACG

#9: 5'-AATTCGTATACCCCTACGACGTGCCCGACTACGCCTACCCCTACGACGT

#10: 5'-GGGCACGTCGTAGGGGTAGGCGTAGTCGGGCACGTCGTAGGGGTATACG

#11: 5'-GCCCGACTACGCCTACCCCTACGACGTGCCCGACTACGCC<u>TAA</u>T

#12: 5'-CTAGA<u>TTA</u>GGCGTAGTCGGGCACGTCGTAGGGGTAGGCGTAGTC

#13: 5'-GATTACGAATTCGTAGATCTACCTGAAGAAGCTGGGCGG

#14: 5'-CACGTCGTAGGGGTAAGCCGACGCACCGGCCACCGC

#15: 5'-CAGCAAGGTGAACCGGCGCTGCAAGCGG

#16: 5'-TCTCCATGGTACAGGCGGTCCAGCTGCTG

Thylakoid membrane reparation and affinity purification.

Cells grown to the density of 5×10^6 cells mL^{-1} were collected by centrifugation (2,860 x g at 25 °C for 5 min) and were resuspended in the buffer containing 25 mM HEPES-NaOH (pH 7.5), 1 mM MgCl$_2$ and 0.3 M sucrose. Cells were broken by French Pressure Cell at 170-190 psi, and the thylakoid membranes were purified by floating method using stepwise sucrose gradients as previously described (Chua and Bennoun 1975). Thylakoid membranes (400 µg Chl mL^{-1}) were solubilized with 1% α-DDM at 4°C for 10 min. The solubilized thylakoid extracts (500 µL) were incubated in a spin column followed by the addition of anti-HA beads (HA-tagged protein purification kit, MBL) for 1 h with constant end-over-end mixing. Non-adsorbed proteins to the HA-beads in the column were removed by centrifugation (Nellaepalli et al. 2018). The columns were washed with the washing buffer (500 µL) 8-14 times. Alb3.1-HA and Alb3.1-3HA preparations were eluted two times with 20 µLelution buffer.

Pulse-chase labeling of proteins.

Pre-cultures from cAlb3.1-1HA strain were grown to a cell density of 2-3 x 10^6 cells mL^{-1} in 100 mL of TAP medium containing reduced amounts of sulfur. The cells harvested by centrifugation were resuspended in TAP media containing no sulfur (25 µg Chl mL^{-1}) and were starved for sulfur under the light of 50 µmol photons m^{-2} s^{-1} for 2 h. Total cellular proteins were labeled with $Na_2{}^{35}SO_4$ (5 µCi mL^{-1}) (American Radiolabeled Chemicals) under the light of 50 µmol photons m^{-2} s^{-1} at 25°C for 20 min. The labeled proteins were chased by the addition of cold 10 mM Na_2SO_4 for 4 h. The radio-labelled cells were immediately subjected to thylakoid preparation as reported (Nellaepalli et al. 2021; Nellaepalli et al. 2018).

SDS-PAGE and Immunoblotting.

Polypeptides from total cellular proteins, thylakoid membranes and affinity purified samples were separated by SDS-PAGE (Fling and Gregerson 1986; Kashino et al. 2001; Laemmli 1970). The separated polypeptides were visualized by staining with a fluorescent dye Flamingo (Bio-Rad, USA). Nitrocellulose membranes were used for blotting and probed with several antibodies raised against, HA, PSI, PSII, LHCI, and LHCII polypeptides. Immunoblotting signals were detected by enhanced chemiluminescence (ECL) with a luminescent image gel analyzer (LAS-4000 mini, Fuji film). An antibody against HA was purchased from Medical & Biological Laboratories, Tokyo, Japan. Antibodies against PSI and LHCI subunits were described previously (Ozawa et al. 2018) and antibodies against LHCII subunits (Bujaldon et al. 2020) were described previously. Antibodies against CP43 and D1 are kind gift from Jean-David Rochaix and Masahiko Ikeuchi, respectively.

