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Review

Regulatory factors for the assembly of thylakoid membrane protein complexes

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Major multi-protein photosynthetic complexes, located in thylakoid membranes, are responsible for the capture of light and its conversion into chemical energy in oxygenic photosynthetic organisms. Although the structures and functions of these photosynthetic complexes have been explored, the molecular mechanisms underlying their assembly remain elusive. In this review, we summarize current knowledge of the regulatory components involved in the assembly of thylakoid membrane protein complexes in photosynthetic organisms. Many of the known regulatory factors are conserved between prokaryotes and eukaryotes, whereas others appear to be newly evolved or to have expanded predominantly in eukaryotes. Their specific features and fundamental differences in cyanobacteria, green algae and land plants are discussed.

Keywords: regulatory factors; biogenesis; assembly; thylakoid membrane protein complex; chloroplast

1. INTRODUCTION

Thylakoids are flattened vesicles found in cyanobacteria and chloroplasts that probably arose in photosynthetic bacteria, appearing in close correlation with oxygenic photosynthesis [1]. The most advanced and efficient thylakoids are present in the chloroplasts of land plants, in which they are arranged into extensive, highly interconnected networks of grana stacks connected by stroma lamellae [2]. By contrast, the thylakoids in cyanobacteria consist mainly of single layers formed by long lamellae, and extensive stacking of the grana lamellae is not observed [3]. Thus, thylakoid membrane systems evolved in cyanobacteria but became more complex in algae and plants [2], and the ultrastructure and composition differences of the thylakoids in cyanobacteria, algae and plants correspond well with their evolutionary positions [3].

In the chloroplasts of green algae and plants, four photosynthetic protein complexes are embedded in the thylakoid membranes and are functionally connected in a series through a photosynthetic electron transport chain. These complexes include photosystem II (PSII) and photosystem I (PSI) as well as their associated light-harvesting antennae, the cytochrome *b₆f* complex (cyt *b₆f*), and the proton-translocation ATP synthase (ATPase). In cyanobacteria such as *Synechocystis* PCC6803, cyt *b₆f* is also a part of the respiratory electron transport chain [4]. Three-dimensional structures of several multimeric protein complexes for oxygenic photosynthesis have been reported at atomic resolution [5]. However, our

knowledge of the molecular mechanisms involved in the biogenesis of thylakoid membrane protein complexes is still limited. In addition, PSII, PSI and cyt *b₆f* complexes also contain accessory factors such as chlorophylls and xanthophylls as well as haems, quinones and iron–sulphur centres. The processes of pigment synthesis, transport and final incorporation remain largely unknown.

Genetic and biochemical studies have revealed that multistep assembly processes are responsible for the formation of functional photosynthetic protein complexes. Each assembly step is likely to be regulated by a number of factors [6–12]. In the last few decades, analysis of photosynthetic mutants in oxygenic photosynthetic organisms such as *Synechocystis*, *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*, maize and tobacco has identified a considerable number of regulatory proteins involved in these processes and has provided insights into the molecular mechanisms underlying the assembly of thylakoid membrane protein complexes. Some of these factors appear to be newly evolved or to have proliferated predominantly in plants, whereas others are of eubacterial origin, often having gained new functions. In this review, we provide a brief overview of the present knowledge of regulatory factors involved in the assembly of PSII, PSI and cyt *b₆f* complexes in photosynthetic organisms, particularly in three photosynthetic model organisms: the cyanobacterium *Synechocystis* PCC6803, the unicellular green alga *Chlamydomonas* and the vascular plant *Arabidopsis* (table 1).

2. REGULATORY FACTORS FOR PSII ASSEMBLY

PSII catalyses light-induced electron transfer from water to plastoquinone, with the concomitant production of molecular oxygen. In general, PSII can be

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Table 1. Regulatory factors for PSII, PSI and cyt *b₆f* assembly. M, thylakoid membrane; L, thylakoid lumen; S, stroma; —, not identified yet.

