Novel chloroplast membranes are involved in protein synthesis and protein import for the biogenesis of the photosynthetic thylakoid membranes in *Chlamydomonas reinhardtii*

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Abstract

Novel chloroplast membranes are involved in protein synthesis and protein import for the biogenesis of the photosynthetic thylakoid membranes in *Chlamydomonas reinhardtii*

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The cytological organization of *de novo* photosystem II biogenesis in chloroplasts was examined using a unique technique of cellular subfractionation. The cellular subfractions were biochemically examined for three chloroplast membrane compartments. The results revealed membranes that are specialized in the synthesis of photosystem II proteins that are encoded by the chloroplast genome and synthesized by bacterial-like ribosomes within this semiautonomous organelle. Furthermore, a novel class of envelope membrane was identified which has a higher density than envelope membrane that was previously described. The localization of newly synthesized proteins and lipids after cellular subfractionation were also determined. Similarly, intermediates of chlorophyll synthesis were localized in the cellular subfractions, and it was confirmed that they are not only localized to the envelope membrane. The results reveal novel membranes that are involved in the synthesis of chloroplast genome-encoded subunits of photosystem II and the import of chloroplast proteins from the cytosol. They also contribute to the current realization in the field that organelles are highly compartmentalized and that certain compartments are specialized in biogenesis processes.

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List of Abbreviations

β-DM	n-dodecyl-β-d-maltoside
DGDG	Digalactosyldiacylglycerol
LHCII	Light Harvesting Complex II
MGDG	Monogalactosyldiacylglycerol
OEC	Oxygen Evolving Complex
pT-ZAM	putative T-ZAM
PSI	Photosystem I
PSII	Photosystem II
T-zone	Translation zone
T-ZAM	T-zone associated membrane
TIC	Translocon of the inner membrane of chloroplasts
TLC	Thin Layer Chromatography
TOC	Translocon of the outer membrane of chloroplasts
VIPP1	Vesicle inducing protein in plastids 1

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Introduction

The thylakoid membranes of plants and algae chloroplasts, and cyanobacteria, house the photosynthetic machinery, which catalyzes the oxidation of water for the production of ATP and NADPH via the light-driven electron transport chain (Nevo et al. 2012). In plants and green algae, the chloroplasts evolved through the endosymbiosis of a cyanobactrium after the development of the endoplasmic reticulum and the mitochondria (Goksoyr 1967). Like the mitochondria, chloroplasts are a semi-autonomous organelle type and have maintained their own genetic system, as well as an elaborate membrane system (The Arabidopsis Genome Initiative 2000). The nuclear and chloroplast genomes are coordinated in their activities; this coordination is mediated by the double envelope membrane which encloses the chloroplast (Beck 2005; Block et al. 2007).

The complex cytological organization of chloroplasts includes both the thylakoid membrane network and the envelope membranes. These segregate several aqueous compartments: the lumen within the thylakoid membranes, the stroma of the chloroplasts, and the inter-envelope space of the envelope membranes (Ohad, Siekevitz, and Palade 1967). The lumen is believed to be a contiguous compartment which is enclosed by the thylakoid membranes (Shimoni et al. 2005). The stroma of the chloroplast, analogous to the cytosol of a cell, is the aqueous, proteinaceous matrix between the inner envelope membrane and the thylakoid membranes (Ohad, Siekevitz, and Palade 1967). The inter-membrane space of the envelope membranes is the space between the inner and outer envelope membranes. These membranes and compartments form six locations which house different protein complements. In addition, the thylakoid membranes of chloroplasts are morphologically and

functionally divided into the grana thylakoids, which are appressed within stacks of flattened thylakoid vesicles , and the unappressed, stromal thylakoids, which extend between grana and are located at the stroma-exposed ends of grana (Ohad, Siekevitz, and Palade 1967; Shimoni et al. 2005). Photosystem II (PSII) is a multisubunit complex which is embedded in grana thylakoid membranes and catalyzes the first light absorbing, water oxidizing step of photosynthesis (Nelson and Ben-Shem 2004; Guskov et al. 2009). Stroma thylakoid membranes contain photosystem I (PSI), which catalyzes the second light-absorbing, NADPH-producing step of photosynthesis (Andersson and Anderson 1980). The lipid components of the thylakoid membranes and the inner envelope membrane of the chloroplast are primarily monogalactosyldiacylglycerol and digalactosyldiacylglycerol (MGDG and DGDG, respectively), and these account for 75% of the lipids of the chloroplast (Joyard et al. 1998).

There are several problems posed by the combination of the complex membrane systems and dual genetic systems of chloroplast for the *de novo* biogenesis of the photosystems, particularly PSII. A functional PSII protein complex includes protein subunits which are encoded by both the nuclear genome, such as the light harvesting complex II (LHCII), the oxygen evolving complex (OEC), and many extrinsic proteins of the reaction center complex (RCC). On the other hand, the chloroplasts genome encodes core proteins of the RCC (D1, D2, CP43, CP47, and cytochrome$_{b559}$) (Minagawa and Takahashi 2004; Nelson and Ben-Shem 2004; Rokka et al. 2005; Roose, Wegener, and Pakrasi 2007). The polypeptides of the LHCII and OEC are synthesized on cytosolic ribosomes, translocated across the double envelope membranes of the chloroplast, and integrated into the thylakoid membranes, where they assemble with the RCC of PSII to form the PSII-LCHII supercomplex (Chua and

Schmidt 1978; Nielsen et al. 1997). The RCC is composed of over 20 protein subunits and, similarly, the extrinsic RCC proteins must be integrated into the thylakoid membranes after being synthesized on cytosolic ribosomes and translocated into the chloroplast (Roose, Wegener, and Pakrasi 2007). The core polypeptides of the RCC are synthesized on the ribosomes of chloroplasts, which are similar to bacterial ribosomes, and co-translationally integrated into the thylakoid membranes, where the individual proteins associate with one another and the extrinsic polypeptides of the RCC to form the complete RCC (Siddell and Ellis 1977; Yamaguchi et al. 2003). PSII is not only composed of polypeptides, its catalytic function is dependent on cofactors and pigments which are folded into the polypeptides, particularly chlorophyll (Guskov et al. 2009).

Because the appressed, granal membranes are inaccessible to ribosomes, the core RCC proteins of PSII, and the imported LCHII and OEC cannot be inserted directly into the thylakoid membranes where they are functional in the PSII-LHCII supercomplex (Yalovsky, Schuster, and Nechushtai 1990). Several models for the cytological localization of *de novo* PSII biogenesis have been developed, and recently the cytological organization of the co-translational insertion of the core RCC into the thylakoid membranes has been studied extensively (reviewed in Zerges 2000; Adam et al. 2011; Komenda, Sobotka, and Nixon 2012). One model suggests that the core RCC subunits are co-translationally inserted into the stromal thylakoid membranes (Chua et al. 1973; Margulies and Michaels 1974). The association of ribosomes with the stromal thylakoid membranes presents the clearest evidence for translation at this site (Falk 1969; Alscher, Patterson, and Jagendorf 1978). This, in combination with the lack of ribosomes on the granal thylakoid membranes, has led to the supposition that the stromal thylakoid membranes are the major site of thylakoid membrane

biogenesis. It is possible, however, that the ribosomes on stromal thylakoid membranes are synthesizing proteins for repair of PSII, and not *de novo* complex assembly. The synthesis of PSII subunits on the stromal thylakoid membranes is known to be required for repair synthesis (Tyystjärvi and Aro 1996; van Wijk, Andersson, and Aro 1996). During a light-induced stress condition called photoinhibition, PSII is susceptible to photodamage even under low light conditions. In these conditions, the D1 subunit of the core RCC is repaired on the stromal thylakoid membranes after the damaged PSII complex has migrated out of the granal thylakoid membranes. Therefore, it has been difficult to determine whether stroma thylakoids are where chloroplast mRNAs are translated for thylakoid membrane biogenesis, although this remains the prevailing belief in the research community.

An alternative model suggests that thylakoid membrane biogenesis and PSII biogenesis occurs in the inner envelope membrane of the chloroplast. This model suggests that the site of galactolipid synthesis and the late steps of chlorophyll synthesis are the envelope membranes of the chloroplast (Block et al. 1983; Pineau et al. 1986; Joyard et al. 1990). In addition, thylakoid membrane biogenesis has been suggested to be dependent on vesicle formation and trafficking (Kroll et al. 2001). In this model, the elements composing thylakoid membranes and PSII – protein subunits, lipids, and chlorophylls – would assemble on the inner envelope of the chloroplasts before being transferred to the thylakoid membranes by vesicle transport mediated by VIPP1 (Vesicle Inducing Protein in Plastids 1, or Very Important Protein in Plastids 1) among other proteins. This model has come under increasing scrutiny as the role of VIPP1 in thylakoid membrane biogenesis has been re-examined, and alternative functions to the original designation of VIPP1 have been suggested (Nordhues et al. 2012; Vothknecht et al. 2012).

Recently, PSII biogenesis has been considered using cyanobacteria as model organisms. In the cyanobacteria *Synechocystis* sp. PCC 6803 (hereafter referred to as *Synechocystis*), the protein that cleaves the C-terminal end of D1, CptA, and the core RCC proteins D1 and D2 have been found in membranes containing proteins of the plasma membrane, which is analogous to the inner membrane of the chloroplast (Zak et al. 2001). These authors concluded that the initial steps of PSII biogenesis occur in the plasma membrane, after which the heterodimer of D1 and D2 is transported, possibly by vesicles, to the thylakoid membranes where the remaining steps of PSII biogenesis are completed. More recently, also in *Synechocystis*, an intermediate membrane sub-compartment associated with PSII biogenesis has been identified using a two-step cellular subfractionation procedure (Schottkowski et al. 2009). The membranes identified contained PratA, which binds to D1 and facilitates the processing of D1 by CptA, as well as chlorophyll precursors. This membrane sub-compartment was found in an intermediate region between the thylakoid membranes and the plasma membrane, where the initial steps of PSII biogenesis is hypothesized to take place. Finally, electron tomography studies of *Synechoccocus elongatus* PCC 7942 observed layers of the thylakoid membranes merging to form perforations where ribosomes were concentrated. It was speculated that these perforations might be areas of thylakoid membrane biogenesis independent of the plasma membrane (Nevo et al. 2007).

The site of *de novo* PSII biogenesis has also been studied in *Chlamydomonas reinhardtii*. This unicellular green alga has one large chloroplast (Ohad, Siekevitz, and Palade 1967). The chloroplast of *C. reinhardtii* cells has a basal region which contains the pyrenoid, a structure where the first step of carbon fixation is concentrated (Michael, McKay, and Gibbs 1991). The lobes of *C. reinhardtii* chloroplasts are

finger-like projections from the basal region that envelope the nuclear-cytosolic compartment of the cell (Uniacke and Zerges 2007) (see Appendix 5 for schematic of *C. reinhardtii* cell). Using *in situ* localization under biogenesis conditions of chloroplast ribosomal subunits, mRNAs of the core RCC proteins and IF of a D2 translation factor (RB38/RBP40) identified regions of *de novo* PSII synthesis lateral to the pyrenoid. These regions were termed T-zones for Translation-zones (Uniacke and Zerges 2007).

As the core proteins of the RCC are co-translationally inserted into membranes (Margulies and Michaels 1974; Margulies and Michaels 1974; Margulies 1983; Hurewitz and Jagendorf 1987), T-zones, proposed regions of the chloroplast for the *de novo* biogenesis of PSII, should have a membrane sub-compartment associated with them. These T-zone associated membranes (T-ZAM) should have several defining characteristics predicted from the earlier *in situ* characterization (Uniacke and Zerges 2007). First, T-ZAMs should have both the large and small ribosomal subunits of the chloroplast associated with them. Second, T-ZAMs should also be associated with the translation factor RB38/RBP40 (Barnes et al. 2004; Schwarz et al. 2007). Third, T-ZAMs should not be thylakoid membranes. Fourth, T-ZAMs should be a minor membrane sub-fraction. Fifth, T-ZAMs should be less abundant in the dark than in the light because localization of translation markers to T-zones was not seen during *in situ* studies of dark-adapted *C. reinhardtii* cells (Uniacke and Zerges 2007). Sixth, T-ZAMs should contain intermediates in the assembly of PSII and possibly other photosynthesis complexes. These would be subcomplexes with a few subunits, which have been characterized previously (Rokka et al. 2005). Seventh, T-ZAMs should contain the newly synthesized proteins of PSII. To identify T-ZAMs a cellular

subfractionation technique was developed, the cellular subfractions were analyzed biochemically, and the results were compared to the criteria above.

In addition to the predictions made from the *in situ* localization patterns described previously (Uniacke and Zerges 2007), other experiments addressed the possibility that T-ZAMs are involved more broadly in thylakoid membrane biogenesis, and are not only a PSII biogenesis membrane. As well as have the predicted characteristics, a thylakoid biogenesis membrane should also incorporate the synthesis of lipids and chlorophylls. These functions have generally been assigned to the envelope membranes of the chloroplast, reinforcing the claims that the envelope membranes are the region of thylakoid membrane biogenesis (Joyard et al. 1998). For this reason, and to ensure that the envelope membranes were not mistakenly contaminating thylakoid membrane or T-ZAM fractions, after cellular subfractionation marker proteins for the thylakoid membranes, T-ZAMs, and envelope membranes where used to identify where these membrane compartments were. The envelope membrane was identified by the translocons of the outer and inner chloroplasts envelope, Toc75 and Tic110 respectively (Schleiff and Becker 2011).

Materials and Methods

Strains and Culture Conditions

C. reinhardtii wild-type strain 4A+ was cultured photoautotrophically with stirring by magnetic bar in a light intensity of 100 μE m^{-1} s^{-2} and at 24°C in high salt minimal medium (HSM) (Sueoka 1960) with constant aeration. Where indicated, a cell wall deficient strain was used (CC-503), and grown in the same conditions with the addition of 1.0% sorbitol to the HSM. Dark-adapted cultures where incubated in

darkness for 2 h by wrapping flasks in aluminum foil while in mid-long phase of growth (2-4 x 10^6 cells/ml).

Cellular Subfractionation

Cultures (500ml) in mid-log phase of growth (2-4 x 10^6 cells/ml) were pelleted (4,000 x g for 5 minutes at 4°C) and resuspended in 6 ml ice cold Buffer 1 (25 mM $MgCl_2$, 20 mM KCl, 10 mM Tricine pH 7.5 with 1/100 concentration of plant protease inhibitor cocktail (Sigma)). A sample of whole cells was collected at this step and stored at -80°C. Subsequently all manipulations were carried out on ice or at 4°C. The cells were broken by French Press (1,000 p.s.i.; three passages). Complete breakage was confirmed by light microscopy at 400X magnification as the absence of cells and chloroplast fragments. The lysate was centrifuged at 100,000 x g for 1 h at 4°C to pellet membranes and high molecular weight complexes. The supernatant was collected and the pellet resuspended in 2.5 M sucrose (in Buffer 1) in the same tube. A layer of 2.2 M sucrose (in Buffer 1) was layered on top of the 2.5 M sucrose followed by the formation of a 0.5 M to 2.0 M sucrose gradient (in Buffer 1). The gradients were centrifuged at 100,000 x g for 16 h at 4°C. The gradients were collected as c.a. 0.75 ml fractions, while respecting the borders of the visible band of thylakoid membranes. The pellet of the gradient was resuspended in Buffer 2 (60 mM KCl, 20 mM Hepes pH 7.0, 0.2 mM EDTA, 20% Glycerol).

Immunoblot analyses

Immunoblots were used to analyze the fractions of the gradient for protein composition. Equal portions of each fraction were resolved by SDS-PAGE to determine the fraction of the total cellular pool associated with known chloroplast structures: thylakoid membranes and envelope membranes. These were revealed by

monitoring marker proteins for these compartments. Chlorophyll was also used as a marker for thylakoid membranes. Immunoblots were, otherwise, done according to standard techniques and visualized with chemiluminescence. Antisera dilutions used for the immunoblots were: PsaAp 1:20,000 (gift of K. Redding); D2 1:2000 (gift of J. Nickelsen); RBP40 1:1500 (gift of J. Nickelsen); L7/L12 1:2000 (gift of E. Harris); S20 1:2000 (gift of E. Harris); Tic110 1:1000 (gift of E. Schleiff); Toc75 1:1000 (gift of E. Schleiff).

