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Running head

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Photosystem II Assembly Steps Take Place In The Thylakoid Membrane Of The Cyanobacterium *Synechocystis* sp. PCC6803

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Photosystem II Biogenesis In S*ynechocystis*

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Abbreviations

CN: Clear Native; CP43: PsbC subunit of PSII; CP47: PsbB subunit of PSII; CtpA: C-terminal processing protease; D1: PsbA subunit of PSII; D2: PsbD subunit of PSII; DGDG: digalactosyldiacylglycerol; EM: electron microscopy; GFP: green fluorescent protein; Hlip: high-light inducible proteins; HL: high light; iD1: processing intermediate form of D1; mD1: mature form of D1; NL: normal light; OD: optical density; pD1: unprocessed, premature form of D1; PG: phosphatidylglycerol; PM: plasma membrane; PVDF: Poly(vinylidene fluoride); RC: reaction center; RF: Restriction-free; SQDG: sulfoquinovosyldiacylglycerol; TM: thylakoid membrane; WT: wild type

Abstract

Thylakoid biogenesis is an intricate process requiring accurate and timely assembly of proteins, pigments and other cofactors into functional, photosynthetically-competent membranes. Photosystem II (PSII) assembly is especially studied as its core protein, D1, is very susceptible to photodamage and has a high turnover rate, particularly in high light. PSII assembly is a modular process, with assembly steps proceeding in a specific order. Using aqueous two-phase partitioning to separate plasma membranes (PM) and thylakoid membranes (TM) we studied the subcellular localization of PSII biogenesis early assembly steps in a *Synechocystis* sp. PCC6803 cyanobacterium strain lacking CP47 antenna. This strain accumulates the early D1-D2 assembly complex which was localized in TM along with associated PSII assembly factors. We also followed insertion and processing of the D1 precursor (pD1) by radioactive pulse-chase labelling. D1 is inserted into the membrane with a C-terminal extension which requires cleavage by a specific protease, the C-terminal processing protease (CtpA), to allow subsequent assembly of the oxygen evolving complex. pD1 insertion as well as its conversion to mature D1 under various light conditions was seen only in the TM. Epitope-tagged CtpA was also localized in the same membrane giving further support for the thylakoid location of pD1 processing. However, Vipp1 and PratA, two proteins suggested to be part of the so-called “thylakoid centers”, were found largely accumulated in PM. Together, these results suggest that early PSII assembly steps occur in TM or specific areas derived from them, with interaction with PM needed for efficient PSII and thylakoid biogenesis.

Keywords: aqueous two-phase partitioning; cyanobacteria; Photosystem II biogenesis; *Synechocystis* sp. PCC6803; thylakoid biogenesis

Introduction

Oxygenic photosynthesis occurs in specialized membranes in plants, algae and cyanobacteria, the thylakoid membranes. These are the home of several large complexes, with photosystem II (PSII) being one of the key elements for the photosynthetic process. PSII is composed of 16 integral and 3 peripheral membrane proteins, several β-carotene, chlorophyll a and pheophytin molecules (Guskov et al. 2009; Loll et al. 2005) and its assembly has been the object of many studies (Boehm et al. 2011; Nixon et al. 2010; Stengel et al. 2012). Unlike photosystem I (PSI), which is rather stable and assembled extremely fast (Duhring et al. 2007), PSII assembly is more complex, requiring the association of several different functional "modules" in a specific order (Boehm et al. 2012; Komenda et al. 2012b).

One of the most important protein subunits of PSII is the D1 protein. It binds many co-factors involved in the PSII electron transfer including those of the primary charge separation and water oxidation. In the model cyanobacterium *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*), D1 is inserted into the membrane as a precursor containing a C-terminal extension (pD1), which is matured in a two-step process (Inagaki et al. 2001; Komenda et al. 2007). The insertion of *Synechocystis* pD1 was also shown to require the YidC (Slr1471) membrane insertase (Ossenbuhl et al. 2006; Spence et al. 2004). During or shortly following the insertion, pD1 associates with PsbI (Sml0001) (Dobakova et al. 2007) and together with other components (see below) forms the D1 module. This module then associates with the D2 module, consisting of (at least) the D2 protein and subunits PsbE (Ssr3451) and PsbF (Smr0006) of cytochrome (cyt) b_{559}, forming the PSII reaction center assembly complex (RCII) (Komenda et al. 2008; Komenda et al. 2004). At this stage of PSII assembly pD1 is cleaved by the specific C-terminal processing protease CtpA at the residue A352, yielding a D1 processing intermediate iD1 (Komenda et al. 2007). The assembly process continues by attachment of the CP47 module resulting in an assembly subcomplex termed RC47. This attachment promotes the cleaving-off of the remaining eight amino acids of pD1 to yield the mature D1 protein. After subsequent attachment of the CP43 module, binding of the oxygen-evolving complex (OEC) subunits and dimerization, the complete PSII complex can participate in the photosynthetic light reactions (Komenda et al. 2004; Komenda et al. 2012b).

Owing to its highly complex nature, the precise assembly of fully functional PSII requires the involvement of various auxiliary protein factors that optimize individual assembly steps but are not part of the final complex (Nickelsen and Rengstl 2013). These factors were mostly identified in specific PSII assembly intermediates that accumulate in specific mutants lacking a component essential for the next assembly step (Komenda et al. 2008;

Komenda et al. 2004). Several factors have been identified to assist in early PSII biogenesis, including Ycf48 (Slr2034), a Ycf39 (Slr0399)–high-light inducible proteins (Hlip) complex, and PratA (Slr2048). The first of these factors, Ycf48, stabilizes the D1 module and the RCII assembly intermediate (Komenda et al. 2008). The Ycf39-Hlip complex, associating specifically with both of these early intermediates, has been implicated in their photoprotection and in chlorophyll delivery to newly synthesized D1 (Knoppova et al. 2014; Staleva et al. 2015). The Mn^{2+}- binding PratA protein was found to accumulate in a specific membrane subfraction that also contains substantial amounts of pD1, which has been suggested by Nickelsen and co-workers (Rast et al. 2015; Schottkowski et al. 2009; Stengel et al. 2012) to be the site of initial steps of PSII biogenesis (the so-called thylakoid centers). In this context, a putative role of the vesicle-inducing protein 1, Vipp1 (Sll0617), as a constituent of the thylakoid centers has also been discussed (Rutgers and Schroda 2013) and its importance for thylakoid membrane formation in chloroplasts and cyanobacteria previously described (Aseeva et al. 2007). The later steps of PSII assembly are accompanied by the Psb28 (Sll1398) and Psb27 (Slr1645) factors. Psb28 stabilizes the RC47 assembly complex (Dobakova et al. 2009), while Psb27 interacts with CP43 and stabilizes the CP43 assembly module (Komenda et al. 2012a; Liu et al. 2011). Its attachment to the reaction centre core was suggested to block the premature binding of extrinsic proteins, thus preventing Mn-cluster assembly (Liu et al. 2011).

The functional PSII complexes are vulnerable to photoinactivation, the light-induced loss of the PSII photochemical activity. In order to maintain its activity, PSII complexes undergo a repair process which consists of a partial PSII disassembly, selective replacement of the damaged D1 protein by a newly synthesized (functional) D1 copy, reassembly and reactivation (Mulo et al. 2012). This process, known as PSII repair cycle, though sharing some similarities with *de novo* synthesis and assembly of PSII, was suggested to occur in different regions from the latter (Sacharz et al. 2015).

