Minireview

Synthesis, membrane insertion and assembly of the chloroplast-encoded D1 protein into photosystem II

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Abstract Rapid light-dependent turnover of the chloroplast-encoded D1 protein maintains photosystem II (PS II) functional over a wide range of light intensities. Following initiation of *psb*A mRNA translation, the elongating D1 is targeted, possibly by chloroplast signal recognition particle 54 (cpSRP54), to the thylakoid cpSecY translocation channel. Transmembrane domains of nascent D1 start interacting with other PS II core proteins already during the translocation process to ensure an efficient assembly of the multiprotein membrane complex. Here we review the progress recently made concerning the synthesis, targeting, membrane insertion and assembly to PS II of the chloroplast-encoded D1 protein and discuss the possible convergence of targeting and translocation of chloroplast- and nuclear-encoded thylakoid proteins.

Key words: Assembly; cpSecY; Chloroplast signal recognition particle; D1 protein; Photosystem II; Protein translocation

1. Introduction

Photosystem II (PS II), a pigment–protein complex of more than 20 proteins in the thylakoid membrane, catalyses the light-driven water oxidation and reduction of plastoquinone. The reaction centre complex of PS II is composed of the D1 and D2 proteins, the α and β subunits of cytochrome *b*559 and the PsbI protein, all encoded by the chloroplast genome. The D1 and D2 heterodimer binds all essential redox components of PS II (chlorophyll a P_{680}, pheophytin, non-heme Fe^{2+}, quinones) and is a minimum unit for primary charge separation [1]. Oxygen-evolving PS II complexes additionally contain the intrinsic chlorophyll a binding proteins (CP43 and CP47), the oxygen-evolving complex (33, 23 and 17 kDa proteins) and several small proteins of largely unknown function [2]. Recent progress in resolving the three-dimensional crystal structure has allowed an assignment and revealed the spatial distribution of most of the subunits and cofactors within PS II [3,4]. Compared to the present structural information available on PS II, our understanding of the biosynthesis and assembly of this complex is very limited, particularly when considering the biosynthesis, membrane insertion and assembly of chloroplast-encoded subunits.

One approach to study the assembly of PS II is to make use of light-induced damage to PS II, occurring with low quantum yield at all light intensities [5]. The main target of photodamage under visible light is the reaction centre D1 protein [6,7]. To cope with constantly occurring photodamage, an efficient repair mechanism has evolved to replace the damaged D1 protein with a newly synthesized copy [8]. Such efficient PS II repair is comprised of several distinct and well-regulated processes, including interconversion and shuffling of PS II dimers and monomers between the grana and stroma membrane, partial PS II disassembly and D1 degradation, de novo synthesis, targeting, membrane insertion and reassembly of the D1 protein into PS II, as well as a release and ligation of cofactors during degradation of D1 and reassembly of PS II. In this review we focus on synthesis, membrane insertion and incorporation of the chloroplast-encoded D1 protein into PS II in higher plants.

2. Regulation of D1 translation elongation

The D1 protein is encoded by the plastid *psb*A gene and translated on thylakoid membrane-bound ribosomes. Mature D1 has five transmembrane domains (TMs) with the N-terminus on the stromal side of the thylakoid membrane. Light has an important role in regulation of various stages of D1 synthesis. Upon illumination, reducing equivalents generated by PS I electron transport activate a chloroplast protein disulfide isomerase called RB60 [9], which in turn catalyses a reduction of RB47, a homologue of poly(A) binding protein [10]. The regulatory multiprotein complex, including RB60 and RB47, then binds to the 5′-end of *psb*A mRNA activating the translation initiation process [10]. For details of the regulation of D1 translation initiation, mostly studied with the green alga *Chlamydomonas reinhardtii*, we refer to several recent review articles [11–14].

Experiments with higher plants have revealed that, besides translation initiation [13], the elongation of D1 is also strictly regulated. Indeed, no accumulation of full length D1 was found in etioplasts in the dark [15], even though the *psb*A transcripts were abundant and associated with polysomes [16]. Only upon illumination did the full length D1 start ac-

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Abbreviations: LHC II, light harvesting complex II protein; PS, photosystem; SRP, signal recognition particle; TM, transmembrane domain

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cumulating, concomitantly with pigment biosynthesis, without considerable increase of *psb*A mRNA in polysomes [16–18]. During elongation of the D1 protein, ribosomes pause at several distinct sites generating well-defined pausing intermediates of 17–25 kDa [19,20]. These intermediates were detected also in etioplasts [17,21] and in chloroplasts during the diurnal dark phase, though in lower quantities than during the light phase [22,23]. Distinct pausing of ribosomes during D1 translation elongation in mature chloroplasts probably facilitates the binding of chlorophyll to D1 intermediates thereby allowing the stabilization of full length D1 directly upon release from ribosomes [17,18].