Measuring Concentration of Protein and Chlorophyll

Fractions from the gradients were characterized for their concentrations of protein and chlorophyll. The protein concentration was measured using the Bicinchoninic Acid assay (Smith et al. 1985). The pigments of the fractions were extracted in cold (-20^{o}C) methanol. The samples were then centrifuged at 17,000 x g and the pigments of the supernatant were assayed spectrophotometrically. Measurements were taken at 652 nm and 665 nm (Porra 2002). The proportion of chlorophyll and protein in each fraction was determined.

Measurement of the Chlorophyll Intermediates Protochlorophyllide and Chlorophyllide

Equal proportions of each fraction (100 µl) from a gradient were diluted 50-fold in Buffer 1, mixed, and centrifuged (100,000 x g for 1 h at 4^{o}C) to concentrate the membranes. The pellets were then resuspended in Buffer 1. Acetone was added to a final concentration of 80% (vol/vol) to solubilise the pigments and membranes. Hexane was added to separate the polar pigments from the non-polar pigments. The acetone phase (lower) was then collected and its pigment content quantified using an AMINCO-Bowman Series 2 Luminescence Spectrometer at an excitation of 440nm.

Peaks at 633 nm and 677 nm correspond to protochlorophyllide and chlorophyllide respectively (Pineau et al. 1986).

Blue Native (BN)-PAGEs

BN-PAGEs were preformed as described by (Schägger and von Jagow 1991) with slight variations. The membranes from gradient fractions containing 100 µg of protein were diluted in Buffer 1, concentrated by ultracentrifugation (100,000 x g; 1 h; 4°C) and resuspended in ACA 750 (750 mM aminocaproic acid, 50 mM Bis-Tris, and 0.7 mM EDTA pH 7). Membranes were then solubilized on ice in 0.8% n-dodecyl-β-d-maltoside (β-DM) for 30 min. The soluble material was then separated from the insoluble material by centrifugation at 17,000 x g in a microcentrifuge for 25 min at 4°C. The supernatant was collected, transferred to a new 1.5 ml microfuge tube. Loading buffer, a 6X solution of 0.5% Coomassie Brilliant Blue G-250, 750 mM aminocaproic acid and 1.5% β-DM, was added to the supernatant. The protein complexes were then separated on a 4.5% – 12% acrylamide gel, containing 0.5 M aminocaproic acid and 50 mM Bis-Tris HCl pH 7.0, overnight with voltages ranging from 60V – 300V at 4°C. The gels were subsequently stained with Coomassie Blue (Sambrook and Russell 2001).

In vivo radio pulse-labelling of newly synthesized proteins by chloroplast ribosomes

To label the newly-synthesized proteins in the chloroplast, cultures of a cell mutant strain (CC-503) were grown until mid-log phase in HSM (with 1% sorbitol) as described above. The cells were harvested by pelleting (4,000 x g for 5 min at 4°C) and then resuspended in HSM made with HPLC water. The cells were then allowed to recover by exposure to a fluorescent lamp for 15 minutes. Then 750µl of [^{35}S] H_2SO_4 was added (1.5 mCi). The pulse labelling was done for 5 minutes at 12°C before

Buffer 1 was added and the cells were broken by bead beating three times for 50 seconds with 1 minute intervals on ice in a 2 ml conical screw cap microfuge tube. The supernatant of the lysate was collected and the beads were washed with Buffer 1, before the lysate was pelleted (100,000 x g for 1 h at 4^{o}C). The supernatant was then collected and the pellet resuspended in 2.5 M sucrose (with Buffer 1). A gradient was formed above the 2.5 M sucrose cushion as described earlier, and it was centrifuged for 16 h at 100,000 x g at 4^{o}C. The gradient was collected in 0.75 ml fractions with cut-off blue-tip pipettes and, as previously, the pellet of the gradient was resuspended in Buffer 2. Proportional amounts of every fraction were used to and denatured in protein loading buffer (Laemmli 1970) and the proteins were separated by 12-18% SDS-PAGE with 4 M urea which was stained with Coomassie Blue, shrunk in 50% methanol (v/v) and 10% acetic acid (v/v) to concentrate the signal, dried for 1 h at 80^{o}C with gradual heating, and then exposed to a phosphorimager screen and scanned with a Typhoon.

In organello Protein Labelling of Isolated Spinach Chloroplasts

Chloroplasts from spinach (*Spinacia oleracea*) were also used in protein labelling experiments. The leaves of spinach were homogenized by blending three times for 20 sec in a Warring Blender in Buffer 3 (0.35 M sucrose, 25 mM Hepes pH 7.8, and 10 mM EDTA pH 8.0). The homogenate was filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 4,000 x g for 3 min at 4^{o}C to pellet chloroplasts and other organelles and debris. Two cycles of filtering and pelleting were used to separate the chloroplasts from the homogenate. Finally, the chloroplasts were resuspended in 0.33 M sorbitol and 35 mM HEPES pH 7.8 and the chlorophyll content was measured so chloroplasts could be diluted to 200 µg/ml of chlorophyll. The 4X Labelling Mix (Buffer 4) was then added to a final concentration of 0.35 M

sorbitol, 43.5 mM Hepes pH 7.6, 10 mM $MgCl_2$, 1 mM 3-PGA, 0.4 mM spermidine (freebase), 1 mM Mg-ATP, 10 mM DTT, 40 µM of each amino acid excluding methionine and cysteine. In a 1 mL reaction, 10 µL (10 µCi) of a mix of [^{35}S]methionine and [^{35}S]cysteine (PerkinElmer EasyTag Express Protein Labelling Mix) was added, as well as 1/1000 concentration of cyclohexamide to inhibit any translation competent cytosolic polysomes with 80S ribosomes. Protein synthesis by 70S chloroplast ribosomes, which are resistant to cycloheximide, was allowed to continue for 15 min or 5 min at 4^{o}C and halted with the addition of 1/1000 concentration of chloramphenicol. The temperature was maintained at 4^{o}C for the duration of the pulse in an effort to reduce the speed of migration of the newly synthesized subunits. Chloroplasts were then pelleted and resuspended in 5 mM $MgCl_2$, and vortexed vigorously for 2 min to lyse them hypotonically. The lysate was made to a final concentrations of 25 mM $MgCl_2$, 20 mM KCl, 10 mM Tricine-HCl pH 7.5 and 1% of plant protease inhibitor cocktail (Sigma), before the membranes were pelleted by ultracentrifugation (100,000 x g; 1 h; 4^{o}C). The pellet was then resuspended in 2.5 M sucrose (in Buffer 1) and a linear sucrose gradient from 0.5 M - 2.0 M was formed above it. The gradient was then centrifuged for 16 h at 100,000 x g (4^{o}C). The gradient was divided into three fractions: envelope membranes, thylakoid membranes, and T-ZAMs, and the pellet was resuspended directly in SDS-PAGE protein loading buffer. The membranes of the three fractions were diluted in Buffer 1 and re-pelleted (100,000 x g; 1 h; 4^{o}C) before being resuspended in SDS-PAGE protein loading buffer. The proteins were then separated on a 12% acrylamide gel. The gel was stained, shrunk, and dried and then exposed to a phosphorimager screen. Radiolabelled proteins were revealed by a Typhoon phosphorimager.

In vivo pulse [^{14}C] Acetate Labelling of lipids of *C. reinhardtii* cells

In vivo labelling was done on whole cells of *C. reinhardtii* to indentify the site of lipid synthesis and concurrently the site of protein synthesis was examined. 2.5 x 10^7 cells in mid-log phase were collected from HSM cultures, concentrated in 500 µL of HPLC-HSM, and acclimatized under a fluorescent lamp for 15 min. The labelling was allowed to continue for 5 min at 12^{o}C in the presence of 40 µCi of [^{14}C] acetate (Sodium acetate, [1,2-^{14}C], 1 mCi/ml, ViTrax, VC258). Then Buffer 1 was added, and chloramphenicol was added to a 1/1000 dilution to end the reaction. The cells were then broken by two 50 sec agitations in a bead beater at maximum with glass beads and placed for 1 minute on ice during the interim. The supernatant was collected and centrifuged (1 h; 100,000 x g; 4^{o}C) to pellet the membranes. The membranes were resuspended in 2.5 M sucrose (in Buffer 1) followed by isopycnic separation by sucrose gradient ultracentifugation as described above. The gradient was not collected in 0.75 ml fractions, but the different membrane types were collected separately. A methanol:chloroform extraction was then performed on the fractions collected from the sucrose gradient to isolate the lipids and proteins from the sample (Seigneurin-Berny et al. 1999). The lipids from the chloroform phase were concentrated by successive cycles of air drying and resuspension in chloroform. The concentrated lipids were then applied to a silica gel plate for fractionation by thin-layer chromatography (TLC). Plates were placed in a glass tank containing TLC buffer (85 ml chloroform, 15 ml methanol, 10 ml acetic acid, 3 ml water). The proteins were resuspended Laemmli buffer and separated by 12% SDS-PAGE, which was stained with Coomassie Blue (Sambrook and Russell 2001). Both the TLC plate and the gel were exposed to phosphorimager screens and signals were revealed by Typhoon phosphorimager.

Results

Isolation of Membranes by Sucrose Density Gradient Centrifugation

Previously, T-zones were localized to the lateral regions of the pyrenoid in the chloroplasts of *C. reinhardtii* by *in situ* localization of RB38/RBP40 and other translation factors (Uniacke and Zerges 2007). Comparison with PsaAp and HSP70B, marker proteins for thylakoid membranes and stroma, respectively, established that T-zones are contiguous with the stroma but overlapped the thylakoid membranes. To isolate membranes, with the newly synthesized proteins, trials with continuous and discontinuous sucrose density gradient centrifugation were performed. When membranes are floated from a 2.5 M sucrose layer, non-membrane associated proteins either pellet or remain soluble in the 2.5 M sucrose. This ensures that membranes and membrane-associated material are analyzed in the gradient fractions. To ensure that soluble proteins did not mix with the gradient during its formation a layer of 2.2 M sucrose was layered on the 2.5 M cushion prior to the formation of the gradient above. A linear sucrose gradient was then formed above the layer of 2.2 M sucrose. This gradient ranged from 0.5 M to 2.0 M sucrose. Visual inspection showed that the majority of green membranes formed a band located approximately half-way up the gradient. However, a small but significant amount of green membranes remained below the major band (Fig. 1). Even more faintly, above the major green band, there was a faint yellow band of membranes, corresponding to the expected density and colour of chloroplast envelope membranes (Keegstra and Yousif 1986).

Distribution of Marker Proteins for T-zones and Thylakoid Membranes in Gradients from Cells Cultured in Moderate Light

Each fraction from the gradient and the pellet were examined for the presence of thylakoid protein markers PsaAp and D2, to determine where the thylakoid

membranes were in the gradient. These marker proteins were concentrated in fractions derived from the major green band (Fig. 1, fractions 6-9), and from here on is referred to as the thylakoid membranes. Only trace levels of PsaAp and D2 were detected in the fractions 10 - 2.5 M. D2 was detected in the pellet, suggesting that this protein dissociated from the membranes in handling, or there were some unbroken or dense chloroplast fragments. However, this result suggests that fractions 10-12, and the 2.2 M and 2.5 M do not correspond to either granal or stromal thylakoid membranes. The whole gradient was also examined for T-zone marker proteins: both ribosomal subunits and RB38/RBP40. These marker proteins were found in the thylakoid membranes, but also, in almost equal levels, in the gradient fractions 10-12 (Fig. 1). The 2.2 M layer and 2.5 M cushion of the gradient also contained T-zone markers, as did the pellet. These results confirm that fractions 10-12 fulfill with the first three predictions of T-ZAMs: 1) they are enriched for both the large and small the ribosomal subunits; 2) and RB38/RBP40; and 3) they have much lower levels of PsaAp and D2, suggesting they are not thylakoid membranes. Because these three criteria have been met these fractions which are denser than thylakoid membranes will from now be called putative T-ZAMs (pT-ZAMs).

Chlorophyll and Protein Concentration of Gradient Fractions

The chlorophyll and protein concentration of the gradient fractions were used to compare the relative amounts of thylakoid membrane and pT-ZAM. Thylakoid membrane fractions of the gradient contained between 10% and 30% of the total chlorophyll of the gradient, while those of the pT-ZAMs had below 5% (Fig. 1). The protein distribution of the gradients was more even than the distribution of chlorophyll, and in the pT-ZAM fractions the proportion of total protein exceeded the proportion of total chlorophyll. Nonetheless, the pT-ZAM fractions could be

considered minor compared to the thylakoid membrane fraction in accordance with the fourth prediction.

Effect of Dark-Adapting Cells on Protein Distribution

In situ studies showed that localization of the translation marker proteins to the T-zones is most evident in cells under moderate light intensity and rare in cells adapted to darkness for two hours (Uniacke and Zerges 2007). To determine whether the amount pT-ZAMs showed a similarly light-dependent pattern, a culture in mid-log phase under moderate light was shifted to the dark for two hours before cell breakage. Fractions from gradients obtained using the he dark-adapted cells showed the same thylakoid membrane pattern of D2 and PsaAp (Fig. 2, fractions 6-9). However the dark-adapted cells had fewer T-zone marker proteins in both the thylakoid membranes and the pT-ZAMs. Only comparatively small amounts of the L7/L12 or RB38/RBP40 associated with membranes (Fig. 2, fractions 9 and 10). S20 was the only T-zone marker found throughout the thylakoid membranes, and was also concentrated at the interface between the thylakoid membranes and pT-ZAMs (fractions 9 and 10) and in the 2.5 M sucrose fraction. Trace amounts were found in fraction 11 and the 2.2 M sucrose fraction (Fig. 2). The other T-zone markers were found at the interface between the thylakoid membranes and pT-ZAMs (Fig. 2, fractions 9 and 10), and were concentrated in the 2.5 M fraction, with slight amounts in the 2.2 M fraction and in the pellet. This pattern of membrane association of the T-zone markers was reproduced twice in four attempts. It confirms prediction five from the *in situ* studies that the T-ZAMs are inactive in dark-adapted cells.

Protein Complexes of the pT-ZAMs

Blue Native-PAGE (BN-PAGE) was used to analyze the protein complexes of the pT-ZAMs and compare them to the thylakoid membranes. The methodology of BN-PAGEs necessitated that equal amounts of protein are loaded in each lane, therefore, unlike the analysis of gradients described above, fractions are not loaded proportionately and the pT-ZAMs are over-represented. This technique facilitated analysis of complexes by visual inspection and Coomassie stain. A 1-2 M sucrose gradient was used, and the fractions were pooled to ensure there was enough material and that the entire gradient was represented. Visual inspection of the BN-PAGE did not reveal any difference between the thylakoid (Fig. 3, left panel, lanes 2-3 and 4-5) membranes and pT-ZAMs (lanes 9-10 and 12-13). After staining with Coomassie Blue (Fig. 3, right panel) protein complexes, were disproportionately concentrated in either the thylakoid membranes (lanes 2-3 and 4-5) or the pT-ZAMs (lanes 9-10 and 12-13). Though there were many equally shared complexes, complex 1 was shown to be largely concentrated in thylakoid membranes, whereas complex 2 was largely in the pT-ZAMs. This was confirmed by immunoblot analysis of BN-PAGEs and 2D BN/SDS-PAGEs (conducted by Dr. Schottkowski, Appendix 1), suggesting that in accordance with prediction six the pT-ZAMs have free core RCC subunits of PSII.