In spite of our knowledge of many of the details about the PSII biogenesis and repair, the long-standing question regarding the location of both processes in particular membrane regions and related presence or absence of connections between thylakoid membranes (TM) and the plasma membrane (PM) is still not completely clarified. The first report on localization of early steps of PSII assembly pointed to the presence of the newly synthesized D1 protein and its maturation protease CtpA in PM (Zak et al. 2001) while characterization of the D1 early assembly factor Ycf48 indicated its presence in thylakoids (Komenda et al. 2008). Location of early steps in PM and later steps in TM suggested the presence of at least temporary connections between the two membrane systems (Nevo et al. 2007; Nickelsen et al. 2011; Pisareva et al. 2011;

van de Meene et al. 2006). In contrast, other studies have presented data that argued for complete separation of TM and PM (Liberton et al. 2006; Schneider et al. 2007). In this study we have used a *Synechocystis* mutant lacking CP47 to show the accumulation of the RCII assembly complex and its associated assembly factors in TM isolated by aqueous two-phase partitioning. Using radioactive pulse-chase and epitope-tagging we also demonstrate that the newly synthesized pD1 is inserted and processed within TM under high irradiance and that the CtpA protease is also present in TM. Thus, we show that early steps of the PSII assembly occur either in *bona fide* thylakoid membrane or in specific domains within it. At the same time we confirmed the localization of PratA and Vipp1 in the purified PM, which points to the need for at least a partial connection between the two membrane systems.

Results

Photosystem II reaction centre assembly complexes lacking CP43 and CP47 and associated assembly factors accumulate in thylakoids

PSII biogenesis in the knock-out strain lacking CP47 antenna, which is unable to assemble a functional PSII, is arrested at the early stage of the RCII complexes accumulating in the membranes. Our previous studies revealed the presence of two complexes *in vivo*, designated as RCa and RC*, differing in their size depending on the presence of additional protein factors involved in the assembly process (Komenda et al. 2008). These contain the constitutive RCII subunits, D1, D2, PsbI, PsbE, and PsbF, and the assembly factor Ycf48. The “bigger” RC* also binds an additional component, a small Ycf39-HliD (Ssr1789) complex, which is probably involved in photoprotection of RC* (Staleva et al. 2015) and delivery of chlorophyll to the newly synthesized D1 protein (Knoppova et al. 2014). In contrast to the WT, the CP47-less mutant accumulates the RCII complexes in amounts which are easily detectable by immunochemical methods and hence this mutant is especially suited to elucidate whether the processes of early PSII biogenesis take place in TM or PM. The purified TM and PM fractions from the CP47-less strain were subjected to analysis by both one-dimensional denaturing electrophoresis and two-dimensional electrophoresis combining clear native-(CN-)PAGE of native protein complexes and SDS-PAGE in order to analyze the subunit composition of the complexes. The immunochemical visualization of the SDS-PAGE separated proteins (Fig. 1) clearly shows that all main protein components of the RCII complexes as well as the YidC insertase are present predominantly in TM. The Ycf39 factor was never found to interact with PSII assembly complexes larger than RCII (Knoppova et al. 2014) and may therefore be considered a marker of the early steps of PSII biogenesis. Our results show that this protein also accumulates

almost exclusively in the TM. The distribution of Psb27, which was previously described as a factor stabilizing unassembled CP43 (Komenda et al. 2012a), is similar to that of CP43, i.e., mostly present in TM. The two-dimensional analysis of the TM and PM (Fig. 2) enabled us to demonstrate for the first time that the RCII assembly complexes clearly accumulate in the thylakoid membranes. The immunochemical detection revealed the presence of both the partially processed and maturated D1 forms (iD1 and D1, resp.) in the population of the reactions centers. This further supports our idea that the D1 processing occurs in TM, and not in PM. Chlorophyll synthase (ChlG, Slr0056), the last enzyme of Chl biosynthesis pathway, was also mostly detected in the TM fraction. The CP43 subunit, which cannot be assembled to PSII complexes in this mutant, was found to migrate in the region of unassembled proteins (Fig.2, 2D Sypro) in two forms possibly differing in the content of associated low-molecular mass protein components of the CP43 assembly module (Komenda et al. 2012a). Photosystem I (PSI) complexes are also detectable in PM, but this can be ascribed to an unavoidable limitation of the method that leads to a small contamination of the PM with TM membranes (see Discussion).

Membrane insertion and step-wise processing of pD1 occurs in thylakoids

The insertion of the core subunit D1 precursor, pD1, is a prerequisite for the RCII formation and precedes or occurs concomitantly with the RCII assembly. Earlier studies proposed that these initial steps in PSII assembly including pD1 processing take place in plasma membrane (Zak et al. 2001) while later results implied that pD1 insertion (Jansen et al. 2002) and the repair process might happen in TM (Jansen et al. 2002; Komenda et al. 2006). Moreover, further studies showed that the YidC insertase essential for pD1 insertion into the membrane (Ossenbuhl et al. 2006), is also mostly located in TM (Pisareva et al. 2011). These later results raised an important issue: if pD1 is inserted in the TM but processing occurs in the PM this would imply that newly inserted pD1 (in TM) should, either in the unassembled state or as a component of RCII complex, migrate to PM for processing and then return to TM to associate with inner antennae CP47 and CP43 and form active PSII complexes. This requires, especially under high irradiance, massive protein trafficking between PM and TM which would necessitate rather extreme mobility of PSII complexes and also extensive contacts between both types of membranes, never observed thus far. To clarify this possibility we tracked pD1 synthesis and processing using radioactive pulse-chase, which allows us to follow the newly synthesized proteins and their subsequent fate. The cells were labelled under high irradiance and low temperature, with the chase initiated by addition of chloramphenicol to stop D1 synthesis and performed under standard light and temperature conditions. The TM and PM were then separated and analyzed by SDS-PAGE combined with autoradiography

and immunodetection (Figs. 3A and B, resp.). In agreement with YidC localization, radio-labelled pD1 appeared only in TM at time zero (Fig. 3A), and then a clear conversion from pD1 to iD1 and finally to mD1 occurred. Again, both these forms were detected only in TM. The traces of labelled proteins seen in PM can be considered an unavoidable contamination, as mentioned earlier. We conclude that the insertion of pD1 and its subsequent two-step maturation happens only in TM (Fig. 3A). It should be mentioned that there were virtually no radioactivity counts (data not shown) from the low-density PM fraction (PM1, (Pisareva et al. 2011)) and as such we assume pD1 insertion and processing does not require re-localization to PM1 either.

CtpA-FLAG is localized in the thylakoid membrane

Based on our pulse-chase results we hypothesized that the enzyme CtpA, catalyzing the pD1 processing, should also be associated with TM. Since we were not able to produce an antibody which would specifically recognize the protease with sufficient sensitivity, we decided to construct a strain expressing the CtpA protease with 3xFLAG tag at the C-terminus expressed from its native locus under the native promoter (see Fig. S1). For construction we utilized the *ctpA* null mutant as a background strain so as to have biological evidence that the 3xFLAG tag added to the protease did not interfere with its native function in the pD1 maturation. Indeed, unlike the parental strain (ΔCtpA), which is only able to grow in the presence of glucose, the CtpA-3xFLAG (hereafter CtpA-FLAG) strain was able to grow photoautotrophically, with CtpA-FLAG and WT strains having comparable growth rates under these conditions (Fig. S2). Western blot analysis showed that only (mature) D1 could be detected in the FLAG-tagged strain while pD1 was the only D1 form found in the background *ctpA* null strain (Fig. 4A and B).