<i>Arabidopsis thaliana</i>	<i>Chlamydomonas reinhardtii</i>	<i>Synechocystis</i> PCC6803	location	protein domain	references
HCF136	Chlredraft_112806	YCF48	L	BNR repeat	[13–16]
LPA1	REP27	—	M	TPR	[17,18]
PAM68	Chlredraft_169505	Sll0933	M	DUF3464	[19]
LPA2	—	—	M	unknown	[20]
LPA3	Chlredraft_183275	—	M, S	DUF1995	[21]
AtAlb3	CrAlb3.1, CrAlb3.2	Slr1471	M	OxaA/YidC	[21–27]
LPA19	—	Slr1645(Psb27)	L	unknown	[28,29]
AT4G28660	—	Sll1398(Psb28)	M, S	unknown	[30,31]
AtPsb29	—	Sll1414(Psb29)	M, S	unknown	[30,32]
FKBP-2	Chlredraft_2101108	—	L	PPIase	[33]
CYP38	Chlredraft_196558	—	L	cyclophilin	[34–36]
—	—	Slr0286	M	unknown	[37]
—	—	Slr2013	M	DUF58	[38]
AtYCF3	CrYCF3	Slr0823(YCF3)	M	TPR	[39–41]
Y3IP1	—	—	M	unknown	[42]
YCF4	CrYCF4	Sll0226(YCF4)	M	unknown	[39,43–45]
HCF101	Chlredraft_102098	—	S	MRP-like	[46,47]
APO1	—	—	S	DUF794	[47,48]
AT1G54500	—	RubA	M	Rubredoxin	[49,50]
AT1G49380	CCS1	Slr2087	M	ResB	[51–53]
HCF164	CCS5	—	M	Thioredoxin	[54–56]
—	CCS4	—	M	unknown	[57]

described in terms of three major structural domains: (i) the reaction centre consisting of the heterodimeric D1/D2 proteins, the α and β subunits of cytochrome *b559* and two low-mass polypeptides PsbI and PsbW; (ii) the inner light-harvesting antenna comprising the chlorophyll-containing proteins CP43 and CP47; and (iii) the oxygen-evolving complexes of extrinsic proteins, including the manganese-stabilizing protein bound on the luminal side of the complex [58,59]. PSII intrinsic membrane proteins are largely conserved between cyanobacteria and higher plants, whereas the extrinsic proteins that form the oxygen-evolving complexes of PSII vary significantly [60]. PSII assembly consists of multiple steps (figure 1), including the formation of an initial complex of the PSII reaction centre, the association of CP47/43 with the PSII reaction centre, the integration of the small subunits and finally the dimerization of PSII monomers [61].

The multi-step formation of functional PSII is likely to be assisted by many nuclear-encoded regulatory proteins. *HCF136* (*high chlorophyll fluorescence 136*), which encodes a hydrophilic protein localized in the lumen of stroma thylakoids, was first identified as being involved in PSII assembly in *Arabidopsis* through isolation and characterization of high chlorophyll fluorescence mutants [13]. In the absence of HCF136, PSII proteins are synthesized normally but do not assemble into a stable PSII complex [13]. Under standard illumination, the amounts of both PSII and PSI are reduced, suggesting that HCF136 may be required for photosystem biogenesis in general [13]. However, under low light HCF136 selectively regulates the biogenesis of PSII. HCF136 associates with a PSII pre-complex containing at least D2 and cytochrome *b559* and is essential for the assembly of the PSII reaction centre [14]. An HCF136 homologue, YCF48

(hypothetical chloroplast open reading frame 48), was identified in the cyanobacterium *Synechocystis* PCC6803 [15]. Disruption of *ycf48* slows the formation of PSII complexes and causes a decrease in the final level of PSII core complexes, similar to the effects of the *hcf136* mutation [15]. In addition, the absence of YCF48 leads to a dramatic decrease in the levels of precursor and partially processed D1 protein, and only low levels of unassembled mature D1 are detected. YCF48 is also important for the repair of PSII in *Synechocystis* [15]. Interestingly, a recessive allele of the maize *HCF136* homologue causes a lack of PSII complexes and grana thylakoids in mesophyll chloroplasts, which is consistent with the previously defined function of its *Arabidopsis* counterpart. However, maize *hcf136* is also defective in processing the full-length *psbB-psbT-psbH-petB-petD* polycistronic RNA specifically in bundle sheath cells [16]. Microarray analysis revealed that mesophyll and bundle sheath cell transcript pools are altered in the *hcf136* mutant, but the reason for this has not yet been determined [16].