In gel digestion and LC-MS/MS

In-gel tryptic digestion and followingliquid chromatography tandem mass spectrometry (LC-MS/MS) (LTQ, Thermofisher Scientific, USA) were performed as described in (Ozawa et al. 2009). The acquired mass spectra data were analyzed with the Proteome Discoverer ver. 2.1.1.21 (Thermofisher Scientific, USA) against the protein database in ver. 5.5 Chlamydomonas genome of Joint Genome Institute for nuclear gene products and protein database in NC_005353 of National Center for Biotechnology Information for chloroplast gene products, respectively. Post-translational modifications were considered with dynamic modification; N-terminal acetylation, oxidation of tryptophan and methionine, and phosphorylation of serine, threonine, and tyrosine. False Discovery Rate of 0.01 was calculated by the Decoy database search and filtered for all analyses.**3**

Table
Identification of polypeptides in the Alb3.1-HA and Alb3.1-3HA preparations.
The polypeptides of the Alb3.1-HA and Alb3.1-3HA were separated by SDS-PAGE and stained with silver as shown in Figure 2 (panels B and C). The polypeptides in the gel (Bands 1-20) were excised and digested with trypsin, and the resulting polypeptides were applied to LC-MS/MS. Assigned peptide sequences, position of the identified peptides in the protein sequence (position), and the score for assigned polypeptide sequence (Xcorr) are shown. Red M represents methionine sulfoxide (monooxidized methionine).

Band	transcript gene ID accession ID	gene name	Assigned peptides sequence	position	Xcorr
1	not identified				
2	Cre06.g251900.t1.2	Alb3.1	QQVESAMAVQALKPR	84-98	4.49
			KLGGANVV**M**NELGPVTKPGSGR	280-301	4.54
			LGGANVV**M**NELGPVTKPGSGR	281-301	3.72
			NGVAAGEWSVWKPATVLTTAEAAK	303-326	4.87
			ARAEAEEAVER	327-337	4
	Q96550	AtpA	G**M**ALNLQADHVGVVVFGNDSLIHQGDLVYR	124-153	4.65
			EVAAFAQFGSDLDAATQYVLER	462-483	3.24
3	Cre11.g479750.t1.2	cpSRP54	VLEGVTPDVQFIK	129-141	3.48
			TVSNELIDLMGGGTGAK	142-158	5.13
			TVSNELIDL**M**GGGTGAK	142-158	4.38
			SAVRPSDTLLVVDAMTGQEAANLVR	274-298	4.74
			SFNEAVDISGAILTK	299-313	4.81
			ANSLLQELVASAGK	509-522	4.16
4	Cre04.g231026.t1.1	cpSRP43	WKDGSDSTWEVAADLSEDLVR	88-108	3.81
			SALHFAAALGSAECTR	147-162	4.4
			EGYTPLHMAAGYMHTPSMAVLLEAGANPEIK	177-207	3.85
			LYDEVEPGNVLNCR	249-262	4.14
			NVSADVLEDYLAGLEYAVAEEVLDVVQVR	289-317	5.35
5	P10898	PsbC	VITNPTTNAAVIFGYLVK	186-203	5.27
			LGANVASAQGPTGLGK	312-327	4.25
			SPTGEIIFGGET**M**R	332-345	3.65
			AAEY**M**THAPLGSLNSVGGVATEINAVNFVSPR	380-411	3.74
6	Cre07.g346050.t1.2	CRD1 Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase, chloroplastic, Cupper response defect 1 protein	FYTTDFDEMEQLFSK	68-82	4.46
			FYTTDFDE**M**EQLFSK	68-82	4.09
			ASSPEVAEMFLLMSR	157-171	3.54
			ASSPEVAEMFLL**M**SR	157-171	3.68
			ALSDFNLALDLGFLTK	183-198	6.16
			FIIYATFLSEK	209-219	3.91
			FYESLGLNTR	302-311	3.62
			LVELSASSSPLAGLQK	354-369	4.93
			EKDVGSVDIAGSGASR	388-403	3.65
			DVGSVDIAGSGASR	390-403	5.02
7	P23577	PetA	VGNLYYQPYSPEQK	128-141	3.62
			SNNTIYNASAAGK	197-209	3.99
			EGQTVQADQPLTNNPNVGGFGQAETEIVLQNPAR	249-282	6.35
8	Cre16.g673650.t1.1	LHCB5 (CP26)	GWLGGQGGAADLDK	38-51	4.5
			KLFLPSGLYDR	58-68	3.13
			NGTGPAGYSPGIGK	185-198	3.66
			HVADPFGYNLLTVLGAEER	267-285	5.73
			LA**M**VSVLGFAVQSYVTGEGPYANWTK	241-266	4.89
8	Cre17.g720250.t1.2	LHCB4 (CP29)	GSVEAIVQATPDEVSSENR	102-120	5.99
			LAPYSEVFGLAR	121-132	3.14