Analysis of the separated membranes confirmed our initial hypothesis and CtpA-FLAG was detected almost exclusively in TM (Fig. 4B). This was true not only for membranes prepared from cells grown under standard light conditions but also from cells exposed to high irradiance when much more frequent PSII repair and increased synthesis of D1 occurs (Fig. 4B).

PratA and Vipp1 proteins predominate in the plasma membrane

PratA and Vipp1 are well known for their involvement in PSII and thylakoid membrane biogenesis, respectively. The former has previously been suggested as a marker protein of the so-called “PratA-defined membranes” (PDM) originating from “thylakoid centers” (Klinkert et al. 2004; Rast et al. 2015; Schottkowski et al. 2009) in which initial steps of PSII biogenesis were proposed to take place. The latter, Vipp1, was shown to be important

for thylakoid maintenance in *Synechocystis* (Gao and Xu 2009) and was proposed to be a structural component of these "thylakoid centers" as well (Rutgers and Schroda 2013). Previous experiments on localization of these proteins were not conclusive and it is so far unclear whether they reside in PM or in TM. PratA was previously localized in the PDM fraction of *Synechocystis* (Klinkert et al. 2004) using two consecutive sucrose gradient ultracentrifugation steps, and was also identified as a periplasmic protein, isolated from *Synechocystis* cells using cold osmotic shock (Fulda et al. 2000). Our assessment clearly showed almost exclusive presence of PratA in PM of WT (Fig. 5). On the other hand, Vipp1 did not show such an exclusive localization in PM as PratA, with minor amounts also detected in TM (Fig. 5). Previous structural analyses have revealed that Vipp1 does not display any features of an intrinsic membrane protein and, instead, it was predicted to have helix-coils and lipid binding domains, important for its membrane-binding and curvature-inducing ability (Otters et al. 2013; Pisareva et al. 2011). Previous studies of this protein in plants pointed to a unique dual localization at the two membranes bordering the stromal compartment and a potential involvement in thylakoid membrane biogenesis (Li et al. 1994). Our results suggest that *Synechocystis* Vipp1 may have a similar function to that of its plant chloroplast homologue, perhaps by inducing membrane curvature in the PM (and TM) regions forming connecting points between the two membranes (see below).

Discussion

Early steps of PSII assembly and repair occur in the thylakoid membrane

In the present work, we analyzed membrane fractions purified from the CP47-deletion mutant in which the PSII assembly process is halted at the stage of the RCII complexes, allowing their partial accumulation (Komenda et al. 2004). This enabled us more reliable localization of these complexes than in the WT, in which they are hardly detectable. Immunoblot 1D analysis of membranes purified from the CP47-less strain clearly showed that all main PSII components of the RCII complexes (D1, D2, PsbI and PsbE) are predominant in TM (Fig. 1). Even though small amounts of these proteins are also visible in the PM fraction, we believe that they represent an unavoidable contamination by TM, due to the inherent limitations of biochemical purification methods. Thylakoids are less likely to show contamination by PM proteins (as shown by various blots with typical PM marker proteins) due to their sheer predominance over PM in *Synechocystis* cells. This explanation is also supported by the finding of a weak band of CP43 in the PM, even though this is consensually considered to be a protein exclusively present in the thylakoid membrane (Schottkowski et al. 2009; Smith and Howe 1993; Zak et al. 2001). Moreover, the factors previously identified in association with the RCII complexes (Ycf48,

Ycf39, HliD) were also enriched in TM in agreement with a previous report on the localization of Ycf48 in TM (Komenda et al. 2008). Some of these factors had also been found in previous proteomic studies either exclusively or significantly enriched in TM (Pisareva et al. 2011).

We also analyzed membranes purified from the CP47-less strain by 2D CN/SDS-PAGE and we were able, for the first time, to demonstrate the presence of assembled RCII complexes in the thylakoids prepared by aqueous two-phase separation method. These complexes had identical composition to those observed after solubilization of non-purified cellular membranes of the CP47-less strain (Knoppova et al. 2014) and this further supported our hypothesis that these complexes are formed in thylakoids. We also identified the vast majority of chlorophyll synthase (ChlG), the last enzyme of the Chl biosynthesis pathway, on the 2D gel of the purified TM. This is in agreement with recent reports showing the synthase in association with HliD, Ycf39 and YidC, proteins also predominantly found in TM (Fig. 1) (Chidgey et al. 2014). Identification of ChlG in thylakoids also suggests location of Chl biosynthesis pathway in this membrane fraction. Indeed, the thylakoid-localized YidC insertase and associated protein synthesis machinery was proposed to form, together with associated Chl biosynthesis enzymes, a "megacomplex" for synthesis of chlorophyll-proteins (Chidgey et al. 2014). This "megacomplex" would also be responsible for synthesizing and inserting the D1 protein and should be, therefore, located in thylakoids.

Membrane insertion of pD1 and its association with PSII is a pivotal point during assembly and repair of PSII. The insertion occurs co-translationally, with the assistance of the YidC membrane protein insertase (Ossenbuhl et al. 2006). We have previously shown that this insertase predominates in the thylakoid membrane (Pisareva et al. 2011), and the present study confirms those earlier results (Figs. 1 and 5). Moreover, we also demonstrate that under high irradiance, when efficient PSII repair as well as its de novo assembly is needed to cope with the frequent PSII photodamage, the labeled, newly-synthesized pD1 is almost exclusively present in TM. This is in agreement with previously published data based on pD1 immunodetection (Jansen et al. 2002). Using the pulse-chase approach we also followed pD1 processing, which occurred in TM as well, given that the pD1 signal is weakening while iD1 and later mD1 signals rise during the chase period (Fig. 3A).

Furthermore, using the CtpA-FLAG strain, we were able to demonstrate that CtpA associates to the same thylakoid membrane fraction as YidC and pD1 (Figs 4B and 5). Our present results indicate that CtpA is present in the thylakoid lumen, in agreement with the localization of CtpA in plants and with predictions made by servers evaluating the N-terminal signal peptides (SignalP4.0, http://www.cbs.dtu.dk/services/SignalP/; and TOPCONS, http://topcons.cbr.su.se/). No change in the CtpA localization was detected even when membrane

fractions were purified from cells exposed to high irradiance (Fig. 4B). The presence of CtpA allows the nascent pD1 to be processed *in situ* without the need for massive pD1 and D1 shuffling between TM and PM. This might be especially important under high irradiance when insertion and maturation must be extremely fast to cope with the rate of photodamage. Should CtpA be associated to the plasma membrane as originally proposed (Che et al. 2013; Zak et al. 2001), newly synthesized pD1, which is inserted by TM-localized YidC, would be required to move to PM for processing before returning to TM. Such trafficking would significantly extend the life-time of unassembled pD1 and should also be easily detected on 2D autoradiograms of proteins from WT cells labelled under high irradiance which is not the case (Komenda et al. 2006; Komenda et al. 2008). Such trafficking was equally not observed in the present pulse-chase experiment (Fig. 3A) as none of the different forms of [^{35}S]-labelled D1 were detected in the PM during the 20 minutes' chase. On the other hand, a quite substantial iD1 signal was seen in TM after just 5 minutes' chase (Fig. 3A). The original study reporting on the occurrence of CtpA in PM (Zak et al., 2001) relied on a weak immunochemical detection of CtpA using an antibody which may not have been sufficiently specific, thus leading to detection of what could be an incorrect signal. Moreover, unlike our present study, Zak and co-workers (Zak et al. 2001) did not show any other data in support of pD1 maturation occurring in PM.