Site of Protein Synthesis in Chloroplasts

The site of protein synthesis in chloroplasts was examined by radio-labelling the newly synthesized proteins with [^{35}S] methionine and [^{35}S] cysteine in both whole *C. reinhardtii* cells and isolated spinach chloroplasts. After labelling, the cells or chloroplasts were treated similarly, both were broken, the membranes pelleted, and then separated on sucrose gradients. Proteins in the gradient fractions were denatured and resolved by SDS-PAGE. In isolated spinach chloroplasts this revealed enriched levels of radiolabelled signal in the T-ZAMs compared to their low abundance (Fig.

4). After two successful attempts, this result could not be repeated, nor could it be reproduced using *C. reinhardtii* cells. *C. reinhardtii* cells failed to show any pattern of radiolabel enrichment in the pT-ZAMs. This lack of newly synthesized proteins in the T-ZAMs invalidated the seventh prediction from the *in situ* identification of T-Zones. Despite this, because six of the seven predictions were corroborated, from here on, the pT-ZAMs will be considered T-ZAMs.

Site of Lipid Synthesis in Chloroplasts

To examine where the major acyl lipids of the chloroplast membrane bilayers (MGDG and DGDG) are synthesized in *C. reinhardtii*, radio pulse labelling experiments were carried out with [^{14}C] acetate. These membrane types were found in the envelope membranes, the thylakoid membranes, and the T-ZAMs. The 2.2 M sucrose fraction, the 2.5 M sucrose cushion and the pellet were also collected for analysis. The lipids were isolated from the fractions, and resolved on a TLC plate (Fig. 6). Exposing the TLC to a phosphorimager screen revealed a distinct shift in the concentration of newly synthesized lipids between the pulse and pulse-chase (Fig. 5, see Appendix 2 for quantification). In the pulse the envelope membranes, thylakoid membranes, and T-ZAMs had stronger MGDG bands than DGDG. After an hour long chase period all three membrane fractions (envelope membranes, thylakoid membranes, and T-ZAMs) had stronger DGDG bands than MGDG bands. As MGDG is the precursor to DGDG synthesis (van Besouw and Wintermans 1978), this suggests that the synthesis of lipids is taking place in all three membrane fractions, which are able to supply themselves with the lipids necessary for maintenance. Similarly, the shift seen in the envelope fraction suggests that galactolipids of these membranes are synthesized locally. In the T-ZAMs there is a slight shift between MGDG and DGDG from the pulse to the pulse-chase, which suggests that some

synthesis, necessary for biogenesis, could be talking place, but there is no enrichment of newly synthesized lipids in the T-ZAMs. It should be noted, because envelope protein markers were later found in the thylakoid membranes in is not clear if there is enrichment of MGDG and DGDG in the thylakoid membranes. This is considered later in the discussion (Fig. 9, 10, and see below)

Chlorophyll Precursors in Gradient Fractions

To consider if T-ZAMs had a wider role in thylakloid biogenesis and confirm the site of chlorophyll synthesis protochlorophyllide and chlorophyllide, the precursors of chlorophyll were measured. After an acetone:hexane extraction, the non-polar lipid phase was excited with light of 440 nm wavelength and the peaks at 633 nm and 677 nm, representing protochlorophyllide and chlorophyllide, were measured with a spectrofluorometer. The results revealed that most of the chlorophyll precursors are in the thylakoid membrane fractions with lesser amounts found in the T-ZAMs (Fig. 8). None were found in the fractions above the thylakoid membranes which have the density of chloroplast envelope, the accepted location of chlorophyll synthesis (Joyard et al. 1998). Some chlorophyll precursors were found in the thylakoid membrane fractions contaminated with envelope (Fig. 9, 10, and see below). This later result caused a reassessment of the chlorophyll precursor localization (see discussion).

Thylakoid Membrane and T-ZAMs Association of Envelope Proteins

Immunoblot analysis was also used to determine the location of Tic110 and Toc75 in the gradient fractions. It was revealed that these envelope proteins markers cofractionated with the densest thylakoid membranes (Fig. 9), with little or none found in the fractions of lower buoyant density above the thylakoids membranes

where envelope membranes are expected (Keegstra and Yousif 1986; Becker et al. 2004; Eggink et al. 2004). This localization of Tic110 and Toc75 to the lower thylakoid membrane fractions raises doubt about the confidence of the results regarding the locations of lipid synthesis and chlorophyll precursor. It should also be noted that in rare and poorly understood conditions Tic110 and Toc75 cofractionate with both the thylakoid membranes and T-ZAMs, as well as in the expected envelope location (Fig. 10). This will be considered more in the discussion.

Discussion

The cytological localization of PSII biogenesis and the organization of thylakoid membrane biogenesis, have been examined using a unique cellular subfractionation technique to identify membranes, which were characterized by immunoblot analysis, BN-PAGE, and other techniques. Immunoblot analysis showed that the high density fractions of the sucrose gradient contained membranes enriched in T-zone markers, suggesting that these membranes are T-ZAMs. The T-ZAMs were not thylakoid membranes, because the protein marker for thylakoid membranes were found in lighter sucrose density fractions (Fig. 1). The T-ZAMs were present in moderate light condition, and absent in dark-adapted cells in which T-zones are absent (Fig. 2). Further analysis by Blue Native-PAGE revealed that these dense membranes contained free PSII subunits and PSII assembly intermediates, suggesting that the T-ZAMs are the site of *de novo* PSII synthesis and insertion into membranes (Fig. 3 and Appendix 1). Furthermore, immunoblot analysis following cellular subfractionation showed the cofractionation of envelope membranes and thylakoid membranes (Fig. 9 and 10). The localization of chlorophyll synthesis was also determined to be at the thylakoid membranes, though some may also take place in the T-ZAMs (Fig. 8). The major site of lipid synthesis in chloroplasts was also identified as the thylakoid

membranes, though lipids are synthesized in minor amounts in both the T-ZAMs and envelope membranes of chloroplasts (Fig. 5). Together, these results support a need to re-examine where the components of PSII are synthesized and assembled for the *de novo* assembly of the complex.

Recent work suggested that PSII biogenesis occurs in a compartment that is distinct from the thylakoid membranes (Uniacke and Zerges 2007; Nevo et al. 2007; Stengel et al. 2012; and reviewed in Komenda, Sobotka, and Nixon 2012). The isolation and identification of a compartment located near the plasma membrane where PSII biogenesis takes place has been achieved in *Synechocystis* (Schottkowski et al. 2009). Using similar techniques in *C. reinhardtii* a membrane compartment, called Low-Density Membranes, associated with mRNA translation has been identified (Zerges and Rochaix 1998). By extending the techniques of these studies we have isolated novel membranes with T-zone characteristics, which may be involved in *de novo* PSII biogenesis (Fig. 1). These novel membranes, the T-ZAMs, contain chlorophyll but lack the thylakoid marker proteins D2 or PsaAp. Conversely, there is an abundance of a translation factor specific to the chloroplast encoded *psbD* gene encoding the D2 subunit of PSII, RB38/RBP40 (Barnes et al. 2004; Schwarz et al. 2007), and ribosomal proteins in the T-ZAMs, all of which were found co-localized in the T-zones (Uniacke and Zerges 2007). The absence of thylakoid protein makers and the presence of T-zone protein markers in the biogenesis conditions of moderate light fulfill the first three criteria for T-ZAMs: that they should have the large and small chloroplast ribosomal subunits as well as the translation factor RBP40/RB38, but shouldn't be thylakoid membranes (Fig. 1). The fifth prediction of T-ZAMs, that T-ZAMs shouldn't be present in the dark, is fulfilled since in dark-adapted cells the T-zone protein markers are reduced in these novel, high density membranes (Fig. 2).

The lack of thylakoid membrane protein markers in the T-ZAMs indicates that the thylakoid membranes and T-ZAMs are distinct membrane types. A protein marker unique to the T-ZAMs has not been identified, however under conditions of photoautotrophic growth in moderate light, T-ZAMs are enriched in T-zone markers relative to other membrane fractions (Fig. 1). The levels of translation markers in T-ZAM fractions are approximately the same as those in thylakoid membrane fractions, despite T-ZAM fractions containing less protein and chlorophyll compared to thylakoid membrane fractions (fulfilling the fourth prediction of T-ZAMs) (Fig. 1). It should be emphasized that there is a drastic enrichment of T-zone markers on the basis of mass amount of membrane, as between 2- and 8-fold less protein of the T-ZAM fractions was analyzed than thylakoid membrane fractions when proportional amounts of material were analyzed from the gradient fractions. It is possible that some T-ZAMs co-fractionate with thylakoid membranes. This does not imply a strong contamination of T-ZAMs with thylakoid membranes as only trace amounts of thylakoid membrane markers were detected in the lower part of the gradient where the T-ZAMs have been identified. Another possible reason that markers of translation are found in the thylakoids is that many of them are localized to the stromal thylakoid membranes where repair synthesis of photodamaged of D1 occurs even at low light intensity (Tyystjärvi and Aro 1996; van Wijk, Andersson, and Aro 1996). After 2 h of darkness the few markers of translation that remained associated with membranes were concentrated in the gradients at the transition between T-ZAMs and thylakoid membranes (Fig. 2).

The sixth prediction, the presence of PSII assembly intermediates in the T-ZAMs, a strong prediction of a PSII biogenesis membrane compartment, was confirmed using BN-PAGEs (Fig. 3 and Appendix 1). This result was first suggested

by staining of BN-PAGEs, and confirmed by immunobloting of BN-PAGEs and BN/SDS-PAGEs (conducted by Dr. Schottkowski). The immunoblots revealed that the T-ZAMs had free subunits of PSII which were not present in thylakoid membranes, though both T-ZAMs and thylakoid membranes had PSII assembly complexes.

Finally, as protein radio-labelling experiments where successful in determining the location of D1 repair synthesis (van Wijk, Andersson, and Aro 1996), a seventh prediction was made to resolve where PSII biogenesis and, possibly, lipid and chlorophyll synthesis takes place in chloroplasts. Protein radio-labelling experiments were conducted followed by the newly-developed cellular subfractionation system, to determine the location of *de novo* PSII subunit synthesis. Using chloroplasts from spinach, results were initially encouraging. Despite little apparent protein in the T-ZAMs when stained, autoradiography revealed an enrichment of newly synthesized proteins in the T-ZAMs (Fig. 4). This was repeated twice in spinach chloroplasts, however, these were results were not reproducible, in either spinach chloroplasts or *C. reinhardtii*. Nor were the newly synthesized proteins chased out of the T-ZAMs to the thylakoid membranes, thus the assembly pathway between T-ZAMs and thylakoid membranes could not be confirmed in either spinach or *C. reinhardtii*. Further studies however suggested that only the initial steps of PSII biogenesis take place in the T-ZAMs (Appendix 1 and 4). These translation and assembly steps maybe on the order of 2-3 min (Rokka et al. 2005), thus seeing the newly synthesized proteins in the T-ZAMs before they migrate out may be prohibitively difficult.

Although determining the site of *de novo* protein synthesis through protein pulse-radio-labelling remained elusive, the site of lipid synthesis in chloroplasts was illuminated using a similar analysis, and suggested a new location for broader

thylakoid membrane biogenesis. The radio-labelling of newly synthesized lipids showed the majority of newly synthesized MGDG in the thylakoid membrane fraction of chloroplasts. After a chase, the majority of DGDG, which is the subsequent step of the biochemical pathway, was also in the thylakoid membranes (Fig. 5). The lower part of these membranes are contaminated with the translocon apparatus of the envelope membranes (Fig. 9 and 10). This suggests that the lipid synthesis in the thylakoid membrane could be from the envelope membrane. The alternative location of lipid synthesis to the thylakoid membranes seems more likely. This location corroborates previous evidence for the location of radio-labelled galactolipids in chloroplasts which only considered the envelope membranes and thylakoid membranes (Joyard et al. 1980). The authors concluded that synthesis was taking place in the envelope membranes, despite the signal first appearing in the thylakoid membranes, as either the reaction was too fast to capture or the envelope membranes were contaminating the thylakoid membranes. The T-ZAMs however are not a major site of lipid synthesis, and are unlikely to supply the thylakoid membranes with either MGDG or DGDG, and thus not a region of broader thylakoid biogenesis (Fig. 5).

The original location of chlorophyll intermediates to the envelope membranes of chloroplasts was previously established with only cursory examination of thylakoid membranes, but has been used to support the hypothesis that chlorophyll precursor are synthesized in the envelope membranes and transported to the thylakoid membranes, where the last step of synthesis occurs (Pineau et al. 1986; Block et al. 2007). Recently this model has been reconsidered by exhaustive analyses, which examined the chlorophyll intermediates of the envelope membranes, the thylakoid membranes, and the soluble stroma phase. In both *Beta vulgaris* and *Cucumis savitus* 98.5-99% of the protochlorophyllide was found in the thylakoid membranes, with the remainder, 1-

1.5%, found in the envelope membrane fractions (Mohapatra and Tripathy 2003; Mohapatra and Tripathy 2007). This suggested to the authors that chlorophyll synthesis occurs in the thylakoid membranes. The authors further concluded, due to the lack of intermediates in the soluble stromal phase, that it is unlikely there is trafficking of intermediates between membrane compartments in the chloroplasts (Mohapatra and Tripathy 2007). The presence of chlorophyll intermediates in the thylakoid membranes was largely confirmed by our own results, though some intermediates were also found in the T-ZAMs of the chloroplast (Fig. 8). The presence of chlorophyll intermediates in the T-ZAMs suggests that some chlorophyll is synthesized there for the purpose of co-translational insertion into the *de novo* synthesized subunits of PSII. It is unlikely that envelope membrane contamination is affecting this result as chlorophyll intermediates were found in all thylakoid membrane fractions, even those fractions where Tic110 and Toc75 were not seen (Fig. 9, fractions 7-8). Thus, it seems, chlorophyll synthesis is not only localized to the T-ZAMs or envelope membranes, but more broadly distributed throughout the chloroplast in thylakoid membranes as well.

The suggestion that MDGD and DGDG are also synthesized in the thylakoid membranes of the chloroplast is not surprising, because they are the major lipid constituent of the thylakoid membranes and no mechanism of lipid transfer from the envelope membranes to the thylakoid membranes has been identified in the chloroplasts (Kroll et al. 2001; Vothknecht et al. 2012). However, the enzymes which catalyze the final step in both MDGD and DGDG biosynthetic pathway were found in the envelope membranes (Froehlich, Benning, and Dörmann 2001). These contradictions might be resolved by the observation that both enzymes in the galactolipid synthesis pathway were also found in the thylakoid membranes, but, like

previous studies, the authors considered that the thylakoid membranes were contaminated with envelope membranes (Joyard et al. 1980). It should also be noted that acyltransferases used in the synthesis of thylakoid membrane lipids have been found in the thylakoid membranes *in situ* by histochemical staining and EM (Michaels, Jelsema, and Barrnett 1983).

The isolation of chloroplast envelope membranes has long been a settled issue despite the lack of unambiguous evidence. To identify a chloroplast membrane fraction after isolation, proteins of the translocation apparatus are used. These proteins, however, are also found in the thylakoid membrane fractions, despite loading equal protein amounts of the fractions and thus over-representing envelope fractions (Joyard et al. 1982; Schnell, Blobel, and Pain 1990; Froehlich, Benning, and Dörmann 2001). This discrepancy has been ignored because thylakoid membranes are considered to be contaminated with envelope membranes, without further explanation (Froehlich, Benning, and Dörmann 2001). Recently, this hypothesis has begun to be overturned as results suggest a more complex cytological localization of the translocation apparatus. The four isoforms of Tic20, which had been identified as a channel forming protein (Kovács-Bogdán et al. 2011), have been independently found in different membrane sub-compartments (Machettira et al. 2011). This study examined proportional amounts of each fraction of a gradient by immunoblotting and did not over represent the envelope membrane fractions. This has established that a majority of the envelope membrane translocons co-fractionate or have the same density as the thylakoid membranes (Fig. 9 and 10). This contamination led to the re-examination of previous work on envelope localization and chloroplasts membrane organization. Concomitant with this reassessment the *in situ* localization of Tic110 and Toc75 under biogenesis conditions was examined. This revealed that the

translocation apparatus of the envelope localized to regions adjacent to the T-zones (conducted by Dr. Schottkowski, see Appendix 3).