| #	Gene ID	Protein	Peptide	Position	Score
9	Cre04.g232104.t1.1	LHCBM3 (Type I)	QAPASSGIEFYGPNR	31-45	3.42
			YRELELIHAR	86-95	3.58
			AGAQIFSEGGLDYLGNPSLVHAQNIVATLAVQVILMGLVEGYR	125-167	4.13
			AGAQIFSEGGLDYLGNPSLVHAQNIVATLAVQVIL**M**GLVEGYR	125-167	5.28
10	not identified				
11	Cre10.g452050.t1.2	LHCA4	WYAQAEL**M**NAR	95-105	3.02
			YQDFVKPGSANQDPIFTNNK	165-184	4.64
12	Cre11.g467573.t1.1	LHCA3	SKDQLYVGASQSSLAYLDGSLPGDFGFDPLGLLDPVNSGGFIEPK	41-85	5.48
	Cre06.g278213.t1.1	LHCA6	EVESPLGPLGLLAVEFFL**M**HWVEVR	103-127	3.71
			KPGSVDQDPIFSQYK	134-148	2.64
13	Cre12.g548400.t1.2	LHCBM2 (Type III)	YRELELIHAR	78-87	3.58
			AGAQIFAEGGLNYLGNENLIHAQSIIATLAFQVVVMGLAEAYR	117-159	4.57
			AGAQIFAEGGLNYLGNENLIHAQSIIATLAFQVVV**M**GLAEAYR	117-159	5.22
			ANGGPLGEGLDPLHPGGAFDPLGLADDPDTFAELK	160-194	4.7
			LAMFSMFGFFVQAIVTGK	203-220	4.76
			GPIQNLDDHLANPTAVNAFAYATK	221-244	3.91
14	Cre06.g272650.t1.2	LHCA8	YATGAGPVDNLAAHLK	211-226	3.14
15	Cre12.g550850.t1.2	PSBP1	YEDNFDAVNNLVVIAQDTDKK	106-126	3.62
			QAYSGETQSEGGFAPNR	148-164	4.83
			VSAASLLDVSTTTDKK	165-180	4.18
			HQLIGATVGSDNK	202-214	3.68
16	Cre06.g283050.t1.2	LHCA1	FTESEVIHGR	74-83	3.73
			GDAGGVVYPGGAFDPLGFAK	146-165	3.89
	Cre07.g344950.t1.2	LHCA9	TQPIEGLTAHLADPFGK	181-197	2.96
			NITYYLTHLPETLGSA.[-]	198-213	2.52
17	Cre09.g412100.t1.2	PSAF	LKQYEADSAPAVALK	91-105	5.05
			QYEADSAPAVALK	93-105	3.15
			GTLLEKEENITVSPR	213-227	4.27
18	Cre12.g558900.t1.2	PETO	KAEVVESTSGGLDPR	101-115	3.09
			SVALPGALALTIGGFVAASK	116-135	4.12
			DSNNYAGYEATLK	151-163	3.62
19	not identified				
20	not identified				

Figure 1

(A)

(B)