Attachment of the FLAG-tag to the CtpA protease allowed not only a very sensitive detection of the protein in membrane fractions but also permitted the isolation of the protein under native conditions in order to identify possible interacting partners of the protease. Though we were able to use affinity pulldown assays to separate significant amounts of the protease from both *ctpA* null and CP47-less CtpA-FLAG expressing strains, we could not detect any proteins or complexes specifically associated to CtpA (not shown). Thus, CtpA does not seem to stably bind either to other proteins or to their complexes including PSII. It is possible that the efficient processing ability of CtpA will make interactions with pD1 and PSII complexes very transient and difficult to examine using this methodology.

Previous analysis of the accumulation of various unassembled D1 forms in the strains lacking the D2 protein showed a predominance of pD1 over the other two D1 processing forms (Komenda et al. 2004). Since the unassembled D1 species are very quickly degraded (Komenda et al. 2006) it is possible that the rate of degradation exceeds that of processing and therefore the processed forms cannot accumulate. Alternatively, the absence of D2 and other PSII proteins might also directly or indirectly decrease the enzymatic efficiency of the CtpA protease. In agreement with the latter alternative following pD1 association with D2, pD1 is immediately cleaved at the first cutting site and resulting RCII complexes never contain pD1 but are enriched in the iD1

intermediate (Komenda et al. 2004). After attachment of the CP47 module to RCII, iD1 is immediately cleaved to the final mature form and again, no iD1 can be detected in the RC47 assembly complex (Komenda et al. 2004). Altogether, this would indicate that both pD1 maturation steps, which strictly require CtpA (Komenda et al. 2007), seem to be affected by the association of the substrate, pD1, with other PSII components including assembly factors, such as Ycf48. Indeed, in the absence of Ycf48, the maturation of pD1 is extremely fast and the prematurely processed D1 might not be able to associate with D2 (Komenda et al. 2008).

In agreement with the location of CtpA in thylakoid lumen during exposure of cells to high irradiance (Fig. 4B), the PSII repair-related FtsH2 (Slr0228) protease was also previously detected in thylakoids prepared by the aqueous two–phase separation method (Komenda et al. 2008; Pisareva et al. 2011), with confocal microscopy also evidencing its accumulation in thylakoids of strains expressing GFP-tagged FtsH2 (Komenda et al. 2008; Sacharz et al. 2015). During PSII repair, the damaged PSII is partially disassembled to allow access of FtsH2 to the D1 protein. After degradation, the newly synthesized protein is quickly inserted into PSII and simultaneously processed. The D1 replacement is especially efficient and fast in the strain lacking CP43 (Komenda et al. 2006), and during this repair cycle no unprocessed D1 forms can be detected even using radioactive labeling. This implies that CtpA must be in close proximity to the D1-degrading FtsH protease, i.e. in the thylakoids. Based on all these results, we conclude that the PSII repair, including D1 degradation, insertion and maturation, occurs in TM.

From our present results, it seems likely that both the PSII *de novo* assembly (Figs. 1 and 2) and repair (Fig. 3A and 4) occur in thylakoids. The recently characterized D1 module, including the associated assembly factors Ycf48, Ycf39 and HliD, also contained high levels of the FtsH2/FtsH3 (Slr1604) complex responsible for the repair-related degradation of D1 (Knoppova et al. 2014). However, it has been suggested that the PSII *de novo* assembly and repair are two processes that might be separated both spatially and functionally (Komenda et al. 2006; Rast et al. 2015). Given the proven structural heterogeneity of cyanobacterial thylakoids shown by electron microscopy (EM) analyses (e.g. (Nevo et al. 2007)) it is possible that both processes occur in distinct, but neighboring thylakoid regions which cannot be further separated by methods based on membrane density and surface properties alone. Clarification of this point is beyond the scope of this report; nevertheless, it has recently been proposed that the TM contains laterally heterogeneous patches, characterized by a higher local concentration of FtsH2, which may represent specific “repair zones” (Sacharz et al. 2015) .

Where are the PratA and Vipp1 proteins located?

The discussion on whether TM connects to PM or is a separate entity was one of the driving points of the present study. We have shown that pD1 insertion and processing occur in the TM, and that CtpA is also present in TM. This could implicate a model of PSII biogenesis in which none of important assembly steps requires PM. However, this simple model is complicated by the apparent location of protein factors like PratA, which also seem to play an important role in PSII biogenesis, in PM.

Earlier reports have indicated that deletion of the *pratA* gene results in impaired pD1 processing (Klinkert et al. 2004), and a direct interaction between PratA and pD1 was suggested based on results from yeast two-hybrid and glutathione S-transferase pull-down assays (Schottkowski et al. 2009). Later, PratA was shown to have an important role in transport of manganese to PSII (Stengel et al. 2012) from periplasm and therefore it is more likely that it should reside in specific areas of the PM. The limited pD1 processing in the absence of PratA may also be related to the limitation in manganese supply, which can slow down assembly of PSII and inhibit its activity (Salomon and Keren 2011). Our results confirmed that in contrast to the location of pD1 insertion, processing and PSII association processes occurring in TM, PratA accumulates in PM (Fig. 5). The most plausible explanation for these apparently controversial results is that components required for PSII biogenesis/repair reside in specific membrane regions ("thylakoid centers", (Nickelsen and Rengstl 2013)), which could be at the interface between PM and TM. Components of these centers may, upon cell breakage and aqueous two-phase partitioning, separate into both fractions, with some components more closely associated with TM (pD1) and some with PM (PratA). As mentioned above, such membrane lateral heterogeneity in TM has recently been demonstrated (Mullineaux 2014; Sacharz et al. 2015) and could represent the main reason for the apparent discrepancies which exist between various published studies so far.

In cyanobacteria the synthesis of certain membrane components, such as digalactosyldiacylglycerol (DGDG), occurs within PM though DGDG is a component of TM (Selao et al. 2014; Zulkifli et al. 2010). This would imply the need for a (lipid) transfer mechanism between the two membranes. In this respect several studies have investigated the function of the Vipp1 protein, which was proposed to participate in such a transfer of lipids and proteins (Bryan et al. 2014; Hennig et al. 2015; Li et al. 1994; Rutgers and Schroda 2013). Indeed, a reduction in the amount of Vipp1 results in a distorted organization and reduced number of thylakoids in *Synechocystis* (Aseeva et al. 2007; Gao and Xu 2009). More recently, Vipp1 was found to form oligomeric rings with a well-defined geometry *in vitro*, interacting specifically with membranes containing anionic lipids, such as sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Hennig et al. 2015). Interestingly, *Synechocystis* PM is relatively enriched in these two lipids (2–3 times more) in comparison with TM

(unpublished results). This tempts us to speculate that there may be SQDG/PG-enriched domains in PM which would mediate (possibly transient) connections to TM for the transport of lipids and proteins, under Vipp1 assistance. Vipp1 was shown to coalesce into localized "puncta" within the cell upon exposure to high irradiance (Bryan et al. 2014) and these "puncta" were proposed to represent "stress-induced localised protein assembly centres" in which PSII assembly/repair occurs (Bryan et al. 2014). It was suggested that *Chlamydomonas reinhadtii* Vipp1 cooperates with the YidC insertase during integration of proteins into thylakoids (Nordhues et al. 2012). Also, plant chloroplast Vipp1 was recently shown to interact with Alb3 (YidC) (Walter et al. 2015). However, so far there is no direct evidence for a Vipp1-YidC interaction in *Synechocystis*. Vipp 1 also partly associates with TM and our present data rather support its dual location with a majority of the protein in PM and a minority in TM, in agreement with its proposed role in mediating transport from PM to TM.