Early studies of the translocation apparatus of the chloroplast envelope membranes showed a strong association between the inner and outer membranes, which were not dependent on active protein translocation (Akita, Nielsen, and Keegstra 1997; Nielsen et al. 1997). The contact sites between the inner and outer membrane of the chloroplast have long been suggested as the sites of active translocation, and it is speculated they are analogous to membrane contact sites in mitochondria (Dobberstein, Blobel, and Chua 1977; Schnell, Blobel, and Pain 1990). Recently, the membrane contact sites in mitochondria between the inner and outer mitochondria membrane have been characterized at the molecular level. A complex, which includes the mitochondrial translocons, maintains contact sites and determines the cristae architecture, and has been postulated to be involved in mitochondria membrane biogenesis (Harner et al. 2011; Hoppins et al. 2011; von der Malsburg et al. 2011; and reviewed in van der Laan et al. 2012). The *in situ* localization of Tic110 and Toc75 adjacent to T-zones and co-fractionation with thylakoid membranes, suggests that chloroplasts envelope membrane contact sites may be more extensive in *C. reinhardtii* than previously believed and may include thylakoid membranes via a protein bridge. It is also possible that envelope membranes are denser than previously described or our preparation and handling of the membranes affected their density. This connection would facilitate the insertion of membrane proteins, particularly LCHII and OEC, into the thylakoid membranes where they assemble with nascent PSII complexes. The *in situ* examination of the localization of Tic110 and Toc75 suggested that under biogenesis conditions, the translocation apparatus of the envelope membranes forms distinct regions called the ‘import-envelopes’ at the lobe

junctions of *C. reinhardtii*. This import-envelopes, composed of multiple membrane contact sites between in the inner and outer envelope membranes, may then also associate the with the thylakoid membranes, forming protein bridges which are maintained during cellular subfractionation. The formation of import-envelopes and protein bridges near the T-zones would facilitate the assembly of nuclear and chloroplast synthesized proteins in a spatio-temporal pathway into fully functional PSII (see Appendix 6 for further discussion).

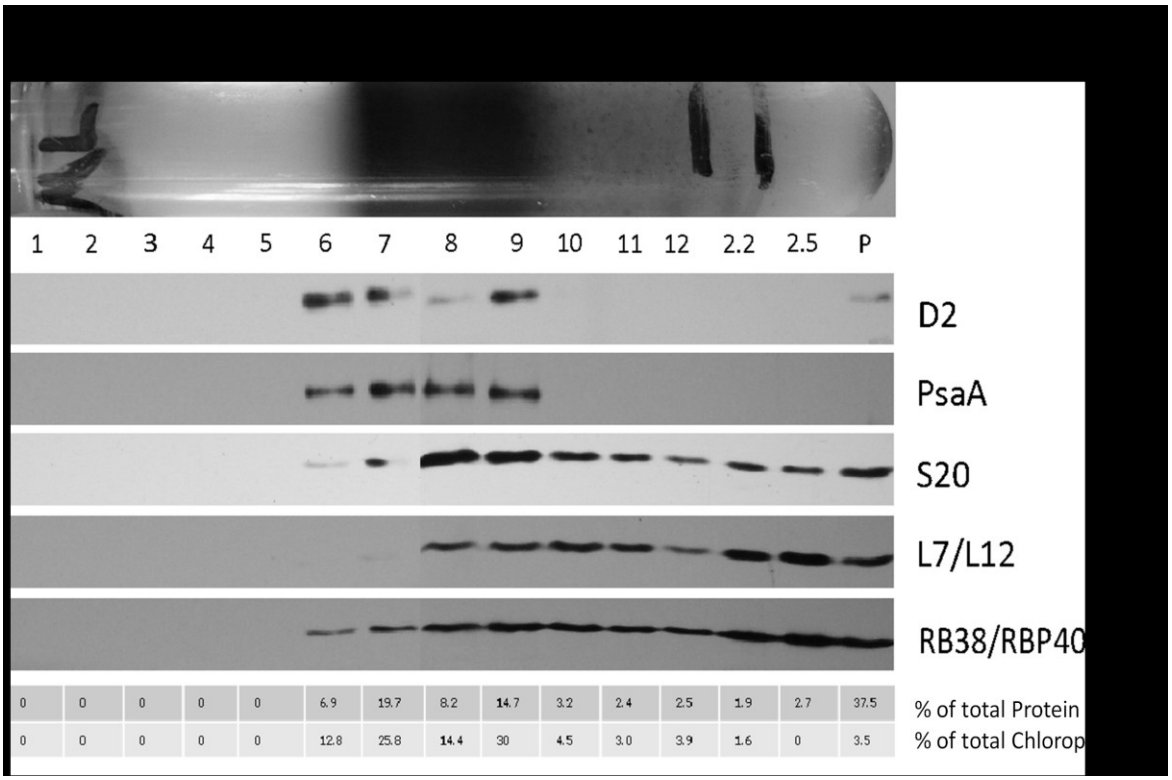

Figure 1: Distribution of Marker Proteins in Cells Maintained in Moderate Light Conditions. A gradient of a culture grown in constant moderate light (top) was examined by immunoblot for marker proteins of the thylakoid membranes (D2 and PsaAp) and T-zones (L7/L12, S20, and RB38/RBP40) after division into 0.75 ml fractions. The fractions were also examined for chlorophyll and protein content, here expressed as a percentage of the total found on the gradient (bottom).

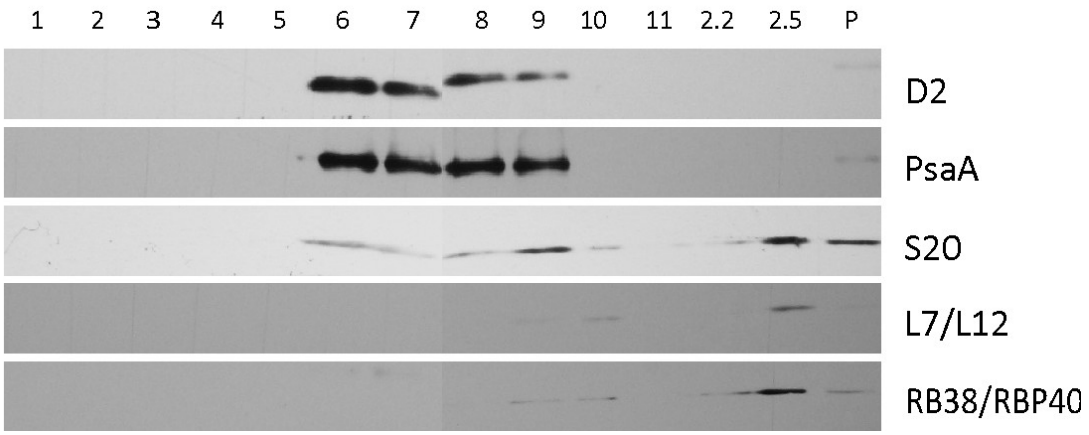

Figure 2: Distribution of Marker Proteins in Membranes from Dark-Adapted Cells. A gradient of a culture treated with 2 h of darkness was examined by immunoblot for marker proteins of the thylakoid membranes (D2 and PsaAp) and T-zones (L7/L12, S20, and RB38/RBP40) after division into 0.75 ml fractions.

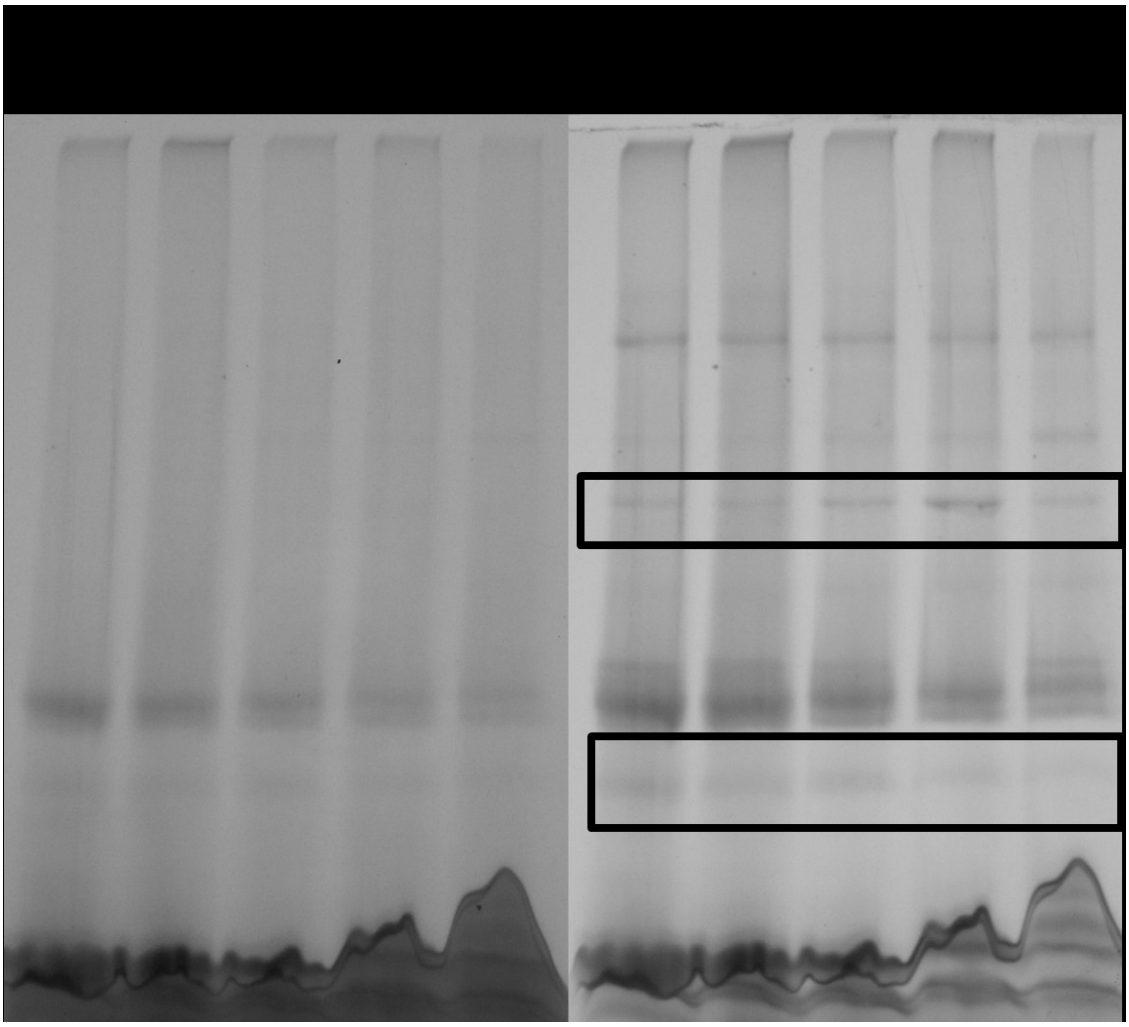

Figure 3: Blue-Native PAGE of Complexes from Fractions of a Gradient. The membranes of cells from a culture grown in constant moderate light was separated on a gradient of 1.0-2.0 M sucrose and the protein complexes of the fractions were examined by BN-PAGE; unstained on left, stained with Coomassie Blue on right. High-lighted are bands which vary through the gradient. The lower band, complex 1, is stronger in the thylakoid membranes. The upper band, complex 2, is stronger in the T-ZAMs. (See Appendix 1 for further confirmation.)

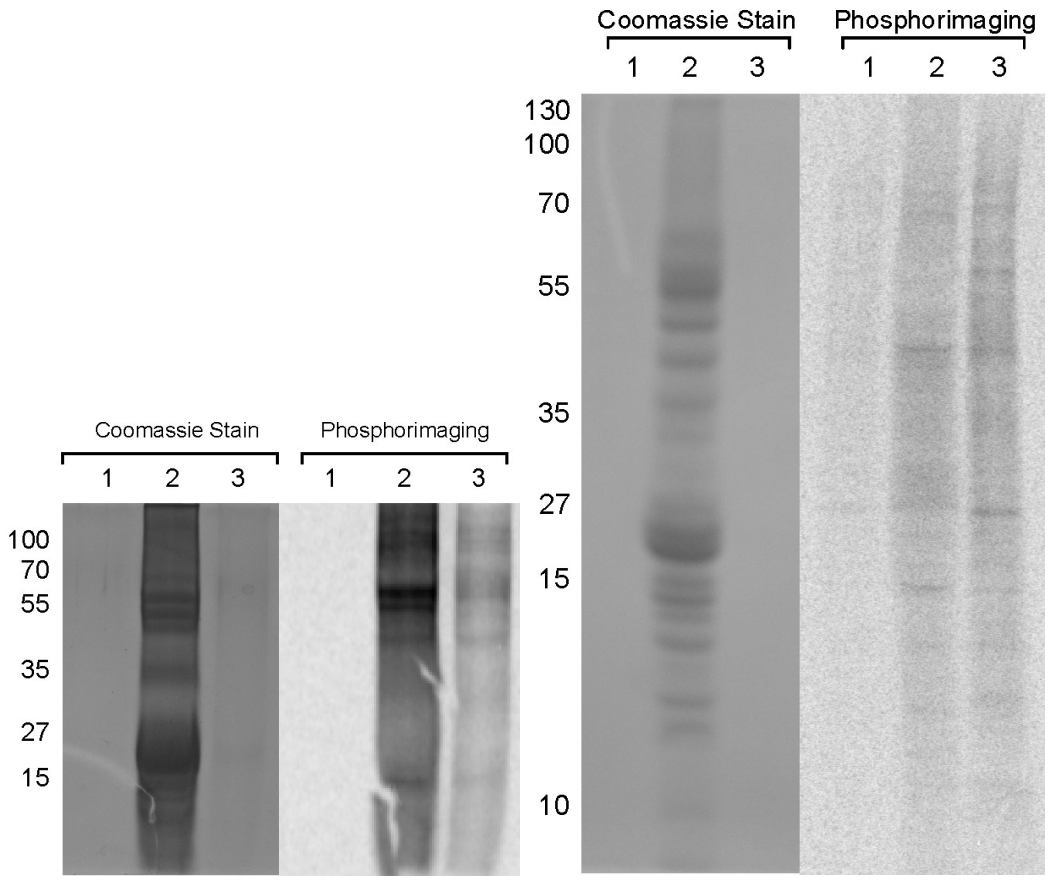

Figure 4: *In organello* Radiolabelling of Proteins from the Chloroplasts of Spinach. Spinach chloroplasts were isolated and then labelled with [^{35}S] methionine and [^{35}S] cysteine for 15 minutes (left) or 5 minutes (right). The membranes of the chloroplasts were then separated by floatation on sucrose density gradient ultracentrifugation. The gradient was divided into envelope membrane (1), thylakoid membrane (2), and T-ZAMs (3) fractions. The proteins from these fractions were then separated by SDS-PAGE, which was stained with Coomassie Blue and then exposed to a phosphorimager screen.