LPA1 (low PSII accumulation 1) appears to be an integral membrane chaperone that is required for the efficient assembly of PSII reaction centres [17]. In *lpa1 Arabidopsis* mutants, the assembly of newly synthesized PSII is less efficient; in addition, the turnover rates of the PSII core proteins CP47, CP43, D1 and D2 are higher in *lpa1* mutants compared with wild-type plants [17]. *LPA1* encodes a chloroplast protein that contains two TPR (tetratricopeptide repeat) domains and interacts with D1, but not with D2, cyt *b₆* or Alb3 (ALBINO3, see below for details) [17]. *LPA1* homologues are present in *Ostreococcus* and in *Chlamydomonas*, in which REP27 (repair-aberrant mutant of PSII) is the *LPA1* homologue. In contrast to the inactivation of *LPA1*, inactivation of REP27

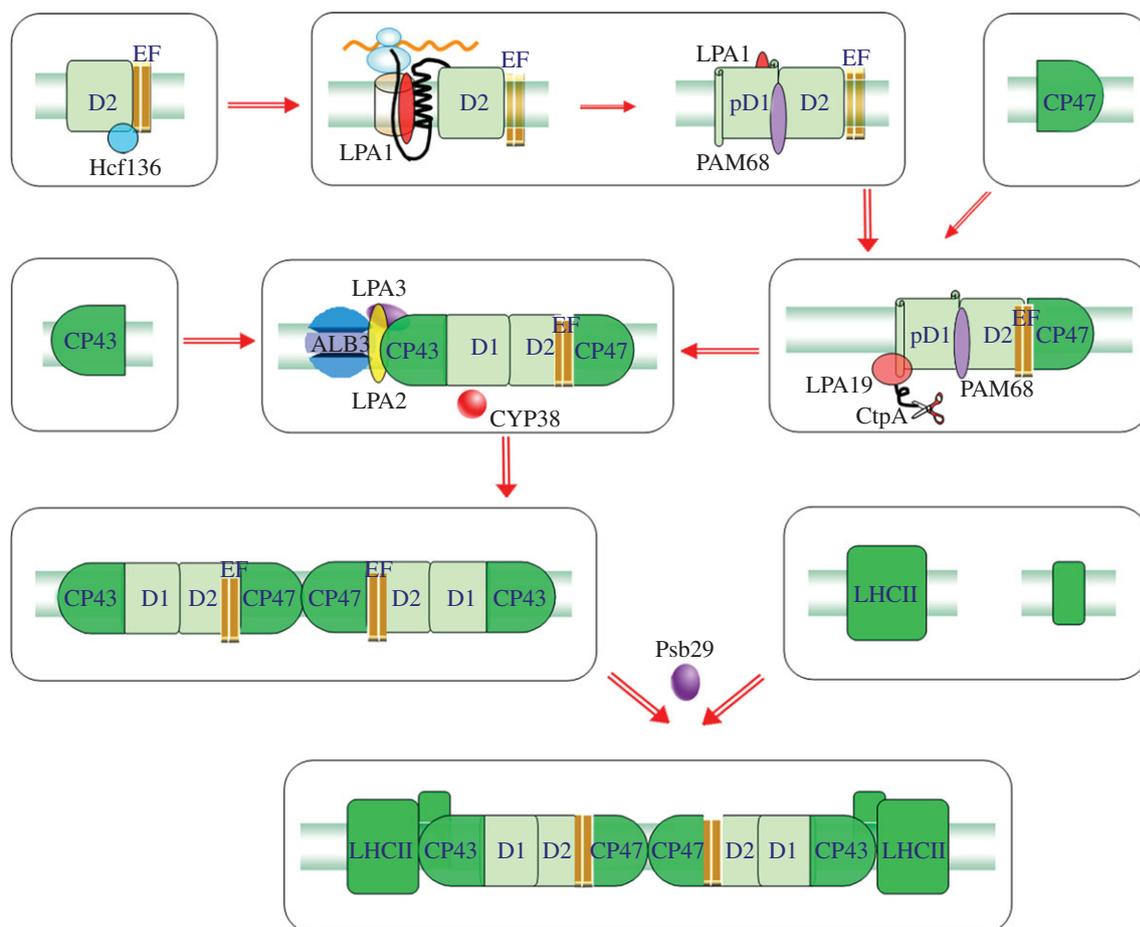


Figure 1. Model for the assembly of PSII in higher plant. The assembly factors for PSII in this model were shown according to currently reported functions, and their association with PSII is only illustrative. For clarity, the low-molecular-mass subunits and the extrinsic subunits are omitted.

does not affect the accumulation of PSII subunits other than the D1 protein [18]. REP27 is specifically required for efficient D1 turnover but is not essential for *de novo* biogenesis and assembly of PSII [18]. Another PSII assembly factor, PAM68 (photosynthesis affected mutant 68), is associated with an early intermediate complex in *Arabidopsis* that may contain D1 and LPA1 [19]. PAM68 is a thylakoid membrane protein and, similar to HCF136 and Alb3, it is conserved in cyanobacteria and photosynthetic eukaryotes. This protein acts at the level of D1 maturation and stability, promoting the transition from the reaction centre assembly intermediate to the larger PSII assembly complexes in *Arabidopsis* [19]. However, removal of PAM68 results in different compensatory mechanisms in plants and cyanobacteria: in *Synechocystis* the transition from the reaction centre to later PSII intermediates appears to be accelerated when PAM68 is absent [19]. The higher-plant-specific HCF243 chloroplast protein has been implicated in maintaining the stability of the D1 protein and promoting subsequent PSII assembly [62].