Efficient translation elongation of *psb*A mRNA is dependent on optimal photosynthetic electron transport, particularly on the production of reducing compounds by PS I [24,25]. Block of electron transfer either with DCMU (keeps plastoquinone pool oxidized) or with DBMIB (keeps plastoquinone pool reduced) prevents the elongation of D1 [25]. Such arrest of D1 protein translation elongation can be partially released by a reducing agent dithiothreitol (DTT) or by electron donors supporting PS I electron transport [24,25]. Optimal D1 protein elongation, however, additionally requires the maintenance of transthylakoidal proton gradient, despite the presence of ATP and the reducing agent DTT [25,26].

Translation of the D1 protein also depends on the availability of assembly partners in the thylakoid membrane. Genetic approaches have revealed that translation of *psb*A mRNA is dramatically slowed down in mutants lacking D2 or CP47 [27]. Hampering the proper D1/D2 protein interaction in in organello translation system also severely inhibited the accumulation of full length D1 protein, and instead generated distinct pausing intermediates which remained associated with polysomes [25]. The assembly-dependent regulation of translation has been addressed in detail for another chloroplast-encoded protein, cytochrome *f*. In the absence of other subunits of the cytochrome b_6/f complex, the synthesis of cytochrome *f* is repressed through interaction of the carboxyl terminus of the protein with the 5′-UTR of the *pet*A mRNA, encoding this protein [28]. Such assembly-regulated translation is particularly important for the coordinated expression of chloroplast- and nuclear-encoded proteins to ensure correct stoichiometric production of polypeptides from each intracellular compartment.

3. Targeting and insertion of nascent D1 protein into the thylakoid membrane

Disclosing the mechanisms of targeting and insertion of chloroplast-encoded D1 protein into the thylakoid membrane is closely related to the progress made with other thylakoid proteins. Particularly interesting was the observation that mutations introduced to the signal sequence of cytochrome *f*, thus preventing the translocation of the protein, not only blocked the accumulation of cytochrome *f* but also interfered with the translocation of D1 and light harvesting complex II proteins (LHC II) [29]. This points to the possibility of a common translocation pathway shared by LHC II, D1 and cytochrome *f* and thus also to a convergence of translocation mechanisms for chloroplast- and nuclear-encoded proteins.

Targeting and translocation of nuclear-encoded proteins in chloroplasts have been extensively studied and are known to

Table 1
Maize and *Arabidopsis* mutants lacking various components implicated in translocation of chloroplast- and nuclear-encoded proteins

Mutant	Species	Phenotype	Mutagen	Protein mutated	Plastid ultra-structure	Effects on chloroplast-encoded proteins	Effects on nuclear-encoded proteins	Ref.
*hcf*106	maize	seedling lethal	Mu transposon	Hcf106		PS II, PS I, cyt b_6/f decreased	delta pH pathway precursors in the stroma increased	[38]
*tha*4	maize	chlorophyll deficiency; seedling lethal	Mu transposon	Tha4		PS II, PS I, cyt $b_6/f \sim 20\%$	delta pH pathway precursors in the stroma increased	[39]
*csy*1	maize	pigment-deficiency; seedling lethal	Mu transposon	cpSecY	little thylakoid membrane	RbcL, D1, PetD $< 10\%$ chloroplast translation blocked	delta pH and Sec substrates $< 10\%$, their precursors in stroma increased	[41]
ffc	*Arabidopsis*	yellow heart/virescent	X-ray	cpSRP54		PS I and PS II affected	LHC II decreased	[42]
*apg*2	*Arabidopsis*	albino	Ac/Ds transposon	TatC	no thylakoid membrane	no D1; RbcL normal	no LHC II	[43]
*tha*1	maize	pale green	Mu transposon	cpSecA		PS II, PS I decreased; cyt *f* diminished and precursor accumulated	Sec pathway precursors in the stroma increased	[44]
*alb*3	*Arabidopsis*	albino; seedling lethal	Ac/Ds transposon	Albino3	very few membrane		*cab* and *rbc*S gene transcripts are detected	[45]
cao	*Arabidopsis*	chlorotic	En/Ds, transposon	cpSRP43	normal	PS II, PS I, cyt b_6/f, ATPase not affected	LHC II decreased	[46]