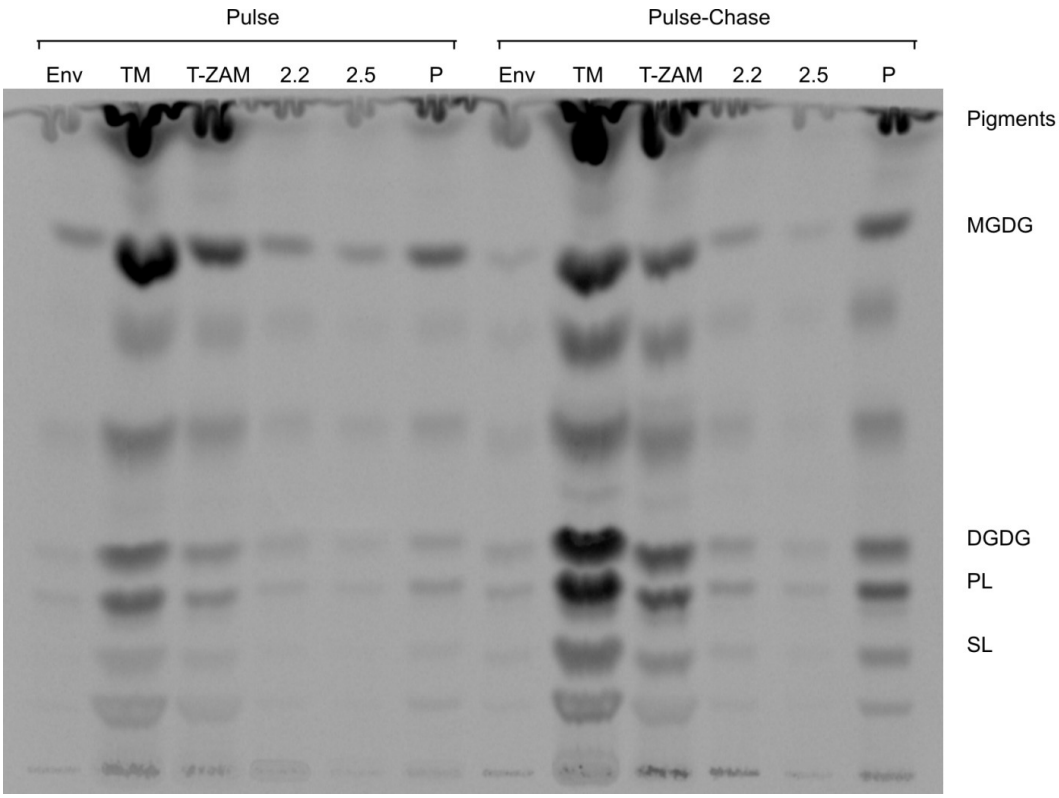

Figure 5: *C. reinhardtii in vivo* Radio-labelling of Chloroplast Lipids, Pulse and Pulse-Chase. Whole cells of *C. reinhardtii* were radio-labelled with [^{14}C] acetate for 5 minutes (left) or radio-labelled for 5 minutes followed by a chased for 1 hour (right) before the cells were broken and the membranes fractionated by sucrose density gradient ultracentrifugation. A TLC plate (see Fig. 6) was used to fractionate the isolated lipids of the gradient fractions before exposing it to a phosphorimager screen. The major bands include: chlorophyll and carotenoids (pigments), monogalactosyldiacylglycerol (MDGD), digalactosyldiacylglycrol (DGDG), phospholipids (PL), and sulpholipids (SL).

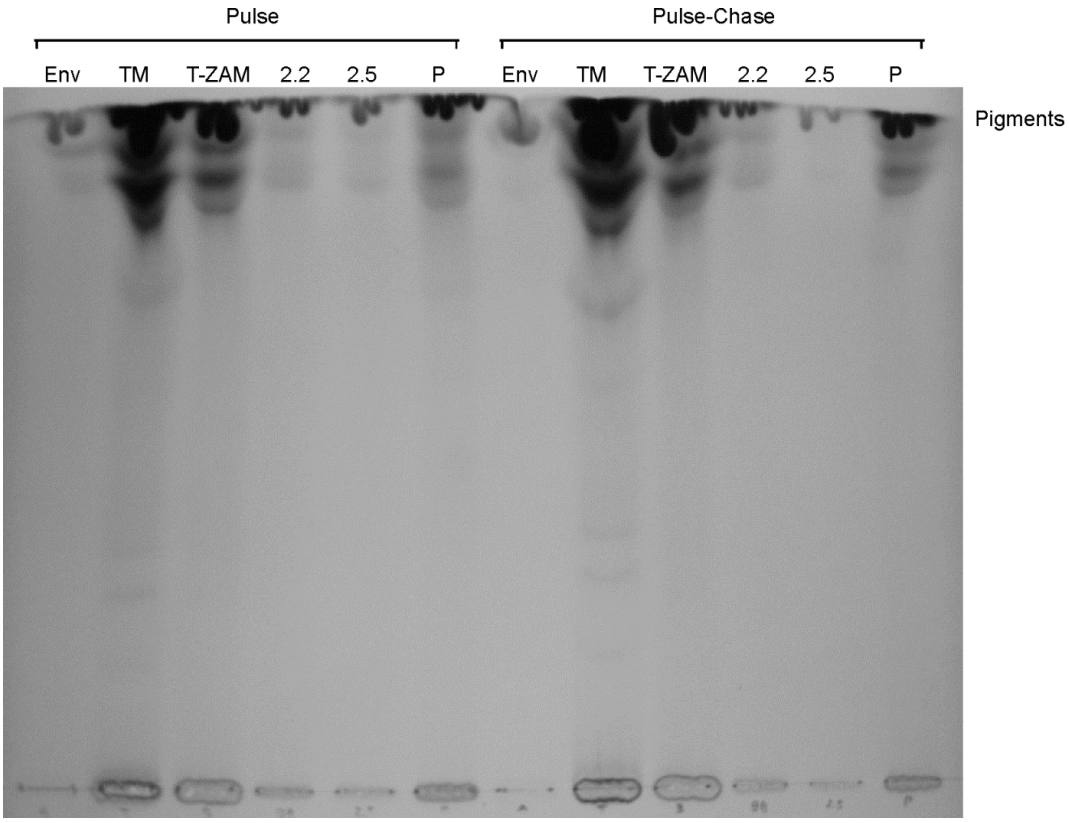

Figure 6: Unexposed TLC plate of *C. reinhardtii in vivo* Radio-labelling of Chloroplast Lipids, Pulse and Pulse-Chase.

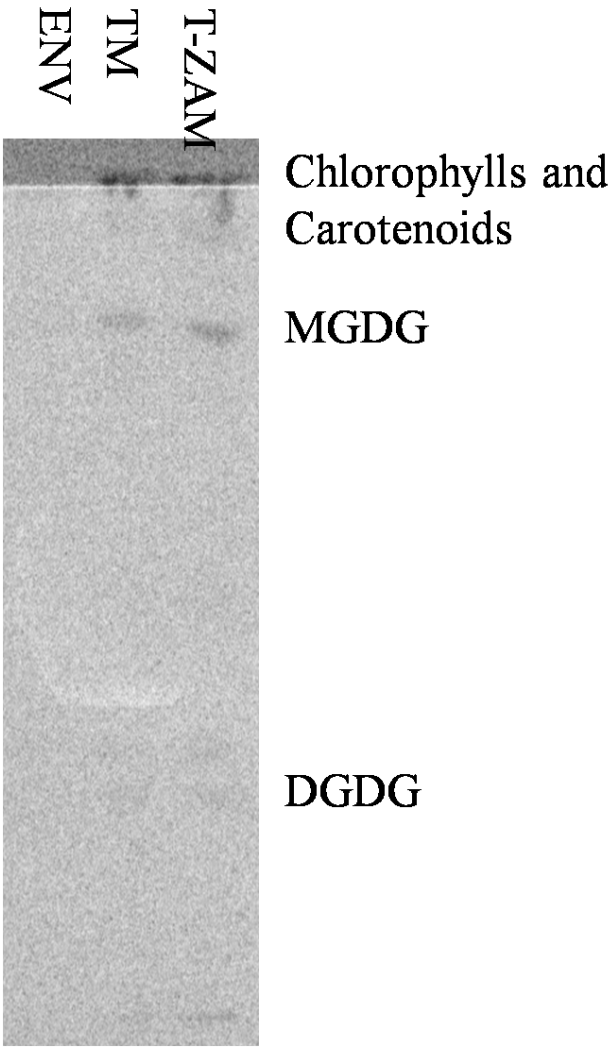

Figure 7: *C. reinhardtii* Whole Cell Radio-labelling of Chloroplast Lipids, Pulse. Whole cells of *C. reinhardtii* were exposed to [^{14}C] acetate for 5 minutes; the membranes were separated by sucrose density gradient ultracentrifugation and divided into the Envelope Membranes (ENV), Thylakoid Membranes (TM), T-zone associated membranes (T-ZAM). A TLC plate was used to separate the lipids before exposure to a phosphorimager screen. The major bands include the chlorophyll and carotenoids, monogalactosyldiacylglycerol (MDGD), digalactosyldiacylglycrol (DGDG).

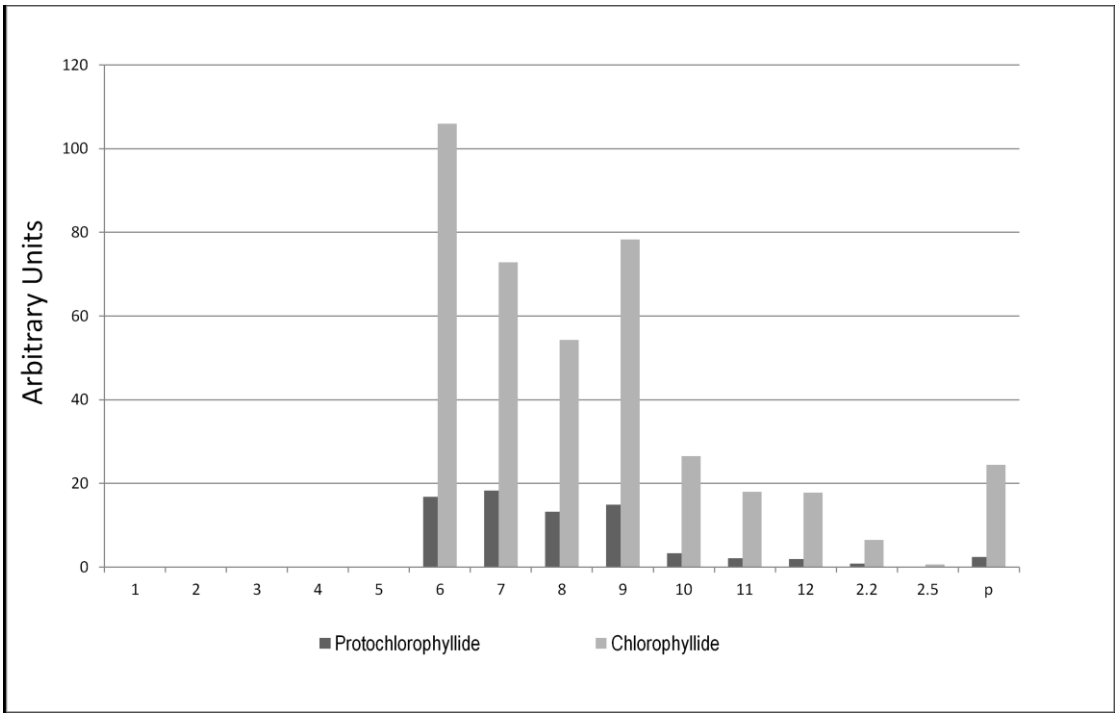

Figure 8: Distribution of Chlorophyll Precursors. The fractions collected following sucrose density gradient ultracentrifugation were tested for the chlorophyll precursors chlorophyllide and protochlorophyllide. Thylakoid membrane fractions (6-9) had the most chlorophyll precursors, while T-ZAM fractions (10-12) contained some chlorophyll precursors.

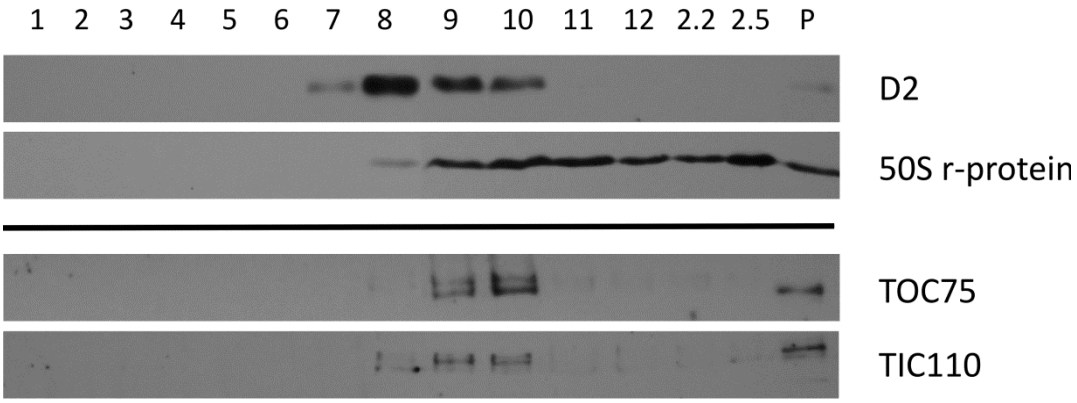

Figure 9: Distribution of Envelope Membrane Protein Markers. Membranes from cells grown in moderate light conditions were separated on a gradient, which, after collection in 0.75 ml fractions, was examined by immunoblot for thylakoid membrane (D2), envelope membrane (Toc75 and Tic110), and T-zone (50S r-subunit) marker proteins.

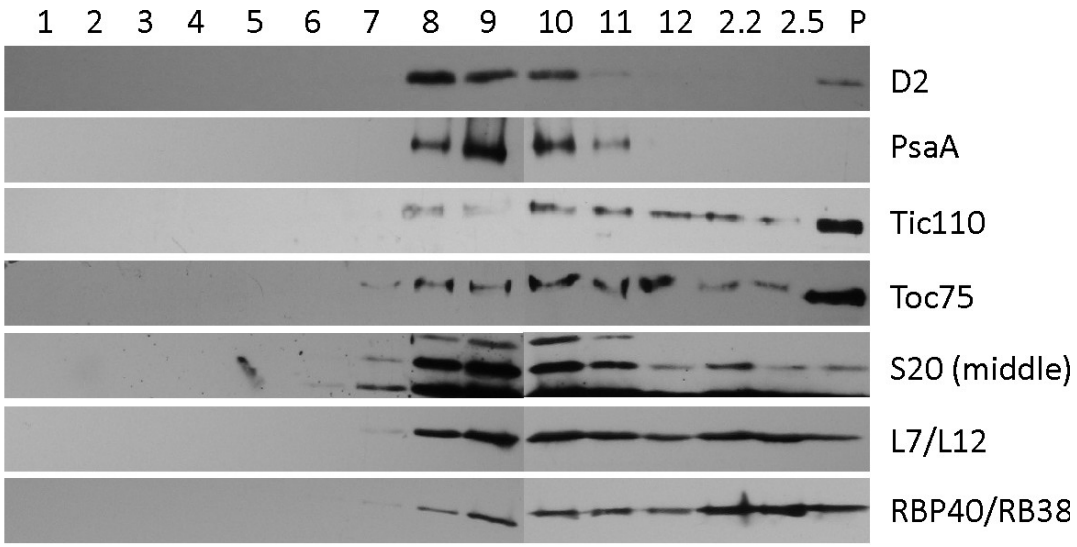

Figure 10: Distribution of Envelope Membrane Marker Proteins II. Membranes from cells grown in moderate light conditions were separated on a gradient which were examined by immunoblot for thylakoid membrane (D2 and PsaA), envelope membrane (Tic110 and Toc75), and T-zone (L7/L12, S20, and RB38/RBP40) marker proteins.