LPA2 and LPA3 (low PSII accumulation 2 and 3), two other thylakoid membrane proteins, have overlapping functions in assisting CP43 assembly, and the cooperation between LPA2 and LPA3 is essential for PSII assembly [20,21]. LPA2 encodes an integral membrane protein containing two transmembrane

domains. Homologues of LPA2 have been identified in other plants, but not in cyanobacteria or *Chlamydomonas* [20]. LPA3 does not have any transmembrane domains, and highly conserved LPA3 sequences have been found in plants, *Chlamydomonas* and *Ostreococcus* [21]. LPA2 may have evolved after the divergence of the land plant lineage or may have been lost in other lineages. Both LPA2 and LPA3 interact with Alb3. This interaction with Alb3 suggests that the function of Alb3 in some PSII assembly processes is most probably mediated through interactions with LPA2 and LPA3 [21].

Alb3 is related to the Oxa1p and YidC proteins from *E. coli*. Such proteins are essential components for insertion and assembly of multi-subunit membrane protein complexes. Two Alb3 proteins (Alb3.1 and Alb3.2) are present in *Chlamydomonas*, whereas only one Alb3 protein is present in *Arabidopsis*. Alb3.1 of *Chlamydomonas* is involved mainly in the insertion of the light-harvesting complex [22], and it is required for the insertion of D1 into functional PSII [23]. Alb3.2 interacts with Alb3.1 and the reaction centre polypeptides of PSI and PSII. The amounts of both PSI and PSII were reduced in *Alb3.2* RNA interference lines, indicating that the level of Alb3.2 is limiting for the assembly and/or maintenance of these complexes [24]. Thus, it appears that Alb3 is a general assembly factor rather than a PSII-specific

assembly factor in *Chlamydomonas*. Indeed, Alb3 interacts with PSII subunits D1, D2 and CP47, as well as with PSI reaction centre PsaA and ATP synthase subunit CF₀III [23–25]. *Synechocystis* contains one Alb3-like protein, Slr1471, which shares 39% identity with the *Arabidopsis* ALB3 protein [26]. One study demonstrated that the thylakoid membrane structure of the merodiploid *slr1471* mutant cell is disorganized, in accordance with the phenotypes of the corresponding mutants in *Arabidopsis* and *Chlamydomonas* [26]. Another study suggested that Slr1471 is important for the *de novo* assembly of the D1 precursor protein into the PSII reaction centre [27].

During PSII assembly, the D1 protein is synthesized in most organisms as a precursor with a C-terminal extension that must be cleaved to allow insertion of the mature D1 protein. A specific endoprotease cTPA (carboxy-terminal processing proteinase) and the TPR protein Prat from *Synechocystis* are involved in the processing of the C-terminal extension of the D1 protein [28,63–65]. Indeed, the Prat protein interacts with the C-terminal region of the precursor D1 protein in yeast two-hybrid assays [65]. Further study revealed that PratA is a Mn²⁺-binding protein and that PratA is required for efficient delivery of Mn²⁺ to PSII *in vivo* [66]. Interestingly, a significantly higher amount of Psb27 is associated with PSII in a cyanobacterial mutant lacking CtpA, suggesting an important role for Psb27 in precursor D1 processing [28]. In *Arabidopsis*, the Psb27 homologue LPA19 (low PSII accumulation 19) plays a role in facilitating the C-terminal processing of precursor D1 [29]. Chloroplast protein labelling assays indicated that the C-terminal processing of D1 protein is impaired in *lpa19* mutants. The LPA19 protein specifically interacts with the soluble C terminal region present in the precursor and mature D1 [29]. A second *psb27* homologue in *Arabidopsis* is required for efficient repair of photodamaged PSII, but not for PSII accumulation [67]. However, in *Synechocystis*, Psb27 has been implicated in the assembly of the Mn₄Ca cluster and the stabilization of unassembled CP43 [68–70].