In summary, our results show that CtpA and pD1 processing occur in TM and that most of the necessary PSII assembly factors are co-localized in the same membranes, with the notorious exception of PratA and Vipp1 which are highly enriched in PM. On the other hand, we have recently shown that the enzyme responsible for DGDG synthesis is specifically located in PM (Selao et al. 2014) and, similarly, CrtO (Slr0088) and CrtQ (Slr0940), two enzymes involved in carotenoid biosynthesis, are also enriched in PM (Zhang et al. 2015). Altogether, we propose that an interaction between TM and PM is necessary for efficient thylakoid membrane biogenesis and PSII assembly, with the TM most likely interacting with the PM at specific points either permanently or through dynamic, temporary connections, formation of which is possibly assisted by the Vipp1 protein.

Materials and Methods

Construction and cultivation of cyanobacterial strains

The wild type (WT) strain used in this study is the glucose-tolerant *Synechocystis* sp. PCC6803 strain (Williams 1988). Other strains used in this study include the CP47 deletion strain, ΔCP47, with the *psbB* gene replaced by a spectinomycin resistance cassette (Yu and Vermaas 1990) and the ΔCtpA strain, in which the *ctpA* gene was also inactivated by a spectinomycin cassette (Komenda et al. 2007).

For generation of a FLAG-tagged CtpA strain, plasmid pCtpA-FLAG was assembled by PCR amplifying (using Phusion polymerase, NEB) a region of the WT genome containing the *ctpA* gene and 1 kbp up- and downstream sequences, by using appropriate primers (see Table S1). The PCR product was cloned into the pCRBluntII-TOPO vector (Invitrogen) following manufacturer's instructions and a 3xFLAG tag was inserted in-frame, immediately before the last (stop) codon of the *ctpA* gene, by restriction-free (RF) cloning (Bond and Naus 2012; van den Ent and Lowe 2006), using a synthetic dsDNA as megaprimer (see Table S1). For clone selection, a chloramphenicol resistance cassette was amplified from plasmid pSK9 (Kuchmina et al. 2012), a kind gift from Annegret Wilde, using the primers stated in Table S1 and introduced in the same orientation as the *ctpA* gene, after the 3xFLAG tag, also by RF cloning. The resulting plasmid, pCtpA-FLAG, was used to transform ΔCtpA cells. The transformants were selected by growth on chloramphenicol-containing plates and their complete segregation was achieved on plates containing increasing amounts of chloramphenicol (Eaton-Rye 2011).

For protein localization studies, three independent liquid cultures were grown in 1 liter batches of BG11 medium in glass bottles, aerated by bubbling with sterile air, at a constant temperature of 30 ºC. In the case of the ΔCP47 and ΔCtpA strains, BG11 was supplemented with 5 mM glucose, and antibiotics (50 μg mL^{-1} spectinomycin and 30 μg mL^{-1} chloramphenicol, respectively) were added. Illumination was provided continuously, by warm white light bulbs, at an irradiance of 50 μmol photons m^{-2} s^{-1}. Cultures were grown to exponential growth phase (OD_{730} between 1.0 and 1.5) and then harvested at 6000 g, 15 min, 4 ºC. Cell pellets were washed once in 20 mM potassium phosphate buffer, pH 7.5, and stored at - 80 ºC until further usage. Solid media contained 1.5% agar, 0.3% sodium thiosulfate and the necessary antibiotics. After complete segregation (Fig. S1), CtpA-FLAG cells were also propagated in medium lacking glucose but supplemented with chloramphenicol.

For growth curve measurements, 20 mL of three independent exponential growth phase cultures of WT, CtpA-FLAG and ΔCtpA were centrifuged at 3000 g, for 10 min at 20 ºC and the pellet resuspended in 5 mL fresh BG11, with or without glucose, as indicated. Cultures were then diluted to an OD_{730} of 0.1 and incubated in a shaking incubator at 30 ºC, with illumination by warm white light bulbs, at an irradiance of 50 μmol photons m^{-2} s^{-1}.

Photoinhibition and pulse-chase labelling

Pulse-chase labelling was performed essentially as previously described (Kanervo et al. 1997), with some modifications. Two independent WT 1 liter cultures at an OD_{730} of 1 were harvested by centrifugation at 5000 g,

15 min at 20 ºC, each pellet was resuspended in 50 mL of fresh BG11 medium and divided into 4 aliquots of equal volume. These aliquots were once again centrifuged and each resuspended in 40 mL of fresh BG11 medium (to an OD_{730} of 8). These aliquots were incubated at 30ºC, with sterile air bubbling and under an irradiance of 50 µmol photons m^{-2} s^{-1} for 2 hours. They were then transferred to a water bath at 10 ºC and subjected to an irradiance of 1000 µmol photons m^{-2} s^{-1}, with sterile air bubbling, for 45 min. At this point, 160 µCi of [^{35}S]methionine (L-[^{35}S]methionine, 10.2 mCi/mL, 1175 Ci/mmol, Perkin-Elmer) were added to each culture aliquot and cells were incubated under the same conditions for an additional 10 min, at which point (T=0) cultures were transferred to a chamber at 30 ºC and irradiance of 50 µmol photons m^{-2} s^{-1} and chloramphenicol (to a final concentration of 400 µg mL^{-1}) was added to all aliquots. Cultures were harvested at the time points indicated by quickly cooling in liquid nitrogen so as to halt metabolic reactions and centrifuging at 6000g, 15 min at 4 ºC. Cell pellets were immediately frozen in liquid nitrogen and stored at -80ºC until further use.

Photoinhibition experiments using CtpA-FLAG cultures followed a very similar protocol, with the exception that no radioactive isotopes were added, and lincomycin (at a final concentration of 125 µg mL^{-1}) rather than chloramphenicol, was utilized to inhibit protein synthesis due to the intrinsic chloramphenicol resistance of the CtpA-FLAG strain. Only one time point (T=0) was collected.

Cell breakage and membrane fractionation

Cells were broken by vortexing in the presence of glass beads and membrane fractionation by aqueous two-phase partitioning was performed according to previously established protocols (Huang et al. 2002; Selao et al. 2014), with the following modifications: pellets of high light treated samples were initially resuspended in phosphate buffer containing protease inhibitors and either 400 µg mL^{-1} chloramphenicol (in the case of radioactively-labelled WT cells) or 125 µg mL^{-1} lincomycin (for high-light treated CtpA-FLAG cultures). Protein content was measured using the Peterson method (Peterson 1977) and radioactivity in each sample was measured by scintillation in a Beckman LS6500 scintillation counter.