(C)
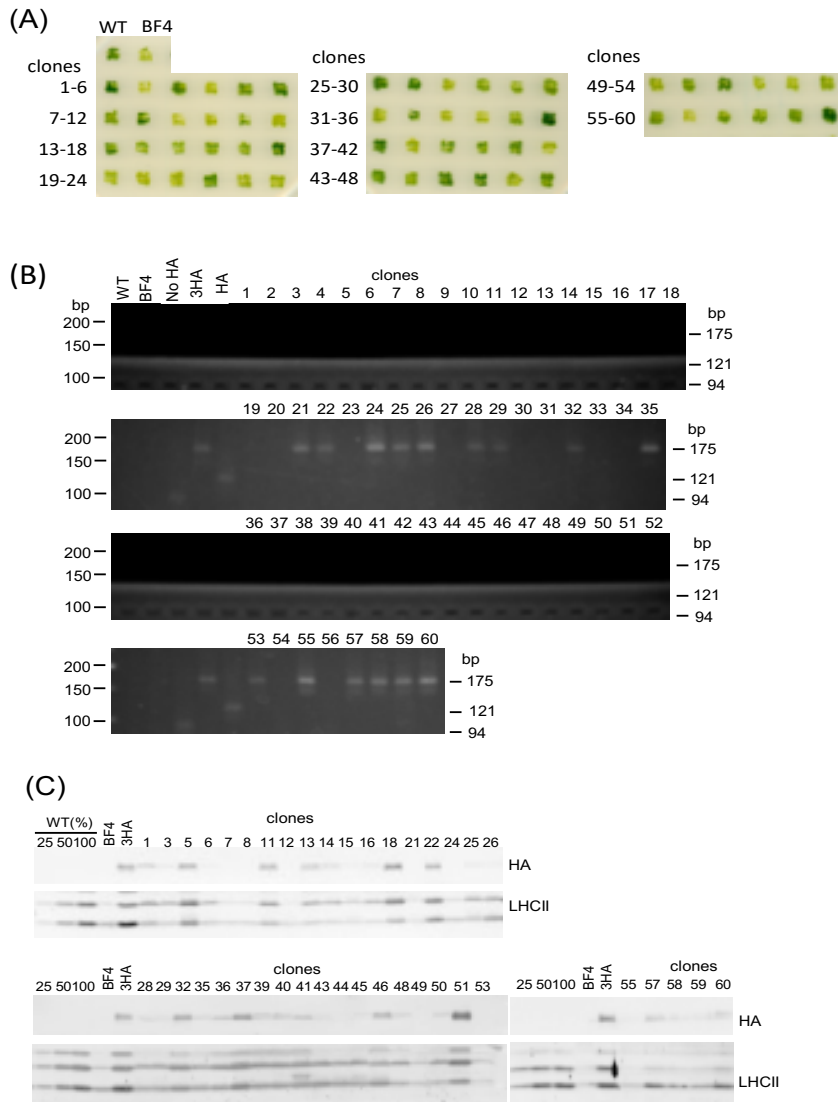
Figure 1

Screening of clones after transformation of BF4 cells with the complementation vector to express Alb3.1- 3HA. Putative complemented clones were first selected on TAP agar plates containing 10 µg/mL paromomycin. (A) The cells of 60 putative complemented clones as well as WT (dark green) and BF4 (pale green) cells were grown on TAP agar plates. (B) Agarose gel electrophoresis of DNA fragments amplified using primers #15 and #16 as described in Materials and Methods and Figure 1A. No DNA fragment was amplified from WT and BF4, fragments of 94, 121, and 175 kb were amplified from the complemented cells expressing Alb3.1, Alb3.1-HA, and Alb3.1-3HA. DNA fragment of 175 bp was amplified from the putative complemented clones (1, 3, 5-8, 11-18, 21-22, 24-26, 28-29, 32, 35-37, 39-41, 43-46, 48-52, 53, 55, 57-60). (C) Immunoblots of total cell proteins of the clones (1, 3, 5-8, 11-16, 18, 21-22, 24-26, 28-29, 32, 35-37, 39-41, 43-46, 48-51, 53, 55, 57-60) using antibodies against HA tag and LHCII polypeptides (types I-IV). The signal intensity of HA tag corresponds to the accumulation of Alb3.1-3HA.