Along with Psb27, Psb28 and Psb29 proteins were also identified as substoichiometric components of His-tagged CP47 preparations isolated from *Synechocystis* PCC6803 [30]. In *Synechocystis* PCC6803, Psb28 is not a component of the fully assembled dimeric PSII core complex, but it is preferentially bound to PSII assembly intermediates containing the inner antenna CP47, probably by attachment to the cytoplasmic side of CP47 [31]. In *psb28* null mutants, the functional properties of PSII are not affected but synthesis of CP47 and PSI subunits PsaA and PsaB is reduced. In addition, inactivation of Psb28 results in inhibition of chlorophyll synthesis at the cyclization step, which suggests that Psb28 functions in regulating the synthesis of chlorophylls [31]. A Psb28 homologue is also found in the *Arabidopsis* genome but its role remains elusive. *Arabidopsis* and *Synechocystis psb29* mutants show increased light sensitivity and an increase in the proportion of uncoupled antenna with increasing light intensities [32]. It is supposed that Psb29 is required for proper assembly of PSII supercomplexes or efficient disassembly of photodamaged PSII complexes [30,32].

Several luminal immunophilin proteins have been found to be associated with PSII assembly. The immunophilin family includes FKBP (FK-506 binding protein) and cyclophilin proteins originally identified as receptors for immunosuppressive drugs (FK506 and cyclosporin A) [71,72]. Most immunophilin proteins have PPIase (peptidyl-prolyl *cis-trans* isomerase) activity that catalyses the *cis-trans* conversion of X-Pro peptide bonds, a rate-limiting step in protein folding [71,72]. This association with protein-folding capability suggests that these enzymes play a central role in the biogenesis of protein complexes, probably including PSII complexes. Indeed, Lima *et al.* [33] found that a redox-active FKBP-type immunophilin FKBP-2 functions in the accumulation of the PSII supercomplex in *Arabidopsis*, although its mechanism remains undetermined [33]. TLP40 (thylakoid lumen PPIase of 40 kDa), a cyclophilin-type immunophilin identified in spinach, is associated with and regulates the activity of a PSII-specific protein phosphatase within the thylakoid membrane [73,74]. Cyclophilin 38 (CYP38), the *Arabidopsis* orthologue of the TLP40 protein, was shown to be important for assembly and maintenance of PSII, particularly the supercomplexes [34]. CYP38 assists in the proper folding and insertion of D1 and CP43 into PSII and the correct assembly of the water-splitting Mn₄-Ca cluster [35]. However, CYP38 was shown not to possess PPIase activity, although it can interact with the E-loop of CP47 through its cyclophilin domain [36]. Crystal structural analysis of CYP38 revealed the presence of N-terminal helical bundle and C-terminal cyclophilin β -sheet domains. This study also uncovered a unique and previously uncharacterized domain, which may provide further understanding of the auto-inhibition mechanism of CYP38 function [36]. There are more than 50 gene models in *Chlamydomonas* with similarity to immunophilin proteins [75]. However, their possible functions in PSII assembly await further investigation.

At least two PSII assembly factors specific to cyanobacteria have been described. One is Slr0286 of *Synechocystis*, which appears to facilitate functional assembly and stability of the water splitting system of PSII [37]. It lacks known motifs and no homologues have been found in other organisms, not even in other cyanobacteria [37]. The other is Slr2013, which is involved in functional assembly of PSII by regulating the folding of D2 protein [38]. Slr2013 is annotated as a hypothetical protein with a DUF58 (domain of unknown function 58) domain, and its homologues are found in other cyanobacteria but not in eukaryotes [38]. In addition, a chloroplast-localized Deg1 protease has been shown to assist in PSII assembly in *Arabidopsis*, probably through interaction with the D2 protein [76].

3. REGULATORY FACTORS FOR PSI ASSEMBLY

PSI mediates light-induced electron transfer from plastocyanin on the luminal side of thylakoids to ferredoxin on the stromal side. The central components and structures of PSI complexes from cyanobacteria and chloroplasts are remarkably similar. Nevertheless, some differences do exist between them. The PSI

complex of chloroplasts exists as a monomer and is associated with membrane-bound light-harvesting complexes [77], whereas that of cyanobacteria is present as a trimer and is connected to phycobilisomes on top of the thylakoid membrane as additional antenna systems [78]. The PSI complex of cyanobacteria consists of 12 proteins per monomer, whereas that of plants is composed of at least 15 subunits plus at least four light-harvesting proteins [8]. Among these 15 subunits, four (PsaG, PsaH, PsaN and PsaO) are newly evolved in chloroplasts, whereas one cyanobacterial subunit (PsaM) was lost in higher plants [8].