follow at least four distinct routes [30–32]. The thylakoidal Sec pathway is comprised of chloroplast homologues of SecY/E translocon components, cpSecY/E [33,34], with cpSecA probably acting as a motor assisting protein movement during translocation [32]. The chloroplast signal recognition particle (cpSRP) pathway specifically targets LHC II from the stroma into the thylakoid membrane [35], possibly through a channel composed of Albino3 [36]. The delta pH pathway uses proton gradient across the thylakoid membrane as sole energy source for protein translocation [32,37], and it involves *hcf106*, *tha4* and *tatC* gene products [33,38–40]. A TatC–Hcf106 complex has been shown to function as a receptor for the pathway, and subsequent participation of Tha4 leads to translocation [40]. Spontaneous insertion into the thylakoid membrane has also been reported for some proteins [33]. Studies on nuclear mutants lacking components implicated in posttranslational targeting and translocation of nuclear-encoded proteins, like cpSRP54, TatC or cpSecY, have revealed strong pleiotropic effects on chloroplast biogenesis [41–43]. A summary of such various effects in mutants of maize and *Arabidopsis* is presented in Table 1 [38,39,41–46]. From these results it can be argued that at least some of the components involved in nuclear-encoded protein targeting and translocation are also essential for chloroplast-encoded protein targeting and insertion into the thylakoid membrane.

Recent development of the chloroplast S30 translation system [47] has greatly facilitated the search of translocation components common for posttranslational translocation of nuclear-encoded proteins and cotranslational insertion of chloroplast-encoded proteins. It was recently demonstrated that cpSRP54 can be efficiently crosslinked to nascent D1 chains which are still linked to ribosomes [48]. This is in line with the observation of two populations of cpSRP54 complexes in thylakoids [49]. One was shown to be associated with ribosomes and thus probably functions in cotranslational targeting of the D1 protein into the thylakoid membrane. Indeed, interaction of D1 nascent chain with cpSRP54 only occurs during the targeting process, and no longer exists after the nascent D1 has been inserted into the thylakoid membrane (unpublished data). The other cpSRP54 population forms a complex with chloroplast specific cpSRP43 and is involved in posttranslational targeting of LHC II into the thylakoid membrane [49]. No interaction of cpSRP43 with nascent D1 chain was detected [48], indicating that cpSRP43 is involved specifically in the posttranslational targeting process.

By analogy with cotranslational protein translocation in endoplasmic reticulum and in *Escherichia coli* [50,51], it is reasonable to speculate that cpSRP54 brings the nascent D1 chain to the thylakoid membrane, where cpSRP54 is released after interaction with a putative SRP receptor (Fig. 1, I). Recently, a chloroplast SRP receptor cpFtsY was identified and shown to target LHC II, together with cpSRP54 and cpSRP43, from stroma to the thylakoid membrane [52,53]. Possible function of cpFtsY in targeting of chloroplast-encoded D1 protein to the thylakoid membrane remains to be elucidated.

After being targeted to the thylakoid membrane, the D1 protein elongation and membrane insertion occur concomitantly. Due to cotranslational insertion of the D1 protein into the thylakoid membrane, the distinct ribosome pausing intermediates of D1 [19] can also be regarded as translocation intermediates. This, together with preferential labeling of the D1 protein during in organello translation [54], made it possible to demonstrate the involvement of thylakoid cpSecY translocon channel in D1 protein insertion and translocation (Fig. 1, II) [55]. Two populations of cpSecY complexes were detected in the thylakoid membrane by blue-native gel analysis, one about 100 kDa and the other about 900 kDa [55]. In the latter complex cpSecY was associated with ribosomes and nascent D1 chains suggesting the involvement of this cpSecY population in cotranslational membrane translocation and insertion of the D1 protein. More direct evidence for a close vicinity of cpSecY with D1 nascent chains was obtained by crosslinking and immunoprecipitation experiments [55]. CpSecY was shown to interact only transiently with D1 elongation intermediates during insertion into the thylakoid membrane, whereas after termination of translation, no interaction could be detected [55].