Figure 2
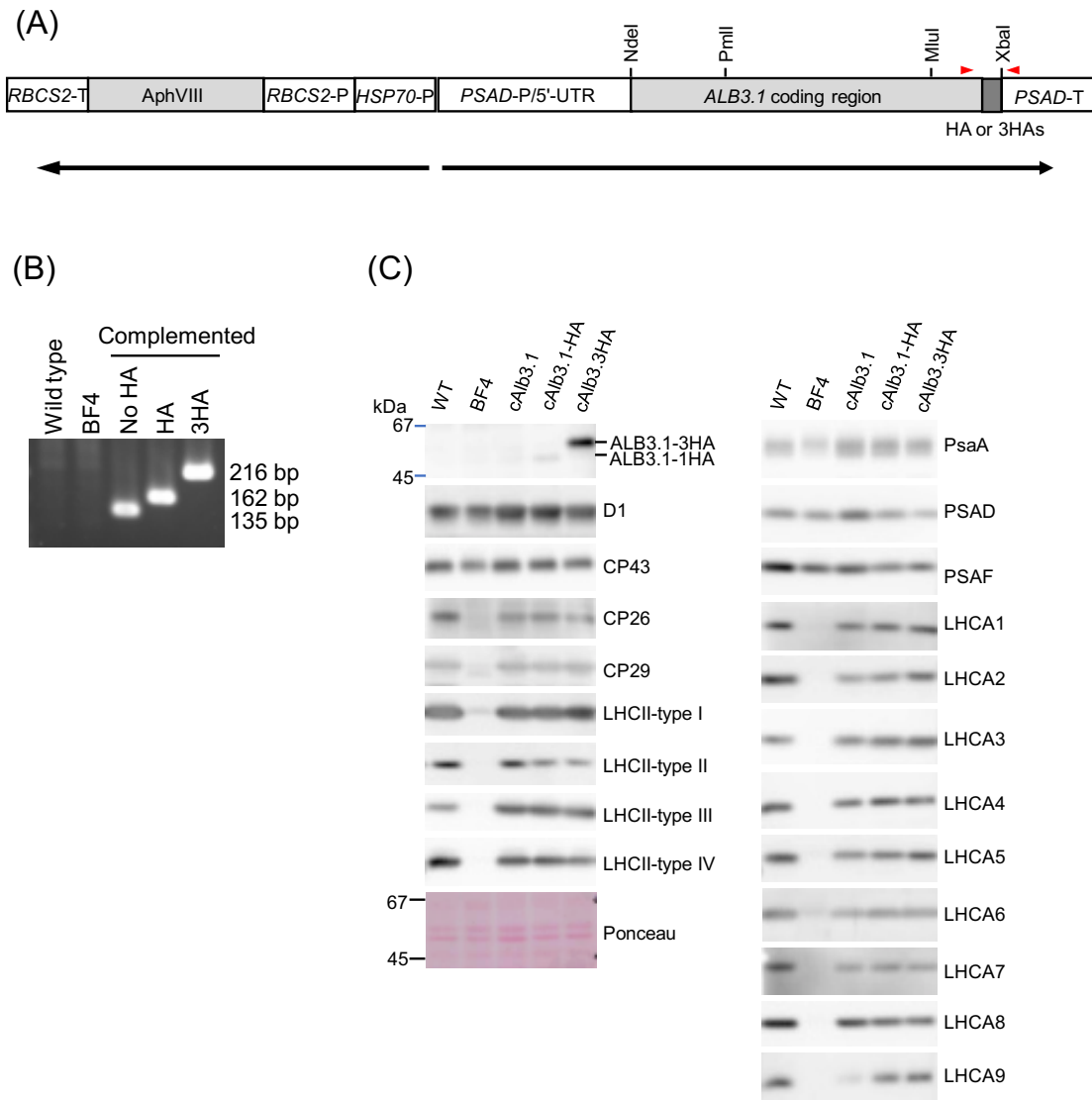
Figure 2

Complementation of BF4 cells.