The assembly of the PSI complex initiates with the co-translational membrane insertion of PsaA and PsaB. Following the binding of co-factors to the two reaction centre proteins, the PsaA/B reaction centre heterodimer is formed [79]. Subsequently, relatively small subunits with a maximum molecular mass of 18 kDa are attached to the heterodimer [80]. During this step, three PSI subunits (PsaC, D and E), which are positioned on the stromal side, form the ferredoxin-binding site. These three PSI subunits do not contain any transmembrane domains and constitute the so-called 'stromal ridge' of PSI, which can form a large ternary complex with ferredoxin and the FNR (ferredoxin-NADP(+) reductase) enzyme on top of PSI [81]. The final formation of the mature PSI complex through the binding of LHCI (light harvesting complex I) and the PsaG, PsaK, PsaL, PsaN, PsaO and PsaP subunits appears to be a slow process; however, the sequence of events is currently unclear [8]. Several regulatory factors involved in PSI assembly have been identified using genetic approaches.

YCF3 (hypothetical chloroplast open reading frame 3), a small chloroplast-encoded protein with three TPR domains, was first identified in tobacco and *Chlamydomonas*, and its role in PSI assembly was subsequently investigated in *Chlamydomonas* using a reverse genetics approach [39]. Using random and site-directed mutagenesis of *yfc3*, Naver *et al.* [40] demonstrated that YCF3 is required for PSI assembly, but not for its stability. YCF3 interacts directly with the PSI subunits PsaA and PsaD, suggesting that YCF3 may function in the assembly of the stromal ridge of the PSI complex [40]. In a temperature-sensitive barley mutant in which *yfc3* mRNA is impaired at high temperature, PSI accumulation is also affected. In this barley mutant, the reduction in PSI is related to the decrease in YCF3 protein content at different temperatures [41]. Thus, it appears that YCF3 may mediate a rate-limiting step in PSI assembly.

Y3IP1 (YCF3-interacting protein 1), which specifically interacts with the YCF3 protein, was identified by co-immunoprecipitation with a FLAG-tagged YCF3 in transplastomic tobacco [42]. Subsequent reverse genetics analysis of Y3IP1 function in tobacco and *Arabidopsis* revealed that the knockdown of Y3IP1 leads to a specific deficiency in PSI but does not result in loss of YCF3. Y3IP1 is loosely associated with thylakoid membranes and exists in two distinct protein complexes [42]. Unlike all other PSI assembly factors known to date, Y3IP1 appears to be newly evolved in photosynthetic eukaryotes. This observation suggests

that it may function in a step of PSI assembly that differs between cyanobacteria and chloroplasts. Such a difference could be in the formation of the stromal ridge, as this step requires import and sorting of PsaD and PsaE into the thylakoid membrane from the cytosol in chloroplasts, a step that is not required in cyanobacteria [8]. Y3IP1 most probably functions as a receptor for (one of) these proteins before their integration into the PSI complex [8].

YCF4 (hypothetical chloroplast open reading frame 4) a 22-kDa protein with two putative transmembrane domains, is another chloroplast-encoded assembly chaperone for PSI. Compared with YCF3, it is more stably associated with the thylakoid membrane and accumulates more abundantly [39]. A tandem affinity purification tagged version of YCF4 was used to purify a stable YCF4-containing complex of approximately 1500 kDa from *Chlamydomonas*, which also contained the opsin-related COP2 and the PSI subunits PsaA, PsaB, PsaC, PsaD, PsaE and PsaF [43]. Pulse-chase protein labelling revealed that the PSI proteins associated with the YCF4-containing complex were newly synthesized and partially assembled as an intermediate assembly subcomplex, indicating that the YCF4 complex may act as a scaffold for PSI assembly [43]. YCF4 is highly conserved amongst photosynthetic organisms from cyanobacteria to higher plants. It appears to be essential for PSI complex assembly in *Chlamydomonas*, whereas a cyanobacterial mutant deficient in YCF4 is still able to assemble the PSI complex, although at a reduced rate [44]. It was recently found that YCF4 may function as a non-essential assembly factor for PSI also in higher plants, based on results from tobacco [45].