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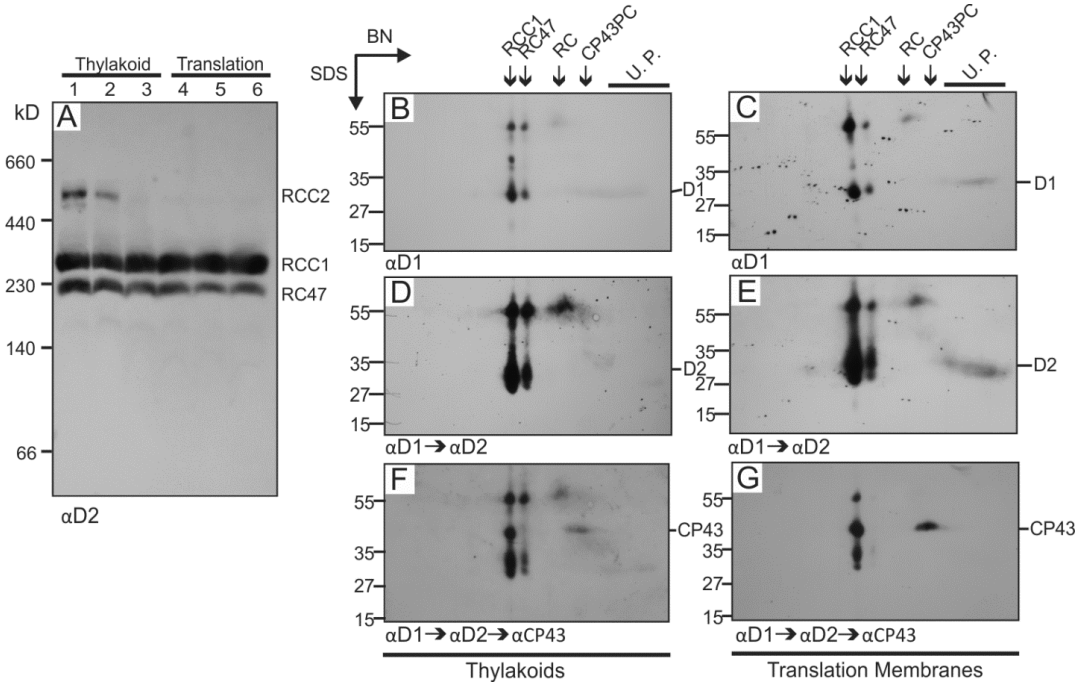
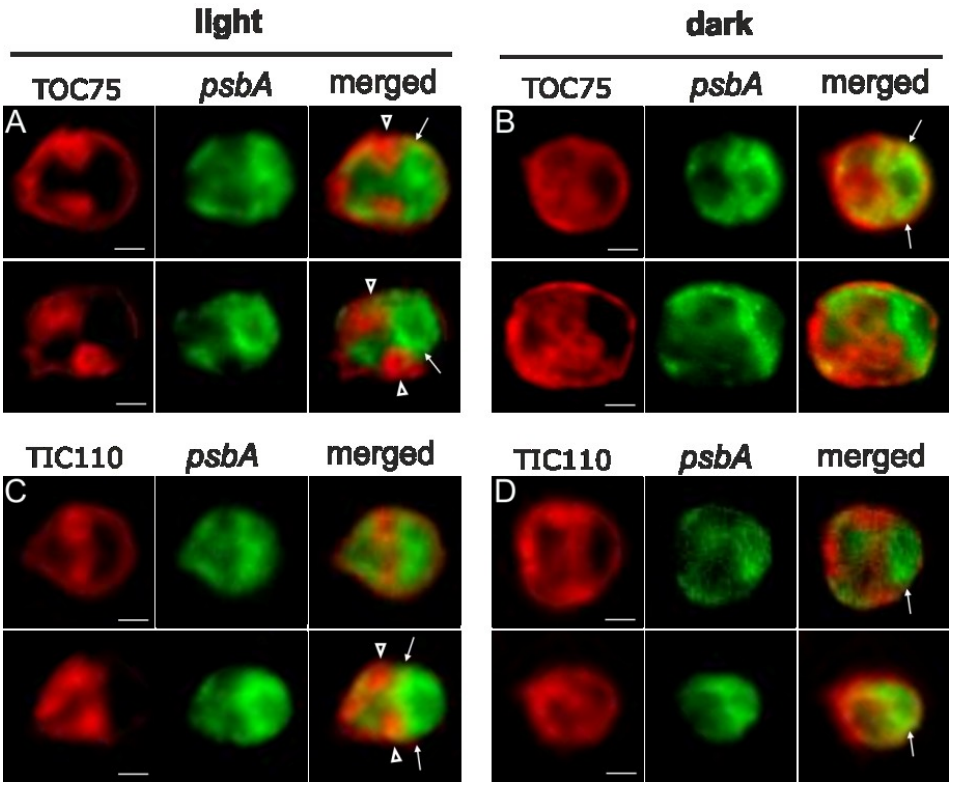


Appendix 1: 2D BN/SDS-PAGE of Thylakoid Membranes and T-ZAMs. A) A BN-PAGE showing the qualitative difference between thylakoid membranes and T-ZAMs (Translation). The T-ZAMs don't accumulate the photosynthetically active PSII dimer. B, D, and F) A blot of a 2D gel of thylakoid membranes sequentially probed for D1, D2, and CP43. C, E, and G) A blot of a 2D gel of T-ZAMs sequentially probed for D1, D2, and CP43. Free D1 and D2 accumulate in the T-ZAMs, and there is more of the CP43 pre-complex (conducted by Dr. Schottkowski).

Appendix 2: Quantification of Fig. 5 Lipid Pulse and Pulse-Chase.

	MGDG		DGDG	
	Pulse	Pulse-Chase	Pulse	Pulse-Chase
Env	40	23	17	22
Thy	157	118	61	115
BT	89	73	35	75
2.2 M	36	32	18	28
2.5 M	32	19	16	18
P	64	68	23	54



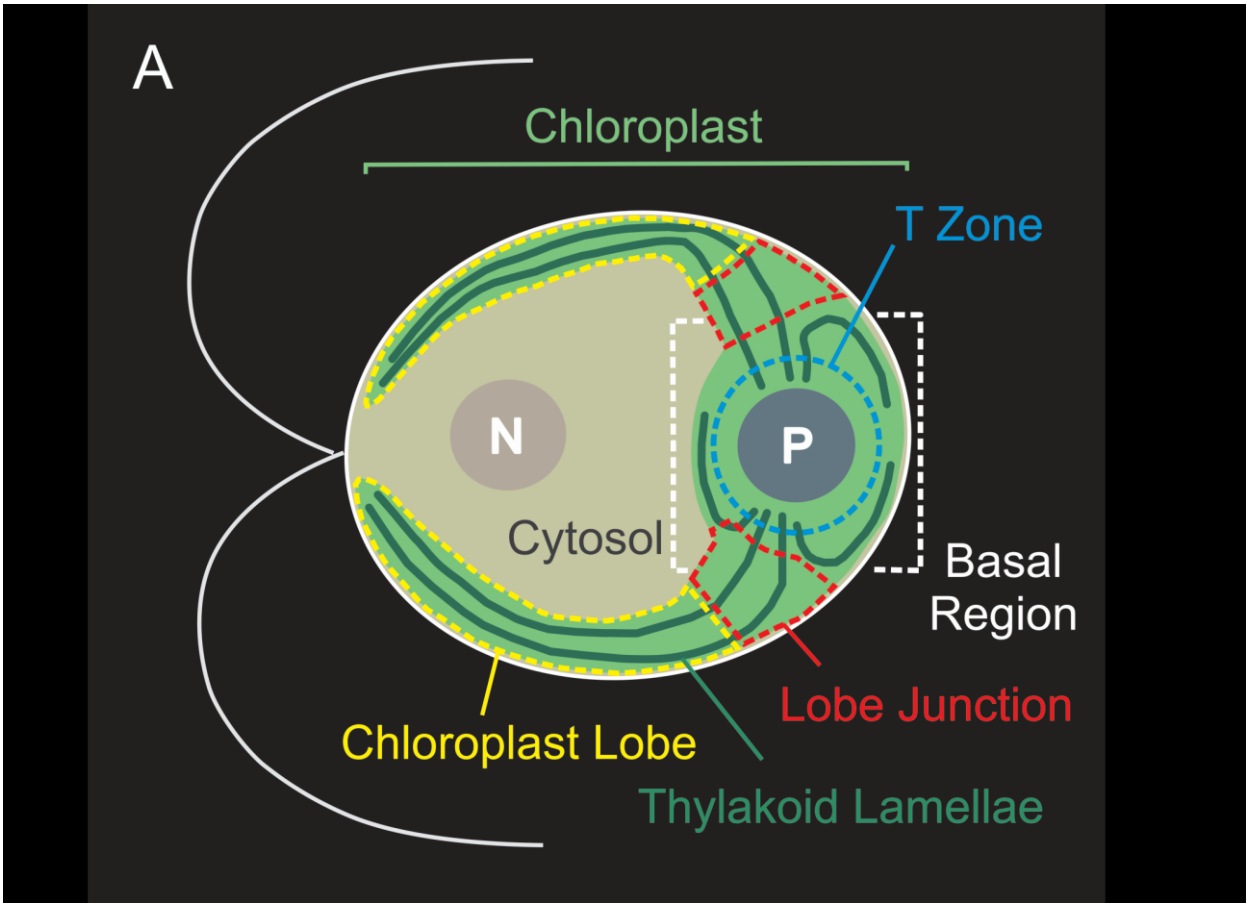
Appendix 3: *In situ* Localization of Tic110 and Toc75. The import apparatus of envelope membranes was localized to regions adjacent to the T-zones in moderate light conditions (A and C) termed the 'import-envelope'. In dark-adapted cells neither the T-zone nor the import-envelope pattern appeared (B and D) (conducted by Dr. Schottkowski).

Appendix 4:

The lysis conditions for the chloroplasts isolated from spinach cells were determined empirically, and a light microscope at 400X magnification was used to confirm lysis. The methods of lysis considered were: Yeda Press, Dounce Homogenizer, 5 mM $MgCl_2$ with and without vortexing, and 2 mM EDTA with and without vortexing. The conditions which lysed the chloroplasts most thoroughly were 5 mM $MgCl_2$ and 2 mM EDTA both with vortexing. As ribosome subunit attachment is partially dependent on Mg^{2+} concentration 5 mM of $MgCl_2$ with vortexing was used for the lysis of chloroplasts (Chua et al. 1973).

In addition, the use of chloramphenicol before the breakage of *C. reinhardtii* cells was considered because it is an antibiotic which stops translating ribosomes on RNA, and might affect the distribution of ribosomes in the gradient. This was done by adding 1/1000 concentration of chloramphenicol to cultures for 10 min before the breakage of cells. The breakate was then treated as previously and the samples collected from the gradient as previously. There, however, was no effect of chloramphenicol on the distribution of ribosomes through the gradient, nor was there an effect on the distribution T-ZAMs.

Appendix 5: *C. reinhardtii* cell schematic.



Appendix 6: Manuscript.

The cytological organization of photosystem II biogenesis

in Chlamydomonas

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Running title: cytological organization of PSII biogenesis

Classification: BIOLOGICAL SCIENCES/ CELL BIOLOGY

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Abstract

The photosynthetic electron transport system of chloroplasts is composed of complexes of polypeptides which are encoded by two genomes. Nuclear genome-encoded polypeptide subunits are synthesized in the cytoplasm, imported across the chloroplast envelope, and assembled with subunits encoded by the plastid genome. The latter include integral membrane subunits synthesized directly into chloroplast membrane by plastid ribosomes. Previous work revealed translation zones in the chloroplast and cytoplasm as privileged locations of the synthesis of PSII-LHCII polypeptides. Our results reveal two novel chloroplast membranes in these regions. A "PSII translation membrane" in the chloroplast was revealed by a cellular subfractionation scheme to be a platform for the synthesis and membrane insertion of chloroplast genome-encoded subunits. "Import envelope" was identified is a specialized domain of the chloroplast envelope by immunofluorescence microscopy. Light-responsive changes in the localization of the protein import machinery reveal the chloroplast envelope is dynamic. The locations and protein compositions of these membranes support a model for the spatiotemporal organization of PSII-LHCII biogenesis. The findings build upon growing evidence for complex organization of biogenesis processes and provide a cytological context and biochemical methods for research into photosystem II biogenesis and other problems of chloroplast biology.

\body **Introduction**

Membrane biogenesis requires the concerted synthesis and localization of the correct lipids, proteins, and cofactors. In the cytosol of eukaryotic cells, the endoplasmic reticulum organizes these processes to form new membranes, which are then routed to the correct intracellular compartment: e.g. an organelle, the nuclear envelope, or the plasma membrane (1). Chloroplasts and mitochondria, by contrast, have internal membranes that arise within these organelles; the photosynthetic thylakoid vesicles and inner membrane, respectively. This distinction probably reflects the early evolution of these semiautonomous organelles as free-living bacteria prior to endosymbiosis with progenitor eukaryotic cells. While our understanding of the ER is advanced and rapidly expanding, little is known about the spatial organization of the pathways that underlie the biogenesis of the inner mitochondrial membrane and thylakoids (reviewed by 2-4).

Thylakoids are the network of flattened vesicles that harvest light and use the energy to generate ATP and NADPH. Embedded in thylakoid membranes are the four major complexes of the photosynthesis apparatus: photosystem I (PSI), photosystem II (PSII), the cytochrome b_6/f complex, and the chloroplast ATP synthase. PSII is a model used to study of the general principles underlying the biogenesis of photosynthesis complexes (5). The polypeptide subunits of PSII are encoded by two genomes and expressed by the distinct genetic systems of the nuclear-cytosolic compartments and the chloroplast. The nuclear genome-encoded subunits are synthesized in the cytosol by 80S ribosomes, imported across the chloroplast envelope, and transported to thylakoids. These include the subunits of two complexes bound to the PSII periphery; the light harvesting complex II (LHCII) and the oxygen evolving complex (OEC) (6). Other PSII subunits are encoded by the plastid genome and synthesized by membrane-bound 70S bacterial-like ribosomes in the chloroplast stroma (2). These plastid-encoded polypeptides include the polytopic integral membrane PSII subunits D1, D2, CP43, and CP47. The fully assembled PSII-LHCII supercomplex consists of a dimer of two PSII core complexes, each with an OEC on the thylakoid luminal side and two peripheral LHCII's within the membrane (6, 7). Despite our knowledge of the pathways involved in PSII biogenesis, major gaps exist in our understanding of their cytological organization.

Precisely where plastid-encoded subunits are synthesized is under debate (2, 3). The accepted model holds that their nascent polypeptides are inserted into stroma-exposed

thylakoid membranes, based on demonstrations of thylakoid-bound ribosomes by cellular subfractionation and electron microscopy (reviewed by 2, 3).

An alternative model of synthesis and assembly of PSII subunits in a specific region within the chloroplast of *Chlamydomonas reinhardtii* is based on results of fluorescence confocal microscopy (2, 8, 9). These studies revealed that markers for PSII biogenesis colocalized in specific region within the chloroplast; a "translation (T) zone". These marker proteins were r-proteins of both chloroplast ribosomal subunits, the PSII-specific translation factor RBP40, chloroplast mRNAs encoding PSII subunits, and the D1 subunit in a mutant defective in its incorporation into assembling PSII complexes. In this model the abovementioned ribosomes bound to the thylakoid membranes are synthesizing proteins for processes other than *de novo* PSII assembly e.g. the repair of photo-damaged PSII complexes and the *de novo* assembly of other photosynthesis complexes (8). Thus, we predicted the existence of an unknown membrane located specifically in T-zones functions as a platform for the synthesis and membrane insertion of the plastid-encoded PSII subunits. Because this model is based entirely on *in situ* evidence, its further consideration requires biochemical evidence for such "PSII translation membranes".

Here, we identified PSII translation membranes by developing an analytical cellular subfractionation scheme that generates subcellular fractions containing these membranes according to criteria based on the *in situ* results reviewed above. PSII translation membranes were predicted to: 1) have the T-zone marker proteins (r-proteins of both subunits of the chloroplast ribosome, RBP40) but not most other proteins; 2) be a minor chloroplast membrane, because T-zones occupy a small proportion of the chloroplast; 3) be neither thylakoid nor envelope; 4) have free PSII subunits, assembly intermediates, or both; and 5) not be involved in PSI subunit assembly, because a chloroplast mRNA encoding a major PSI subunit was not recruited to T-zones (8). These and other results provide evidence of a novel spatiotemporal pathway of PSII biogenesis beginning in T-zone-localized PSII translation membranes and progresses outwards to contribute to the functional PSII pool in thylakoid membranes throughout the chloroplast.

Results

Cellular subfractionation reveals PSII translation membranes.