Gel electrophoreses, autoradiography and western blotting

Samples from WT radioactively-labelled PM or TM fractions (PM1 samples had no measurable radioactivity) were separated on 12% SDS-PAGE gels containing 7M urea (Laemmli 1970). Two sets of gels were prepared, one set with 5 µg proteins per lane, for membrane purity assessment, and one with 2000 cpm per lane, for labelled pD1/D1 localization. The first set was transferred onto PVDF membranes and probed with antibodies

against the typical proteins of the thylakoid membrane (anti PsaA and CP43 are Agrisera products, item numbers AS06 172 and AS11 1787, respectively; anti-D1, own production, see below) or plasma membrane (anti KtrE, also known as DgdA or Slr1508 – a kind gift from Nobuyuki Uozumi). The second set was stained with Coomassie Blue R250 prior to drying and exposing overnight on a storage phosphor screen. Autoradiograms were obtained by reading the phosphor screen on a PhosphorImager (GE Healthcare).

CtpA-FLAG and ΔCtpA membrane fractions were analyzed by SDS-PAGE and western blotting, as described above. Antibodies against the FLAG epitope (anti-FLAG M2) were purchased from Sigma-Aldrich, YidC and Vipp1 antibodies were a kind gift from Dirk Schneider and PratA antibodies were obtained from Jörg Nickelsen.

The protein composition of the ΔCP47 membrane fractions was analyzed using a one-dimensional denaturing electrophoresis 12 – 20% linear gradient gel containing 7 M urea. Each loaded sample contained 10 µg proteins. The analysis of PSII complexes was performed using two-dimensional CN/SDS-PAGE combining the clean native electrophoresis in the 4 - 14% linear gradient gel with the denaturing electrophoresis, as described in (Komenda et al. 2012a). Each loaded sample contained 60 µg proteins. The separated proteins were electroblotted onto a PVDF membrane and probed using primary antibodies described in previous studies: anti D1, D2, CP43 and PsbE (Komenda et al. 2008; Komenda et al. 2004), PsbI (Dobakova et al. 2007), Psb27 (Boehm et al. 2012; Komenda et al. 2012a), Ycf48, Ycf39, HliD and YidC (Chidgey et al. 2014), and KtrE (see above).

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List of author contributions

TS, LZ and JKno performed the experiments. TS, LZ, JKno, JKom and BN designed the experiments and jointly wrote the article. BN had major responsibility for the project.

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Figure Legends

Figure 1 – Western blot analysis of the distribution of PSII subunits and assembly factors in PM and TM isolated from ΔPsbB (ΔCP47) strain. Each lane was loaded with 5 µg membrane proteins.

Figure 2 – Two dimensional analysis of thylakoid (TM) and plasma (PM) membrane samples purified from the ΔPsbB (ΔCP47) strain. Membranes were analyzed by CN-PAGE in the first dimension, the native gel was photographed (1D color) and scanned by LAS 4000 for fluorescence (1D fluor). After a 2nd dimension SDS-PAGE separation the gel was stained by Sypro orange (2D Sypro stain), electroblotted to a PVDF membrane and sequentially probed with the antibodies specific for D1, D2, Ycf39 and ChlG proteins (2D blot). Bands of the Amt1 transporter (Sll0108) and PilA1 (Ssl1694) designated in the stained gels were identified by MS. Designation of complexes: PSI(3), PSI(2), and PSI(1), trimeric, dimeric and monomeric PSI complexes,

resp.; ATPsynth, ATP synthase; NDH-L, the large NADH dehydrogenase complex; RCII* and RCIIa, PSII RC assembly complexes lacking CP43 and CP47; u.CP43, unassembled CP43. Asterisks indicate two Amt1 bands unspecifically cross-reacting with antibodies. The loaded samples contained 60 µg membrane proteins.

Figure 3 – [^{35}S]methionine pulse-chase labelling of pD1 processing during photoinhibition recovery.

A – Autoradiography image of pulse-chase labelled PM and TM samples. Times indicated are in minutes after changing from 10 ºC and 1000 µmol photons $m^{-2}s^{-1}$ to 30 ºC and 50 µmol photons $m^{-2}s^{-1}$. Each lane contained 2000 cpm of labelled proteins.

B – Western blot analysis of pulse-chase labelled PM and TM samples. Each lane was loaded with 5 µg membrane proteins.

Figure 4 – Western blot analysis of PM and TM samples for CtpA-FLAG. Each lane contains 5 µg membrane proteins.

A – CtpA-FLAG PM and TM samples from samples grown in normal light and temperature (50 µmol photons m^{-2} s^{-1}, 30 °C, "NL-NT") and exposed to photoinhibitory conditions (1000 µmol photons m^{-2} s^{-1} , 10 °C, "HL-LT") probed with antibodies for typical PM and TM proteins.

B – Western blot analysis of (CtpA-)FLAG and pD1 distribution in CtpA-FLAG and ΔCtpA strain PM and TM samples.

Figure 5 – Western blot analysis of the distribution of PratA, Vipp1 and YidC in WT samples of PM and TM. Each lane contains 5 µg membrane proteins.

Supplemental Information

The following materials are available in the online version of this article.

Supplemental Table 1 – Primer sequences used for generation of pCtpA-FLAG and segregation PCR testing

Supplemental Figure 1 – Design of pCtpA-FLAG and genomic DNA PCR demonstrating segregation.

686 A – Simple scheme demonstrating the arrangement of the pCtpA-FLAG plasmid used. The various segments are
687 not to scale. Numbers indicate the primers used for genomic DNA PCR reactions.
688 B – Genomic DNA PCR demonstrating full segregation of CtpA-FLAG strain, using the primers shown in the
689 figure. For primer sequences, please consult Supplemental Table 1.
690
691 **Supplemental Figure 2 – Growth curves for the different strains used in this study.**

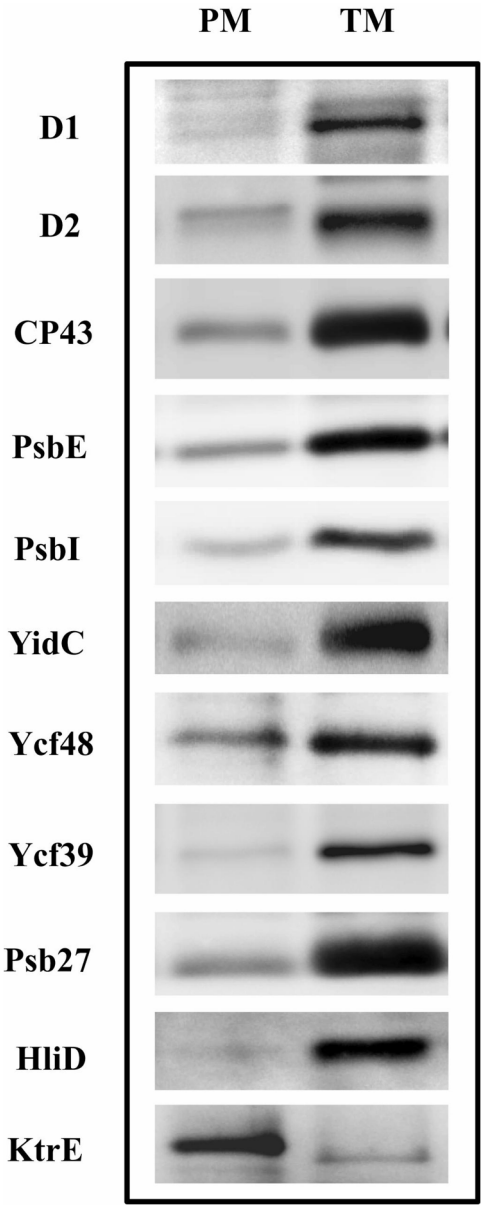

Figure 1 - Western blot analysis of the distribution of PSII subunits and assembly factors in PM and TM isolated from ΔPsbB (ΔCP47) strain. Each lane was loaded with 5 µg membrane proteins.
107x271mm (300 x 300 DPI)