PSI contains three [4Fe-4S] clusters that are associated with the PsaA/B heterodimer and PsaC and that are directly involved in electron transfer. HCF101 (high chlorophyll fluorescence 101) and APO1 (accumulation of PSI 1), two chloroplast proteins, have been genetically identified as essential and specific factors for the assembly of [4Fe-4S]-cluster-containing protein complexes, including PSI complexes [46,82]. HCF101 was identified from the seeding-lethal *Arabidopsis* mutant *hcf101* in which PSI activity is abolished [46]. The *hcf101* plants not only fail to accumulate mature PSI but also have reduced levels of the soluble [4Fe-4S]-cluster-containing complex ferredoxin-thioredoxin reductase in the stroma [46]. HCF101 belongs to an ancient and universally conserved family of P-loop ATPases previously designated as the 'MRP' (metG related protein) family. Schwenkert *et al.* [47] demonstrated that HCF101 might serve as a chloroplast scaffold protein that specifically assembles [4Fe-4S] clusters and transfers them to the chloroplast membrane and soluble target proteins [47]. By contrast, APO1 is conserved only among higher plants. In the *apo1 Arabidopsis* mutant, levels of the PSI core subunits as well as the intrinsic and peripheral PSI subunits are reduced [82]. APO1 had been proposed to be required for the accumulation of [4Fe-4S]-cluster-containing chloroplast complexes and antenna proteins. However, a recent study suggested that APO1 promotes the splicing of several chloroplast group II introns, and these splicing defects

can account for the loss of photosynthetic complexes in the *apo1* mutant [48].

In cyanobacteria, a membrane-associated rubredoxin denoted RubA is required for assembly of functional PSI complexes [49]. *RubA* mutants produce trimeric PSI complexes that are inactive in electron transport to flavodoxin or the artificial acceptor methyl viologen. The PSI complexes from *RubA* mutants lack the stromal subunits PsaC, PsaD and PsaE but contain all of the intrinsic membrane subunits [49]. However, the chloroplast-localized rubredoxin is associated with PSII, but not PSI [50]. Further analysis of the roles of chloroplast rubredoxin is required.

4. REGULATORY FACTORS FOR CYTOCHROME *b₆f* COMPLEX ASSEMBLY

The cyt *b₆f* complex of thylakoid membranes functions as a plastoquinol–plastocyanin oxidoreductase that links PSI and PSII. This complex is also involved in cyclic electron transport around PSI and acts as a proton translocase. In its native form, the cyt *b₆f* complex exists as a dimer with a molecular weight of 310 kDa that may be converted into a 140-kDa monomer with increasing detergent concentrations [83,84]. In plants, the complex is composed of four major subunits (cyt *f* (encoded by *petA*), cyt *b₆* (*petB*), the Rieske-FeS protein (*petC*) and PetD) and four small subunits (PetG, PetL, PetM and PetN) [85]. In addition to these subunits, additional proteins may transiently interact with the cyt *b₆f* complex. In higher plants, cyt *b₆f* has been co-isolated with FNR [86], and the functional coupling of a small phosphoprotein PetO to cyt *b₆f* has been reported [87]. PetP has been proposed as a new cyanobacterial cyt *b₆f* subunit that might be analogous to PetO [88].

The assembly process and the roles of cyt *b₆f* subunits have been studied in *Chlamydomonas* and plants. The assembly initiates with the formation of a mildly protease-resistant subcomplex consisting of cyt *b₆* and PetD, which acts as a scaffold for the subsequent assembly of the cyt *f* and PetG proteins [58,89]. The PetC and PetL proteins then participate in the assembly of the functional dimer [58,89]. Although the roles of the cyt *bf* subunits in the assembly, stability, and dimerization of the cyt *bf* complex have been examined in several studies [89–93], the assembly mechanism of the cyt *bf* complex remains largely unknown.

In the cyt *b₆f* complex, two haem prosthetic groups are covalently bound to the protein moieties: haem *c* of cytochrome *f* and haem *ci* attached to cytochrome *b₆* in the quinone-binding site Qi [94,95]. Covalent haem-binding plays a major role during the assembly of cyt *b₆f*. Two maturation pathways for haem attachment exist in chloroplasts. System II, a maturation pathway, was originally discovered in *Chlamydomonas* through genetic studies of *ccs* (cytochrome *c* synthesis) mutants that exhibited a photosynthetic deficiency and failed to accumulate holo forms of both cytochrome *f* and *c₆*. At least six factors, chloroplast-encoded *CCSA* and nuclear-encoded *CCS1* to *CCS5*, are required for haem attachment to the apo forms of

cyt *f* and cyt *b₆* on the lumen side of the thylakoid membrane [51–53].