(A) Physical map of transformation vector. ALB3.1 coding region amplified from cDNA library is under the control of the promoter/5'-UTR and terminator of *Chlamydomonas* PSAD (PSAD-P/5'-UTR and PSAD-T, respectively). Coding regions of a single HA or a triple HA tag is inserted at the coding region of the C-terminus of ALB3.1. The vector carries a selectable paromomycin resistance gene (AphVIII) under the control of the HSP70 promoter (HSP70-P), RBCS2 promoter (RBCS2-P) and RBCS2 terminator (RBCS2-T). Restriction sites (*Nde*I, *Pml*I, *Mlu*I and *Xba*I) used for plasmid construction are also shown. Arrows indicate the orientation of the genes. Red arrowheads represent primers used for PCR. (B) Confirmation of the insertion of HA tag coding region in the complemented strains by chimeric ALB3.1 transgene without HA (No HA) or with a single or a triple HA (HA and 3HA). Wild type and BF4 strains, which lack chimeric ALB3.1 transgene, were used as negative controls. PCR was carried out as described in Materials and Methods. Estimated sizes of the PCR products are shown. (C) Accumulation of PSI, LHCI, and LHCII proteins in wild type, BF4, and complemented BF4 (cAlb3.1, cAlb3.1-HA, and cAlb3.1-3HA) cells. Total cell proteins (0.5 µg Chl but 0.1 µg Chl for BF4) were solubilized with 2% (w/v) SDS, 0.1M DTT at 100 ℃ for 1 min and protein extracts were separated by SDS-PAGE, electroblotted onto a nitrocellulose filter, probed with specific antibodies against HA tag, PSII proteins (D1 and CP43), LHCII proteins (CP26, CP29, and LHCII-types I-IV), PSI proteins (PsaA, PSAD, and PSAF), and LHCI proteins (LHCA1-9).
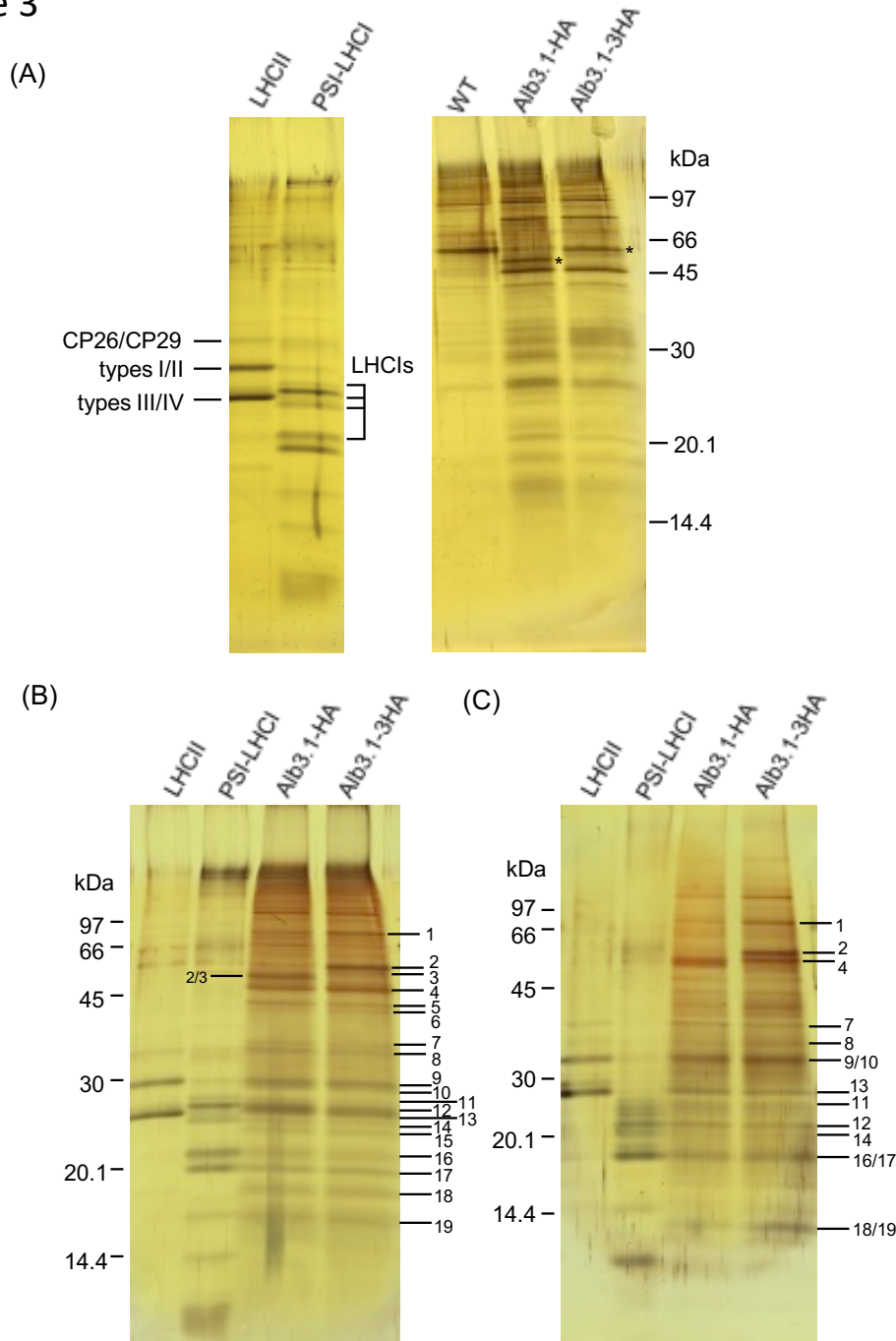
Figure 3

Figure 3

Polypeptide profiles of the affinity purified Alb3.1 preparation.