The analytical cellular subfractionation scheme was established to reveal PSII translation membranes by conducting trails to compare different culture conditions and methods of cell breakage and subfractionation and thereby optimize each step to reveal membrane fractions meeting our first criterion: the presence of marker proteins of T-zones (chloroplast r-proteins, RBP40) but not of thylakoid membranes (chlorophyll and subunits of PSI and PSII, PsaAp, and D2, respectively). Immunoblot analyses were conducted on proportional amounts of each fraction in order to determine the distribution of the cellular pool of each protein relative to markers for known compartments (10).

This procedure revealed membrane fractions that met our criteria for PSII translation membranes. From a representative gradient (Fig. 1A), the fractions with r-protein markers of the 30S and 50S subunits of the chloroplast ribosome (8-13) only partially overlapped the fractions with thylakoid membranes (8-10). (Fraction 14 is the 2.5 M sucrose from which membranes were floated.) Thylakoids were identified by their enrichment in chlorophyll, PsaAp, and D2. Notably, the densest region of the gradient (fractions 11-13) had both r-proteins and RBP40, but only trace levels of the thylakoid membrane marker proteins. A role of the membranes in these fractions (8-13) in PSII translation is supported by the specific role of RBP40 in the translation of a chloroplast mRNA encoding the D2 (11). Stroma-exposed thylakoid membranes, a proposed site of PSII subunit synthesis, were not in these densest fractions because the marker protein for them (PsaAp) was predominantly with the thylakoid membranes (fractions 8-10). PSII translation membranes are minor, our second criterion, because fractions 11-13 contained 13% of total protein and 21% of total chlorophyll, compared to 50% and 80% in the thylakoid membrane fractions, respectively. Therefore, it was striking that these densest fractions had a substantial pool of the membrane-associated ribosome subunits and RBP40 and were distinct from the bulk of the thylakoids. PSII translation membranes probably are not involved in PSI subunit synthesis, our fifth criterion, because only thylakoid membrane fractions contained Ycf4p, a protein that binds newly synthesized PSI subunits for the assembly of this complex (Fractions 7-9, Fig. 1C) (12).

PSII translation membranes are physically associated with these T-zone marker proteins as opposed to simply trapping them within vesicles. First, the stroma marker protein HSP70B would also have been trapped but it was not detected in fractions 10-12 (Fig.

1C). Second, RBP40 and the r-protein of the 50S ribosome subunit could be extracted from PSII translation membranes by incubation with agents that remove peripheral membrane proteins (Fig. 2). The 30S subunit r-protein marker protein was considerably more resistant to extraction; it was weakly extracted only by high ionic strength conditions (2.0 M NaCl). In summary, it is most probable that both subunits are membrane-bound, either as assembled translating ribosomes, individual subunits, or both.

Results of blue-native PAGE reveal free D1 and D2 in PSII translation membranes.

For PSII biogenesis, newly synthesized plastid-encoded subunits associate in specific combinations to form three assembly intermediates; the reaction center (RC) with D1 and D2, a CP43-precomplex, and a CP47-precomplex. The RC and CP47 pre-complex associate to form RC47 (reviewed by 5). RC47 associates with the CP43 pre-complex to form the monomeric PSII core complex RCC1. RCC1 dimerizes to form RCC2 and associates with the nucleus-encoded subunits of the OEC and LHCII to form the PSII-LHCII supercomplex. Considering that this process occurs within a few minutes, we predicted that PSII translation membranes would have free PSII subunits, assembly intermediate complexes, or both (13). However, the use these assembly intermediates as markers of *de novo* PSII biogenesis should consider that the PSII damage-repair cycle also involves the synthesis of D1 and the partial disassembly of PSII (14). Therefore, the valid markers for *de novo* assembly are free PSII subunits other than D1 (e.g. D2), the RC, the CP43- and CP47-precomplexes, as well as the absence of RCC2 and the PSII-LHCII supercomplex.

We therefore analysed the assembly states of D1, D2 and CP43 in thylakoid and PSII translation membrane fractions by blue native (BN) PAGE and immunoblot analysis (15). Analyses of equal amounts of membrane were required to have comparable solubilisation conditions. This necessitated over representation of PSII translation membranes on a per cell basis. Thus, we determined the proportion of PSII subunits that were either free or in assembly precomplexes in each fraction using the level of RCC1 as a standard. When immunoblots of BN gels were immuno-probed with antisera against D2, we detected RCC1 and RC47 in thylakoid and PSII translation membrane fractions, while RCC2 was detected only in thylakoid membrane fractions (Fig. 3A). This result reveals that the PSII translation membranes are qualitatively different from thylakoids in their composition of PSII complexes consistent with early stages in PSII biogenesis.

Lanes with thylakoid or PSII translation membranes were subjected to a second dimension of denaturing SDS-PAGE and immunoblot analysis (Fig. 3B-G). In both thylakoid

and PSII translation membrane fractions, D1 was detected in RCC1, RC47, the RC, and as free subunit. RCC2 was not detected in on our 2D gels for unknown reasons. Some D1 was shifted in the gel to higher molecular mass complexes of approximately 40 and 58 kDa, a result of incomplete denaturation prior to the second dimension of SDS-PAGE. This shift was useful because it resolved the RC (shifted) and the free D1 subunit (not). As mentioned above, free D1 is a marker for both repair and *de novo* biogenesis of PSII (8, 16). When the same blots were reprobed for D2, this subunit was detected in the expected positions corresponding to 33 kDa and as the partial denaturation product of 59 kDa (as described above for D1). Most notably, free D2 was detected the PSII translation membrane sample (Fig. 3E) but not the thylakoid membrane sample (Fig. 3D). By contrast, in both samples, D2 signal was detected in the spots corresponding to RCC1, RC47, and the RC, revealing that PSII translation membranes are not privileged sites for intermediate stages in PSII assembly. When the blot was stripped and reprobed with the antiserum against CP43, the CP43 pre-complex was detected at a higher level in the PSII translation membranes (Fig. 3G) than in the thylakoid membrane sample (Fig. 3F). This result indicates that assembly of the CP43 pre-complex occurs predominantly in the PSII translation membranes. Previous studies have detected newly synthesized CP43 in this complex and not as free subunit (13, 17), consistent with the absence of free CP43 here. Together, these results indicate that PSII translation membranes serve as a platform for the synthesis of plastid-encoded PSII subunits and the assembly of the CP43-precomplex.

Envelope membranes containing the TIC and TOC import machinery cofractionate with chloroplast ribosomes and thylakoid membranes.

The inner membrane of the chloroplast envelope is also accessible to chloroplast ribosomes and RBP40 and it has been speculated that thylakoid membranes arise from the inner envelope membrane (reviewed by 2, 18). We presumed that the PSII translation membranes are not envelope because our previous *in situ* analyses for PSII subunit synthesis in T-zones would have detected such a localization pattern (8). Moreover, there are no reports of chloroplast ribosome association with an inner envelope membrane, to our knowledge. Nevertheless, because any cellular subfractionation study should test markers for all potentially relevant intracellular compartments, the gradient fractions were tested for polypeptide subunits of the TOC and TIC protein import complexes of the outer and inner envelope membranes, respectively (19, 20). Although we expected to find these marker proteins in fractions of low density, as reported for envelope membranes (fractions 4-6) (21), they cofractionated with the chloroplast r-proteins and RBP40 in a high-density region of the

gradient, i.e. with PSII translation membranes (Fig. 1B). However, analyses of other preparations revealed Toc75 and Tic110 primarily in thylakoid membrane fractions with low levels in PSII translation membrane fractions (Fig. 1C). Although we do not know the basis, this occasional separation of PSII translation membranes and envelope membranes reveals that these are distinct membrane types.

The chloroplast protein import machinery localizes to novel envelope domains at lobe junctions

To explore the basis of the unexpectedly high density of the envelope membranes with the protein import machinery, we characterized the *in situ* distributions of Toc75 and Tic110 by immunofluorescence (IF) staining and fluorescence microscopy. T-zones were co-stained for the chloroplast *psbA* mRNA with fluorescence *in situ* hybridization (FISH) while weaker, non-localized *psbA* FISH signal co-stained the rest of the chloroplast (Fig. 4).

The results revealed a dramatic localization of Toc75 and Tic110 where the junctions of the two chloroplast domains; lobes and the basal region (Fig. 4A and C, Fig. 5A). We term these regions “lobe junctions”. These results raise the intriguing possibility that novel domains of chloroplast envelope are specialized in the import of nucleus-encoded polypeptides and localized at the lobe junction, termed here “import envelope”. In light of this finding, the results in Fig. 1B and C suggest that import envelope domains have a higher density than the rest of the chloroplast envelope (21).

Light-responsive changes in the localization of the protein import machinery were observed (Fig. 4, compare A&C with B&D). Dark-adaptation of cells for 2 h diminished the localization Toc75 and Tic110 to lobe junctions (Fig. 4B and D). Statistical analyses revealed that 54% of cells from light conditions showed the T-zone localization of the *psbA* mRNA (n= 278). Of these, strong IF-staining of import envelope domains with Toc75 or Tic110 were observed in 73% (n=120) and 79.5% (n=156), respectively. Of the dark-adapted cells, 41% showed the T-zone localization of the *psbA* mRNA (n=149) and, of these, the localized IF-signals of Toc75 or Tic110 were seen in 15.7% (n=89) and 21% (n=60), respectively. Therefore, the localization of the protein import machinery to lobe junctions appears to be dynamic and responsive to light.

Discussion

Our results reveal a complex cytological organization of the dual pathways that supply the polypeptides for the biogenesis of the PSII-LHCII supercomplex. Biochemical and *in situ* evidence support the existence of two novel membrane compartments of the chloroplast. PSII translation membranes were identified on as a platform for the synthesis of plastid-genome encoded PSII subunits and the assembly of the CP43 precomplex. These membranes have higher buoyant density than thylakoid membranes, a range that has not been resolved previously, to our knowledge. Another novel chloroplast compartment was identified; lobe junctions, where we propose that the nucleus-encoded subunits of PSII, LHCII and OEC are imported and assembled upon RCC1 and RCC2, based on the following results. First, lobe junctions are surrounded by envelope domains enrich in the TOC and TIC complexes (Fig. 4A and C). These complexes import the subunits of the LHCII and the OEC (20). Second, previous *in situ* results support localized synthesis of LHCII subunits in the cytoplasm adjacent to lobe junctions (22). Third, import envelope at lobe junctions appears to be distinct from previously described chloroplast envelope membranes in density (Fig. 1Band C) (23). Fourth, a study of the ultrastructure of the Chlamydomonas chloroplast noted “frequent focal fusions of the two membranes of the chloroplast envelope” at a lobe junction (24). Evidence has also been reported for localized import at contact sites of the inner and outer envelope membranes (25). Therefore, we propose that lobe junctions are where nucleus-encoded subunits are imported and assembled upon the newly generated PSII monomers, dimers, or both to form the supercomplex.

The novel import envelope domains of lobe junctions appear to relationships with T-zones. First, the Toc75 or Tic110 IF signal often extended close to the *psbA* FISH signal in a T-zone, although these signals did not overlap extensively (Fig. 4A and C). Second, there was a strong correlation of appearance of both signals in the same cells. Third, the localization of the TOC and TIC complexes to lobe junctions was enhanced by light, a condition associated with an elevated rates of PSII biogenesis and chloroplast protein import in Chlamydomonas and Maize (8, 26, 27) (Fig. 4, compare A&C with B&D). These results support a role of the “import envelope” domains at lobe junctions in importing the nucleus-encoded subunits of PSII, LHCII, and OEC for the biogenesis of the PSII-LHCII supercomplex (Fig. 5C).

Our model proposes that PSII translation membranes in T-zones and the import envelope surrounding lobe junctions are parts of spatiotemporal organization of the PSII-LHCII supercomplex biogenesis (Fig. 5C). In T-zones, PSII translation membranes are a platform for the synthesis and assembly of the plastid-encoded PSII subunits. Chloroplast ribosome subunits and PSII subunit mRNAs are recruited to these membranes by translation

independent mechanisms, e.g. by tethering by membrane bound RNA-binding proteins (22). The small ribosome subunit binds with remarkably high affinity (Fig. 2). It seems reasonable to suppose that such a high affinity interaction is required by components that nucleate the assembly of a translation competent ribosome, such as the small ribosomal subunit. Then, PSII monomers migrate to the lobe junctions where they dimerize and are built upon by nucleus-encoded subunits of PSII and its LHCII and OEC, imported locally by the import envelope domains of the lobe junctions. Thus, lobe junctions are convergence points for the pathways that provide polypeptides subunits encoded by the chloroplast and nuclear genomes. Assembled PSII-LHCII supercomplexes migrate to photosynthetic membranes of thylakoid lamellae which extend into lobes or around the pyrenoid to occupy the periphery of the chloroplast basal region (Fig. 5A). At each stage, migration occurs by lateral diffusion in thylakoid membranes which extend as continuous, elongated lamellae from T-zones to the ends of the lobes at the anterior cell pole, while lamellae wrap around the pyrenoid to the posterior pole of the chloroplast (28). Generality of this model is supported by the identification of a PSII biogenesis compartment in the cyanobacterium *Synechocystis* sp. PCC 6803 and highly localized GFP-tagged Tic20 paralogue at the periphery of the chloroplast envelope in *Arabidopsis thaliana* (29-31). In addition, mitochondria have a compartment analogous to lobe junctions; the cristae junctions, where the outer and inner membranes make contact via interactions involving the protein import machinery (32, 33). The effect of light on the relocalization of the TIC and TOC import machinery to lobe junctions might be relevant to the light stimulation of chloroplast protein import which has been observed in Chlamydomonas and vascular plants (26, 27). Moreover, the Rubisco holoenzyme might be assembled in the pathways described here because its small subunit is imported via the TOC and TIC pathway and the chloroplast mRNA encoding the large subunit localizes *in situ* in T-zones and is translated in association with membrane (22, 34, 35). Our findings build upon growing evidence of complex cytological organizations of biogenesis processes in chloroplasts and cyanobacteria.

Finally, the preliminary evidence suggests that the pathways underlying thylakoid membrane biogenesis are not organized in a single thylakoid biogenesis compartment analogous to the endoplasmic reticulum. PSII translation membranes are probably not a site of PSI subunit synthesis for two reasons: YCF4p does not cofractionate with them and the chloroplast mRNA encoding the PSI subunit PsaAp is not recruited to T-zones (Fig. 1C) (8). Other thylakoid membrane components, lipids and pigments, are synthesized, at least in large part, at the chloroplast envelope (3, 36-38). Therefore, most evidence indicates that the various pathways that synthesize thylakoid membrane components are dispersed to

different chloroplast compartments. In summary, our findings build upon growing evidence of complex cytological organizations of biogenesis processes in chloroplasts and cyanobacteria.

Methods

Culture conditions. *C. reinhardtii* strains, wild-type CC-4051 or the cell wall mutant CC-503, were cultured photoautotrophically in high-salt-minimal medium (HSM), with aeration, at 24^{o}C, under a light intensity of c.a. 100 µE m^{-2} sec^{-1} until mid-log phase (2-4 x 10^{6} cells ml^{-1}) (39).

Analytical cellular subfractionation. Cells from a 500 ml culture were pelleted by centrifugation at 4,000 x g for 5 min at 4°C, resuspended in MKT-buffer (25 mM $MgCl_2$, 20 mM KCl, 10 mM Tricine-Cl pH 7.5, Protease Inhibitor (Sigma-Aldrich). Cells were broken by three passes through a chilled French Pressure Cell at 1,000 psi. Breakage was verified by light microscopy (400 and 1000X magnification). The lysate was ultracentrifuged at 100,000 g for 1 h at 4° C. The supernatant was collected and stored at -80^{o}C. The pellet was resuspended in 2.5 M sucrose, overlaid with a linear sucrose gradient (0.5-2.2 M). All sucrose solutions were prepared in MKT-buffer. The gradient was ultracentrifuged at 100,000 g for 16 h at 4° C. Fractions (0.75 ml) were collected and the pellet was resuspended in KHEG-Buffer (60 mM KCl, 20 mM HEPES, 0.2 mM EDTA, 20% Glycerol). Gradients contained only membrane and associated material based on the buoyant density of bacterial ribosomes in equilibrium CsCl gradient ultracentrifugation (1.67-1.69 g/ml) would be equivalent to an unachievably high sucrose concentration (4.9 M) (40).