In bacteria, a thioldisulphide membrane transporter of the CcdA/DsbD (disulfide bond D) family and a membrane-anchored, periplasm-facing thioredoxin-like protein CcsX are postulated to act sequentially to reduce the disulphide-bonded CXXCH in apocytochrome *c* before the haem ligation [96], and their orthologues have been identified and studied in chloroplasts. In *Arabidopsis*, the loss of CcdA and HCF164 (high chlorophyll fluorescence 164, an orthologue of CcSX) impairs photosynthesis and results in a cyt *b₆f* assembly defect, suggesting that these proteins are required for cyt *b₆f* biogenesis in plant chloroplasts [54,55]. The role of HCF164 was further investigated through a study of CCS5, its orthologue in *Chlamydomonas* [56]. CCS5 interacted with apocytochrome *f* and *c₆* in a yeast two-hybrid analysis and reduced a disulphide in the CXXCH haem-binding site of apocytochrome *f*. Moreover, the *ccs5* mutant was rescued by exogenous thiols [56]. These results indicate that CCS5/HCF164 is a component of a *trans*-thylakoid redox pathway and operates by reducing the CXXCH haem-binding site of apocytochrome *c* before the haem ligation reaction [56].

Recently, the small novel protein CCS4 was identified as a third component of the system II pathway in *Chlamydomonas*. Although CCS4 does not display sequence motifs suggestive of redox- or haem-binding function, biochemical and genetic complementation experiments suggest a role in the disulphide-reducing pathway required for haem attachment to apo forms of cyt *c* [57]. CCS4 might function in stabilizing CCDA or regulating its activity [57].

System IV maturation pathways, namely the assembly of co-factors on complex C subunit B (CCB), are responsible for the attachment of haem *ci* to apocytochrome *b₆* on the stromal side of the thylakoid membrane [97]. At least four protein factors, CCB1–CCB4, are necessary for this maturation pathway [98]. The CCB proteins of *Chlamydomonas* are conserved amongst all oxygenic photosynthetic organisms [99]. The *ccb1*, *ccb2* and *ccb4 Arabidopsis* mutants all are deficient in the accumulation of cytochrome *b₆f* complex subunits and lack haem covalently bound to cytochrome *b₆* [99,100]. Photosynthesis is impaired in the cyanobacteria *ccb1* and *ccb3* mutants [4]. The cyanobacterial cyt *b₆f* complex is essential because it is involved in the respiratory and photosynthetic electron transfer chains. It is expected that a cyt *b₆f* complex lacking haem *ci* would accumulate to higher levels in cyanobacteria than in *Chlamydomonas* because of lower quality control, as observed for crippled PSII complexes [4].

5. CONCLUSION AND PERSPECTIVE

Many regulatory factors involved in the biogenesis of thylakoid membrane protein complexes have been identified and functionally characterized in recent years through a combination of genetic, transcriptomic and proteomic approaches. However, thylakoid membrane protein complex biogenesis is more complex than originally anticipated. It is expected that the regulatory factors identified thus far represent only a

fraction of those that are involved in these processes; thus, the identification of the remaining regulators represents a goal for future work. In addition, thylakoid membrane protein complexes are dynamic and flexible in response to developmental and environmental inputs. Thus, one of the focuses in this field is to understand how these regulatory factors respond to environmental cues, especially light quality and quantity, and developmental states. Current challenges include elucidating the integration of the actions of different regulatory factors and identifying the signal transduction pathways that link these regulatory factors to internal and external signals.

Some of the known regulatory factors, such as Alb3, seem to have a general function in several steps of thylakoid membrane protein complex biogenesis; in addition, each assembly step may require the participation of many factors that act simultaneously. It appears that there is a dynamic regulatory network, in which the biogenesis of thylakoid membrane protein complexes is mediated by numerous regulatory proteins at different levels. Thus, another challenge is to understand how these factors are coordinated. Some of these factors, such as LPA1 and PAM68, that function in the same step, may form a protein complex mediated by protein–protein interactions [19]; however, the relationships among most of the known factors remain unknown.

To understand the mechanisms of these regulatory factors in more depth, novel techniques must be developed to overcome current obstacles. For instance, a major obstacle to elucidating the assembly of thylakoid membrane protein complexes is tracking of the assembly process *in vivo* and reconstituting it *in vitro*. Most of the assembly intermediates occur only transiently or are unstable and are therefore difficult to dissect using ordinary separation techniques, such as native electrophoresis or density gradient centrifugation. Various types of advanced fluorescent labelling techniques, coupled with the structural information available, may allow the assembly of thylakoid membrane protein complexes to be visualized in real time [101].

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