(A) Comparison of the polypeptides of affinity-purified preparations from WT (wild type), Alb3.1-HA, and Alb3.1-3HA. The thylakoid membranes were solubilized with 1% α-DDM and Alb3.1 preparations were purified by affinity spin column as described in Materials and Methods. The samples were solubilized with 2% (w/v) SDS, 0.1M DTT at 80 ℃ for 1 min and protein extracts were separated by SDS-PAGE and the gels were stained with silver. For further characterization of the polypeptides of the Alb3.1 preparations, the polypeptides were separated by two SDS-PAGE systems; (B) Laemmli system and (C) urea-MES-Tris system. The polypeptides of the preparations were also compared with the polypeptides of LHCII and PSI-LHCI preparations from wild type cells. The polypeptides marked by numbers were subjected to trypsin digestion and the resulting digests were analyzed by LC-MS/MS. The assignments of these polypeptides are summarized in Table 1.

Figure 4
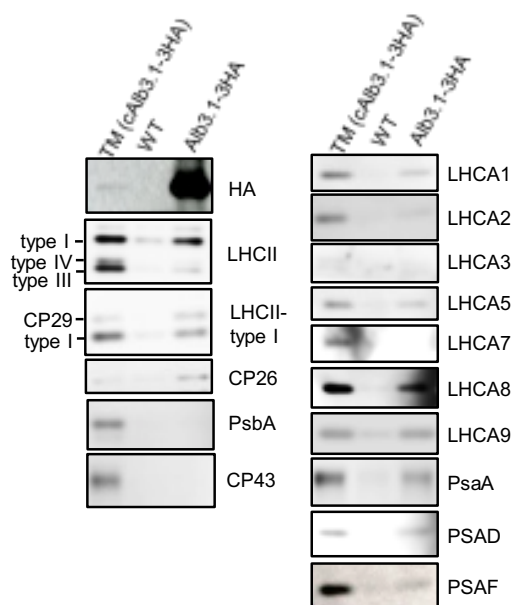
Figure 4

Identification of the interacting proteins with Alb3.1 by immunoblotting. The affinity purified preparations from WT and Alb3.1-3HA thylakoid extracts as well as the thylakoid membranes from cAlb3.1-3HA (TM) were solubilized with 2% (w/v) SDS and 0.1M DTT at 80 ℃ for 1 min and protein extracts were separated by SDS-PAGE, electroblotted onto a nitrocellulose filter, probed with specific antibodies raised against LHCI, LHCII, PSI, and PSII proteins. Alb3.1-3HA was detected with the anti-HA antibody.

Figure 5
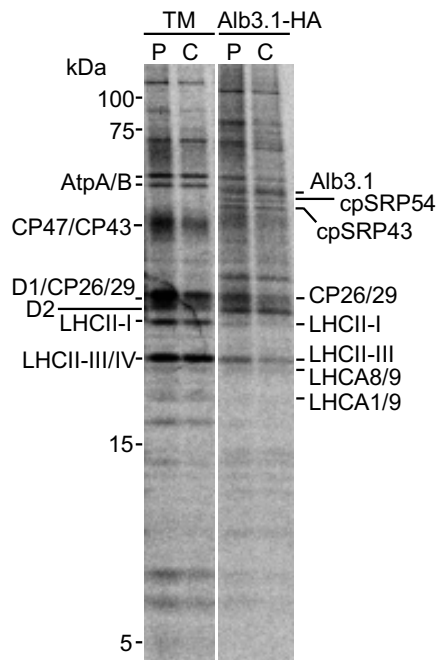
Figure 5

Transient association of LHCPs in the Alb3.1 preparation.Total cellular proteins from cAlb3.1-HA cells were labeled with 5µCi/ml $Na_2{}^{35}SO_4$ for 20 min (P) and chased for 4 h (C). TM; the thylakoid membranes, Alb3.1-HA; the Alb3.1-HA preparation.
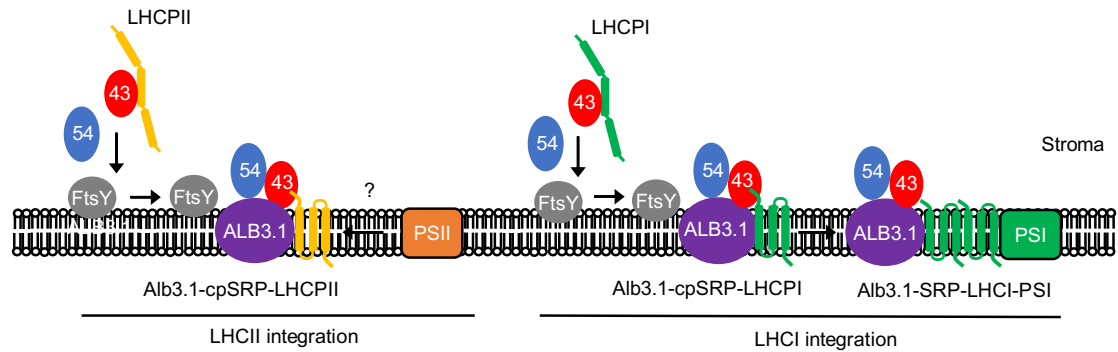
Figure 6