It is likely that the Sec-dependent posttranslational translocation and the SRP-dependent cotranslational protein insertion into the thylakoid membrane converge at the cpSecY translocase. The cpSecY complex of about 100 kDa is apparently involved in posttranslational SecA-dependent translocation of nuclear-encoded proteins [33,34], and the other population of higher molecular mass complex interacts with ribosomes in cotranslational translocation and insertion of chloroplast-encoded proteins [55]. A similar convergence has been previously demonstrated in yeast where Sec61p, a homologue to SecY, is a component of two different translocons, one of which is involved in posttranslational protein translocation, and the other in cotranslational translocation [56].

4. Cotranslational incorporation of the D1 protein into PS II

Replacement of the photodamaged D1 protein with a newly synthesized D1 copy maintains PS II functional over a wide range of light conditions. Functional dimeric PS II that undergoes photodamage is mainly located in appressed grana membranes, and is thus spatially segregated from the site of synthesis and insertion of the newly synthesized D1 protein in the stroma-exposed thylakoid membrane [57,58]. To replace the photodamaged D1, monomerized PS II complex migrates to the stroma-exposed thylakoid domain where coordinated D1 degradation and replacement take place [59,60]. Many lines of evidence suggest that the D2 protein plays an important role in early stabilization of D1 during the biosynthesis and repair of PS II. A D1-less PS II complex has been suggested to function as the receptor for the newly synthesized D1 protein [58,61] though such a complex has never been identified in the thylakoid membrane.

Integration of the newly synthesized D1 protein into PS II has been investigated by combining in organello translation with sucrose gradient fractionation analysis of labeled thylakoid membrane complexes [54,62]. Recent refinings of the solubilization and fractionation conditions to avoid disassembly of protein complexes during experimental analysis indisputably demonstrated that the new D1 copy is cotranslationally incorporated into PS II [20]. Short pulse labeling experiments of only 2.5 min allowed the demonstration that virtually all newly synthesized D1 protein is directly incorporated into PS II reaction center and PS II core complexes with or without CP43 associated [20]. Such rapid association of the newly synthesized D1 protein with other PS II core proteins suggested that the elongating D1 intermediates possibly start