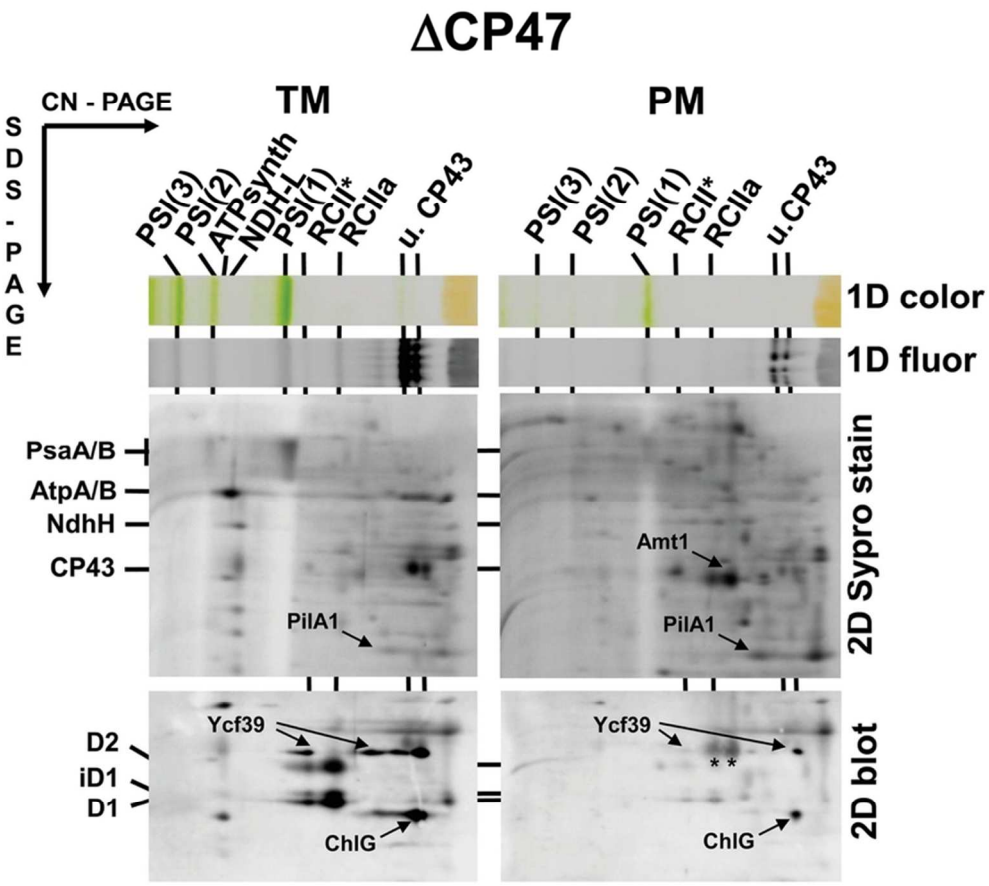

Figure 2 - Two dimensional analysis of thylakoid (TM) and plasma (PM) membrane samples purified from the ΔPsbB (ΔCP47) strain. Membranes were analyzed by CN-PAGE in the first dimension, the native gel was photographed (1D color) and scanned by LAS 4000 for fluorescence (1D fluor). After a 2nd dimension SDS-PAGE separation the gel was stained by Sypro orange (2D Sypro stain), electroblotted to a PVDF membrane and sequentially probed with the antibodies specific for D1, D2, Ycf39 and ChlG proteins (2D blot). Bands of the Amt1 transporter (Sll0108) and PilA1 (Ssl1694) designated in the stained gels were identified by MS. Designation of complexes: PSI(3), PSI(2), and PSI(1), trimeric, dimeric and monomeric PSI complexes, resp.; ATPsynth, ATP synthase; NDH-L, the large NADH dehydrogenase complex; RCII* and RCIIa, PSII RC assembly complexes lacking CP43 and CP47; u.CP43, unassembled CP43. Asterisks indicate two Amt1 bands unspecifically cross-reacting with antibodies. The loaded samples contained 60 µg membrane proteins.
77x69mm (300 x 300 DPI)