Figure 6

A proposed model on the integration and assembly of green algal LHCPs.
Two stromal factors, cpSRP43 and cpSRP54, may form a heterodimer (cpSRP). The cpSRP43 mainly binds LHCI and LHCII apoproteins in the stroma to form a transient cpSRP-LHCP complexes, which is subsequently directed to the chloroplast SRP receptor protein FtsY on the stromal surface of the thylakoid membranes. Then cpSRP-LHCP complex translocates the LHCP to Alb3.1 insertase by forming cpSRP-Alb3.1-LHCP complex. The Alb3.1 assists insertion and assembly of LHCPs into the thylakoid membranes. The integrated LHCI polypeptides forms an LHCI oligomer and associates with PSI core complex (Alb3.1-LHCI-PSI). Finally, the PSI-LHCI subcomplex is detached from Alb3.1. It remains elusive if Alb3.1-LHCII-PSII subcomplex is formed because PSII-LHCII is looser than PSI-LHCI.

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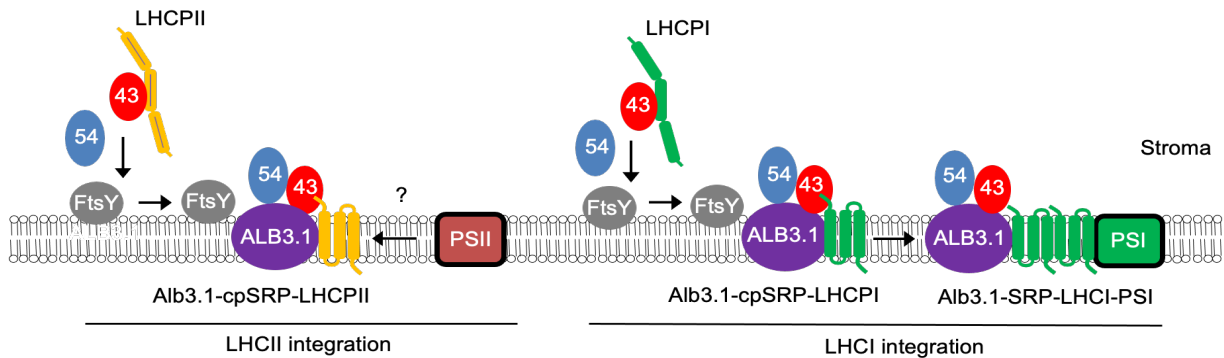
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GENERAL CONCLUSION

In this study, Alb3.1 Integration and assembly of LHCPs process studied at molecular level. A putative Alb3.1 assembly process is proposed in figure. Two stromal factors, cpSRP43 and cpSRP54, may form a heterodimer (cpSRP). The cpSRP43 mainly binds LHCI and LHCII apoproteins in the stroma to form a transient cpSRP-LHCP complexes, which is subsequently directed to the chloroplast SRP receptor protein FtsY on the stromal surface of the thylakoid membranes. Then cpSRP-LHCP complex translocates the LHCP to Alb3.1 insertase by forming cpSRP-Alb3.1-LHCP complex. The Alb3.1 assists insertion and assembly of LHCPs into the thylakoid membranes. The integrated LHCI polypeptides form an LHCI oligomer and associates with PSI core complex (Alb3.1-LHCI-PSI). Finally, the PSI-LHCI subcomplex is detached from Alb3.1. It remains elusive if Alb3.1-LHCII-PSII subcomplex is formed because PSII-LHCII is looser than PSI-LHCI.

Figure

A proposed model on the integration and assembly of green algal LHCPs.

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