Quantification of protein and chlorophyll. Protein concentration was determined using the bicinchoninic acid assay (41). Chlorophyll was quantified as described previously (42).

Immunoblot analysis. Equal proportions of the fractions were solubilized in SDS-PAGE loading buffer, denatured at 42^{o}C for 30 min. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membrane (**Protran, Schleicher** & **Schuell),** and immuno-probed with polycolonal rabbit antisera as described previously (43). The antisera against D1 and CP43 were purchased (Agrisera). The antisera against the r-proteins had been raised against proteins isolated from highly purified *C. reinhardtii* ribosomal subunits (S-20 (30S) and L-30 (50S)) (44). Others were recombinant proteins and reported previously; PsaAp (45), HSP70B (46), RBP40 (11).

FISH, IF staining, and fluorescence microscopy. FISH and IF-staining of cells of strain CC-503 were performed as described previously (8, 47). The *psbA* FISH probes were labeled with Alexa Flour 488 and the IF-staining used Alexa Fluor 568-conjugated anti-rabbit secondary antibody (Invitrogen). Images were captured on a Leica DMI6000B microscope (Leica Microsystems) using a 40x/0.75 objective, a Hamamatsu OrcaR2 camera and Volocity acquisition software (Perkin Elmer). Image analyses used the Colocalization Finder plugin of ImageJ (http://www.rsb.info.nih.gov/ij) (48).

BN/SDS-PAGE. BN-PAGE was performed as described previously with the following minor modifications (15). Aliquots of sucrose gradient fractions containing 6 μg of chlorophyll were concentrated by centrifugation (100,000 g; 1 h; 4 ^{o}C) and resuspended in ACA 750 (750mM aminocaproic acid, 50 mM Bis-Tris, and 0.5 mM EDTA, pH 7.0). Membranes were then solubilized on ice in 0.8% n-Dodecyl-β-D-Maltoside (β-DM) for 5 min. Non-solubilized material and cell debris was pelleted by centrifugation at 13.000 rpm for 30 min in a microfuge at 4^{o}C. The supernatant was added to 1/10 Vol of 5% Coomassie Brilliant Blue G-250 , 750mM aminocaproic acid whereupon protein complexes were then separated by electrophoresis in a 4.5% – 12% acrylamide gel containing 0.5 M aminocaproic acid and 50 mM Bis-Tris HCl pH 7.0. Protein was either transferred to PVDF or used for separation in the 2nd dimension by reducing SDS-PAGE and transferred to nitrocellulose membrane and immuno-probed as described previously (49).

Membrane washing. Aliquots of PSII translation membranes were diluted 25-fold in washing buffer (20 mM KCl, 10 mM Tricine and 2.0 mM EDTA pH 7.2, protease inhibitor cocktail (Sigma-Aldrich)) and pelleted by centrifugation in a microfuge for 1 h at 13,000 x g. Pellets were resuspended in 30 μl of one of the following: washing buffer, 500 mM KCl, 20 mM Na_2CO_3, 1.0 M NaCl, 2.0 M UREA, incubated ice for 30 min, and then centrifuged for 30 min at 13,000 x g in a microfuge. The supernatants were collected and the pellet was washed once to remove residual soluble material and then finally resuspended in 30 μl SDS-PAGE sample buffer. SDS-PAGE and immunoblot analysis were carried out as described previously (43).

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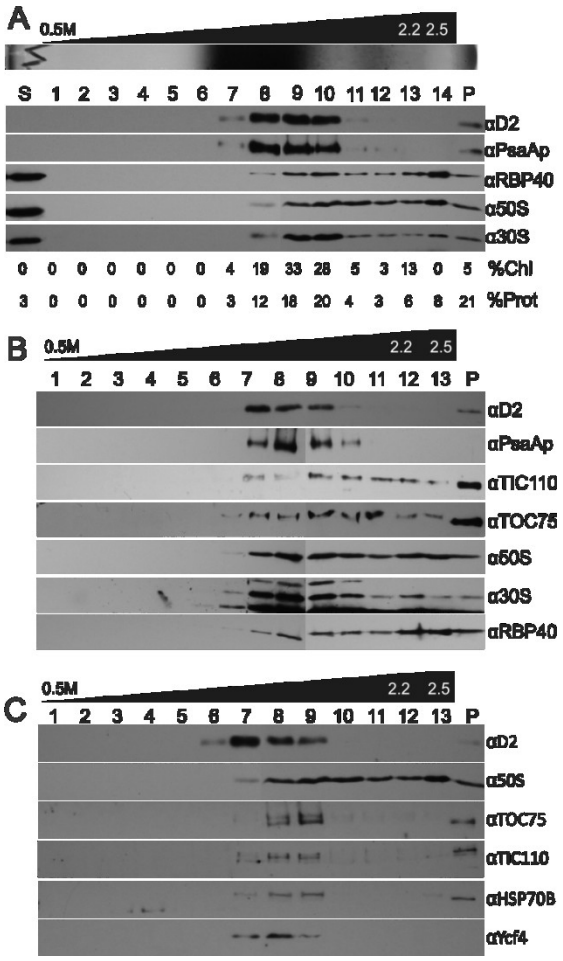


Fig. 1) Immunoblot analyses of cellular subfractions reveals novel membranes with marker proteins for the translation of plastid mRNAs encoding PSII subunits and T-zones.

Each panels shows analyses of a different preparation using the same cellular subfractionation scheme. (A) Fractions were assayed by immunoblot for markers of PSII translation membranes (RBP40, r-proteins for the 30S and 50S subunits of the chloroplast ribosomes) and thylakoid membranes (D2 and PsaAp). Lanes have the supernatant of the initial 100,000 x g centrifugation (SU), fractions from the gradient (1-13), the 2.5M sucrose cushion (14), the pellet (P). The upper image shows the distribution of chlorophyll in the gradient. Percentages of total chlorophyll (%Chl) and protein (%Prot) in each fraction are given. (B) In certain gradients, Toc75 and Tic110 cofractionated with the PSII translation membrane fractions (11-13) while in others (C) they cofractionated with thylakoid membrane (fractions 11-13). Profiles of HSP70B and Ycf4p are shown.

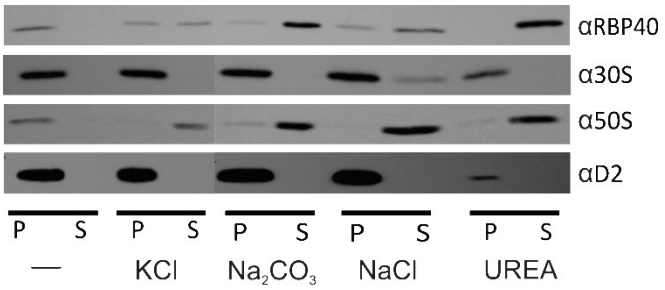


Fig. 2) Membrane association of ribosome subunits and RBP40. Samples of PSII translation membranes were incubated with the agents indicated to allow any release of bound proteins and then membranes were pelleted by centrifugation. The integral membrane protein D2 serves as a marker for membranes and could be detected by long exposure times. Comparisons of the non-membrane supernatant (S) and membrane pellet (P) by immunoblot analysis revealed the degrees of extraction of RBP40 and the marker r-proteins of the 30S and 50S subunits of the chloroplast ribosome.

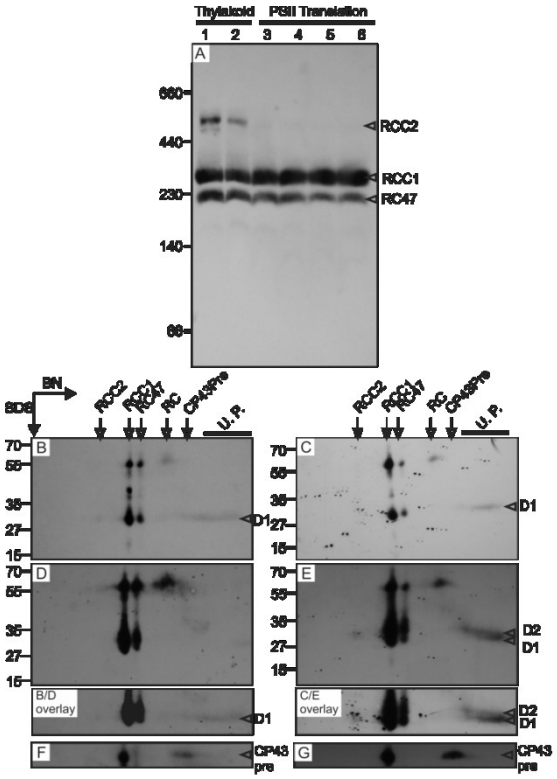


Fig. 3) Blue-Native PAGE reveals PSII subunits in early assembly stated in PSII translation membranes.

One dimensional BN-PAGE analysis of the assembly states of the PSII subunit D2 in sucrose gradient fractions with thylakoids (lanes 1-2) and PSII translation membranes (lanes 3-5). RCC1 levels are similar because samples were loaded on the basis of equal chlorophyll to ensure comparable solubilization conditions and use RCC1 as a standard for qualitative analyses. Two dimensional BN-PAGE-reducing SDS-PAGE reveals the assembly states of D1, D2 and CP43 in thylakoids (Panels B, D, F) and PSII translation membrane membranes (Panels C, E, G). The pair of blots was first immuno-probed for D1 (B and C); then immuno-probed for D2 (D and E), and then stripped and immuno-probed for CP43 (F and G)**.** Overlays of B/D and C/E allow discrimination of the D1 and D2 signals. PSII monomeric complex, RCC1; PSII dimeric complex, RCC2; the monomeric complex lacking CP43, RC47; PSII reaction center, RC, CP43-precomplex, CP43pre; Unassembled subunits, U.P.

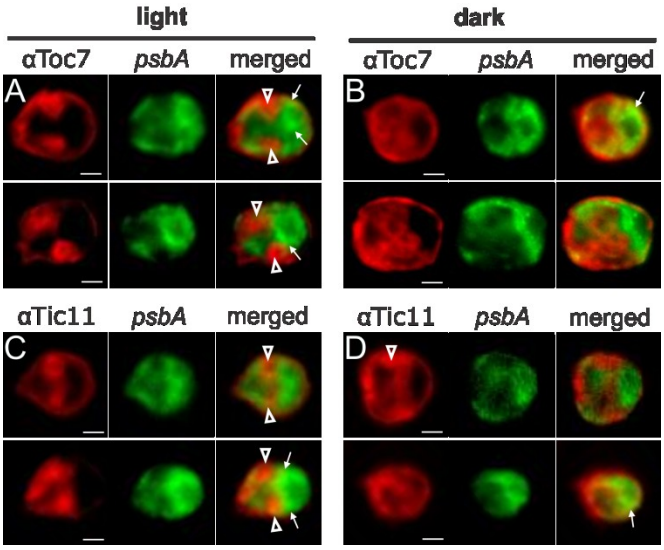


Fig. 4) The TOC and TIC protein import complexes are localized to novel domains of chloroplast envelope at lobe junctions in a light-dependent fashion.

Cells were IF-stained for Toc75 (A and B) or Tic110 (C and D), marker proteins for the TOC and TIC protein import complexes, respectively (red). Co-staining of the *psbA* mRNA by FISH (green) reveals T-zones as strong punctate signal (thin arrows) and the chloroplast as a weaker diffuse signal. IF-signals of Toc75 or Tic110 localized at lobe junctions are indicated with arrowheads (A and C). Cells are oriented with their nuclear-cytosolic compartment is on the left and the basal chloroplast with the pyrenoid on the right. Light-cultured cells (A and C), dark-adapted cells (B and D). Bars = 2μm.

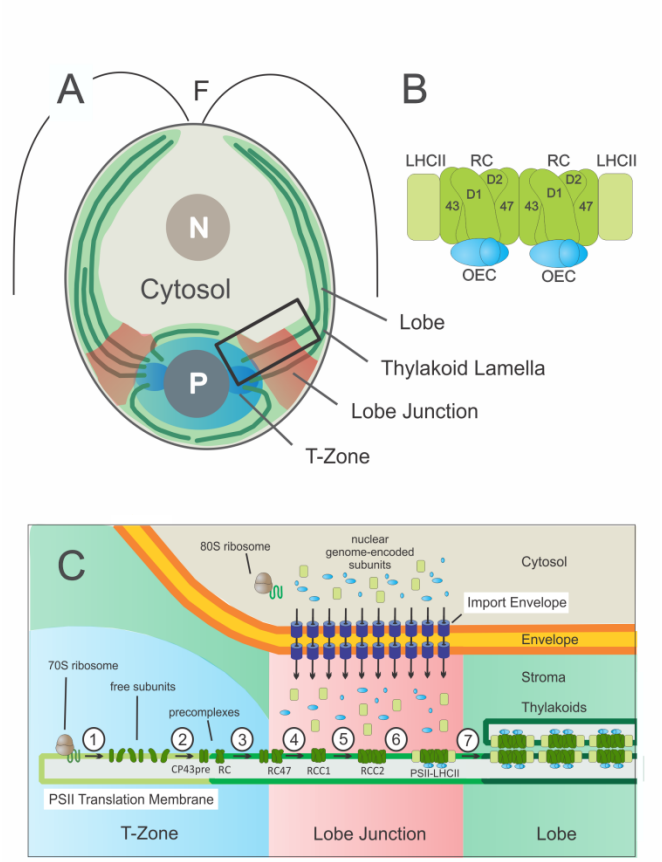
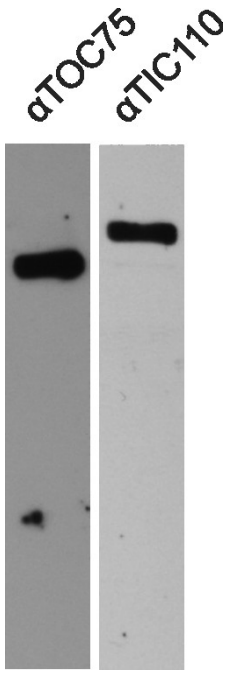


Fig. 5) A model for the spatiotemporal organization of PSII-LHCII supercomplex biogenesis.

(A) An illustration of a Chlamydomonas cell shows the nucleus (N), flagella (F), cytosol, and the chloroplast with thylakoid lamellae (dark green lines), the pyrenoid (P), T-zone (blue), lobes, and lobe junctions (red). The chloroplast lobes are finger-like projections which extend from the globular basal region in the posterior (bottom) to the anterior (upper) poll of the cell thereby encompassing the nuclear-cytosolic compartments (28). (B) A highly simplified illustration of the PSII-LHCII supercomplex shows the plastid-encoded subunits of the reaction center (RC) and the nucleus-encoded subunits of the LHCII and the OEC. See Caffarri et al (2009) for the precise 3D structure (50). (C) An illustration shows the compartments and steps in our model. 1) In the T-zone, plastid-encoded subunits are synthesized into PSII translation membranes. 2) Free subunits assemble to form the PSII RC and the CP43 pre-complex. 3) Intermediates move by lateral diffusion in the membrane to lobe junctions. 4) In thylakoid membranes or an intermediate PSII biogenesis membrane in lobe junctions, the RC associate with the CP47 pre-complex to form the PSII monomeric complex, RCC1. 5) RCC1's dimerize to form RCC2. 6) In lobe junctions, nuclear genome-encoded subunits OEC and LHCII locally imported by the TOC and TIC

complexes (purple) in import envelope and assembled. 7) The PSII-LHCII supercomplex migrates to thylakoids throughout the chloroplast (5).



Supplemental data fig. 1

The antisera against spinach Toc75 and Tic110 are highly specific in Chlamydomonas because each only detects a protein of the expected molecular mass (51). Fractions of another gradient were also assayed for the subunits of the Toc75 and Tic110, markers of the TOC and TIC protein import complexes in the outer and inner membranes of the chloroplast envelope, respectively.