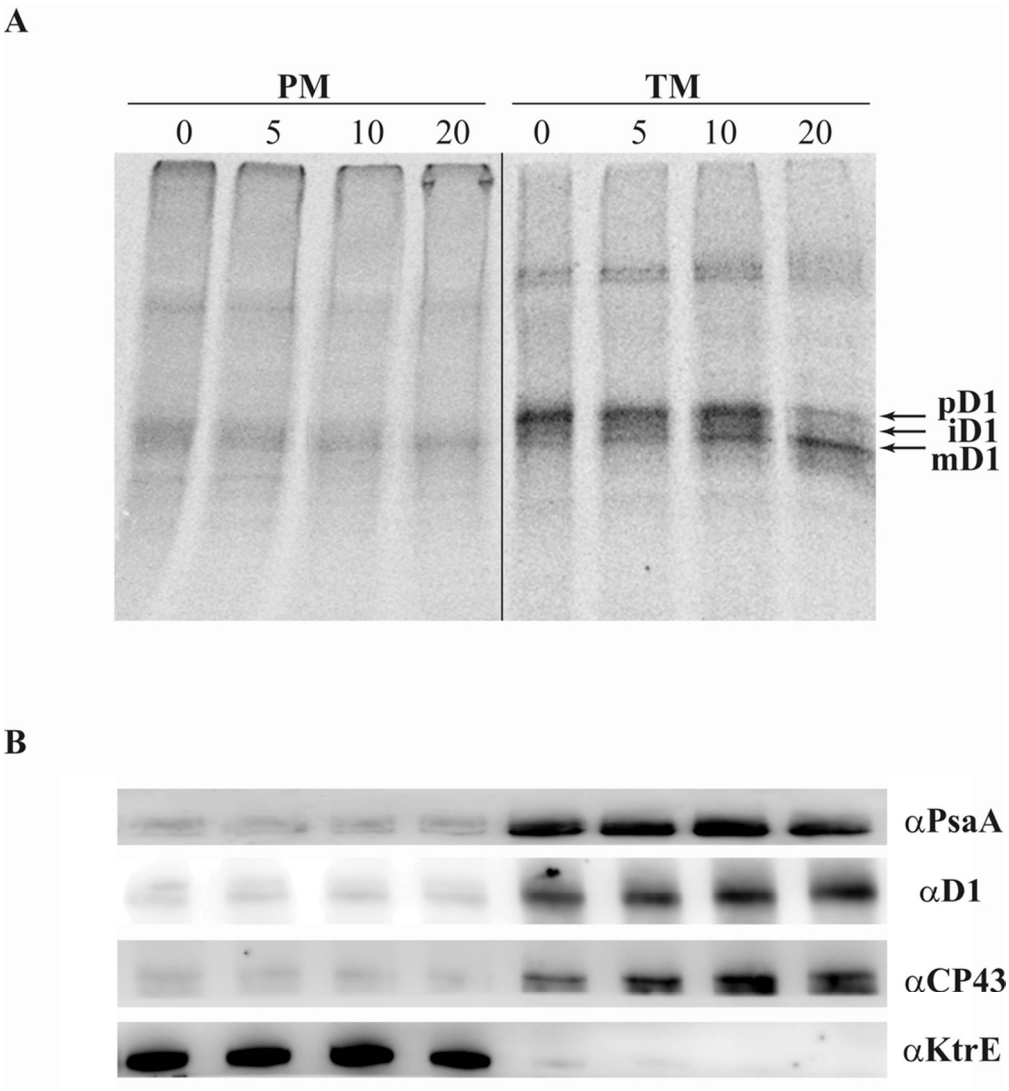

Figure 3 - [35S]methionine pulse-chase labelling of pD1 processing during photoinhibition recovery.
A – Autoradiography image of pulse-chase labelled PM and TM samples. Times indicated are in minutes after changing from 10 ºC and 1000 µmol photons m-2s-1 to 30 ºC and 50 µmol photons m-2s-1. Each lane contained 2000 cpm of labelled proteins.
B – Western blot analysis of pulse-chase labelled PM and TM samples. Each lane was loaded with 5 µg membrane proteins.
93x101mm (300 x 300 DPI)

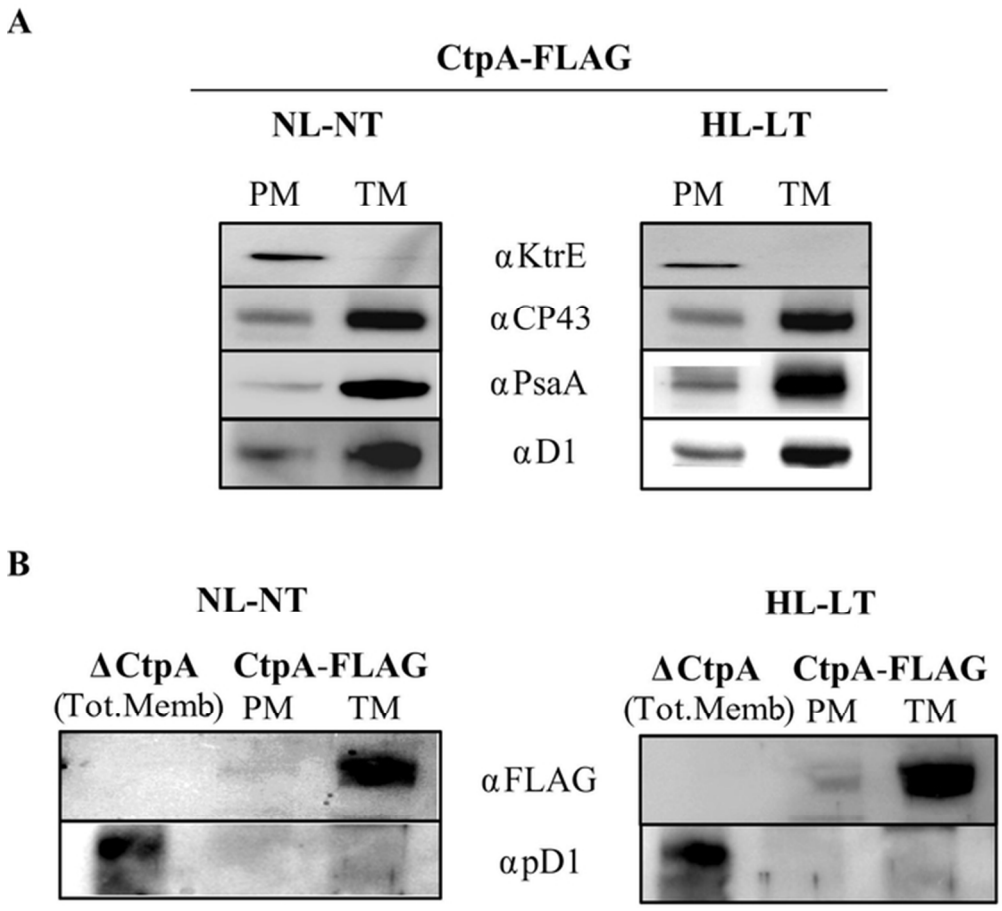

Figure 4 - Western blot analysis of PM and TM samples for CtpA-FLAG. Each lane contains 5 µg membrane proteins.
A – CtpA-FLAG PM and TM samples from samples grown in normal light and temperature ("NL-NT") and exposed to photoinhibitory conditions ("HL-LT") probed with antibodies for typical PM and TM proteins.
B – Western blot analysis of (CtpA-)FLAG and pD1 distribution in CtpA-FLAG and ΔCtpA strain PM and TM samples.
60x55mm (300 x 300 DPI)

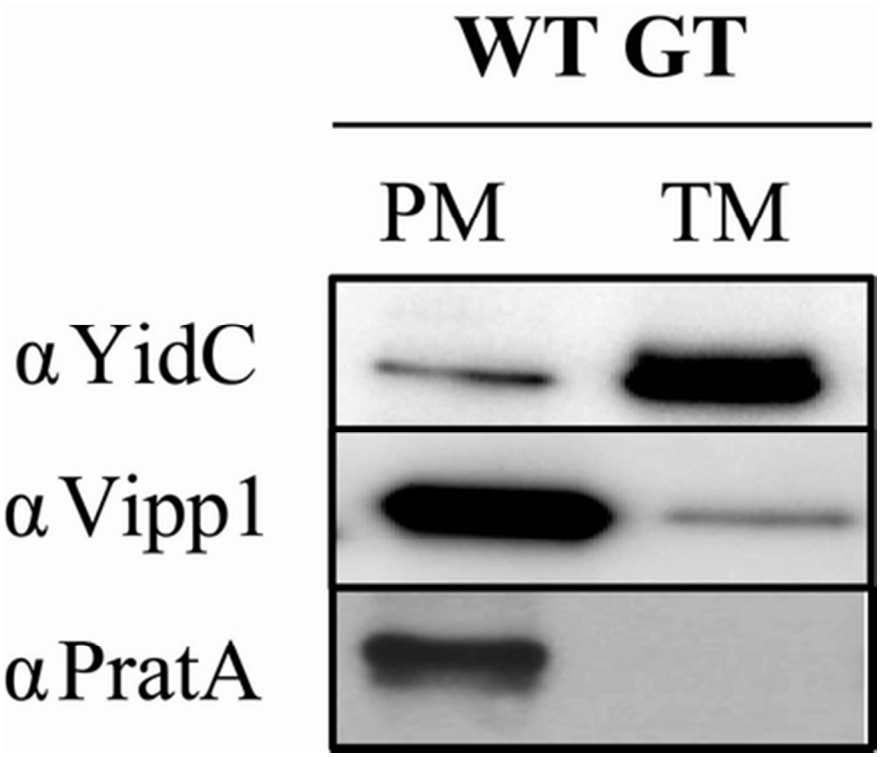

Figure 5 - Western blot analysis of the distribution of PratA, Vipp1 and YidC in WT samples of PM and TM. Each lane contains 5 µg membrane proteins.
36x31mm (300 x 300 DPI)