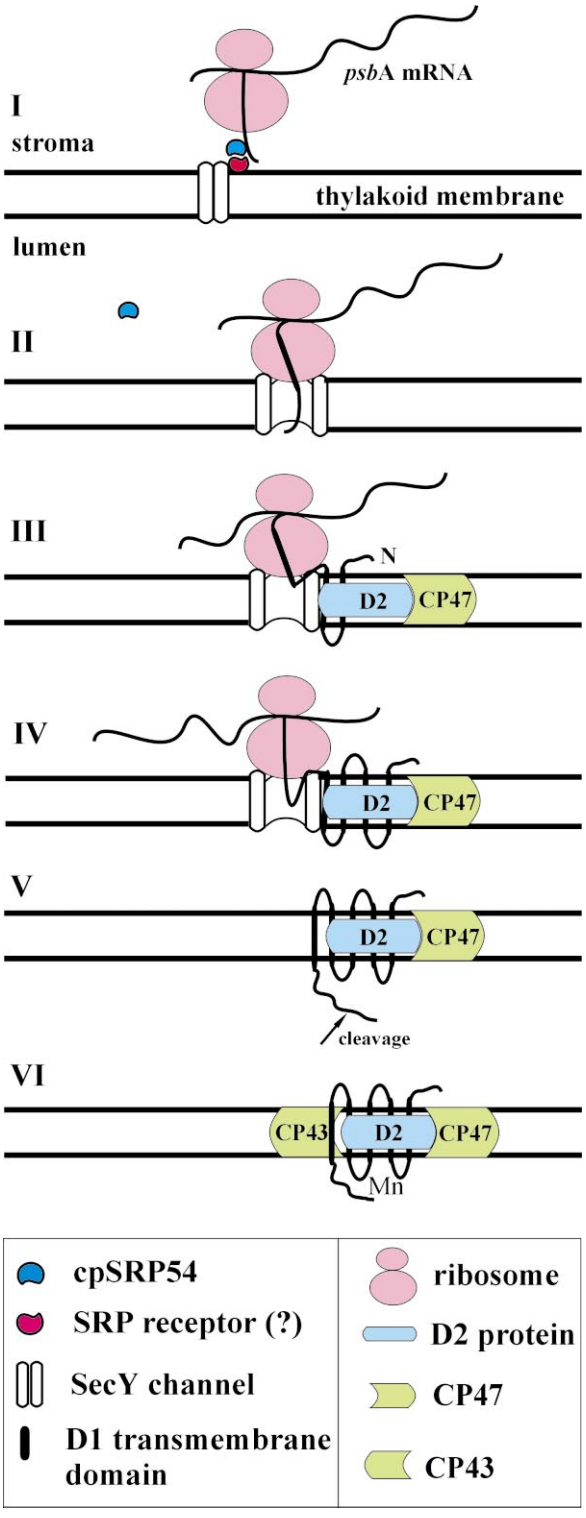

Fig. 1. Model for co- and posttranslational steps in synthesis, insertion and assembly of the chloroplast-encoded D1 protein into PS II. I: Targeting of nascent D1 chain by cpSRP54 to the thylakoid membrane and putative interaction with SRP receptor. II: Insertion of nascent D1 chain into the cpSecY channel of the thylakoid membrane. III and IV: Lateral exit of nascent D1 chain from cpSecY channel and cotranslational interaction with other PS II core proteins. At stage III, the interaction between nascent D1 chain (17 kDa) and D2 can be easily disrupted whereas elongation to 25 kDa (IV) establishes a tight interaction with the D2 protein. V: Termination of translation and C-terminal processing of the precursor D1 protein. VI: posttranslational association of CP43 into PS II. Either a newly synthesized CP43 or the one earlier released reassociates with PS II. Reassociation of CP43 may accelerate the C-terminal processing (indicated by arrow) of the D1 protein or vice versa. ←

to interact with components of D1-depleted PS II already during translation elongation. Crosslinking and immunoprecipitation analysis of thylakoid membrane-bound ribosome nascent chain complexes revealed the interaction of 17 and 25 kDa D1 nascent chains with D2 and provided direct evidence for cotranslational assembly of the new D1 copy into PS II [20,25]. It is likely that the 17 kDa D1 intermediate is comprised of two TMs inserted into the thylakoid membrane, with the third TM having just emerged from the ribosome tunnel. At this stage the interaction with D2 is still rather weak and can easily be disrupted by sodium dodecyl sulfate (Fig. 1, III). Interaction with D2, however, tightens when four TMs have been translated (corresponding the D1 pausing intermediate of 25 kDa) and inserted into the thylakoid membrane (Fig. 1, IV) [20]. Crosslinking experiments further demonstrated a close proximity of cysteine residues in elongating D1 chains with those in D2. Application of a thiol-modifying agent to block the protein disulfide formation markedly inhibited the stable, initial assembly of the D1 protein with D2 [25]. It is thus conceivable that a transient formation of a disulfide bond between one of the cysteine residues in the D1 protein (most probably C-125) and a cysteine residue in D2 is essential for stable integration and cotranslational assembly of D1 during the repair of PS II.

Efficient cotranslational incorporation of the newly synthesized D1 protein into PS II points to a tight coordination between D1 translation and incorporation into the pre-existing core complexes. These D1-depleted PS II complexes function as a receptor for elongating D1 and they contain, in addition to D2, most likely also CP47, PsbI and cytochrome *b*559 [25,61,63,64].

Thus, during the D1 elongation and translocation process, the TMs of elongating nascent D1 protein exit laterally from the translocon and start interacting with other PS II core proteins (Fig. 1, III, IV). The slow rate of translation elongation and pausing of ribosomes are likely to allow the escape of pairs of D1 transmembrane helices from the cpSecY translocon channel, thereby making the interaction of D1 with D2 possible already during translation elongation. Thus the cpSecY translocon seems to play an active role in directing the D1 nascent chains to their final location in the PS II complex. Lateral release of TMs from the translocon before termination of translation is not unique for the D1 protein and assembly of the multiprotein PS II complex in chloroplasts. Indeed similar observations on lateral exit of proteins from the channel during translocation have been made on bacterial membrane protein biogenesis and also on eukaryotic endoplasmic reticulum membrane [65,66]. Such structural and functional flexibility of the translocon probably ensures efficient protein folding and assembly during the biogenesis and repair of multiprotein membrane complexes, such as PS II.

5. Posttranslational assembly steps in the formation of functional PS II

Although the initial assembly of the D1 protein into PS II is

a cotranslational process, the formation of functional PS II complexes also depends on several posttranslational assembly steps, including reassociation of CP43 and C-terminal processing of the precursor D1 protein (pD1) (Fig. 1, V, VI). Formation of disulfide bonds seems to be particularly important for the reassociation of CP43 [25]. Conditions favoring disulfide bond formation have a stimulatory effect on the reassociation of CP43, while thiol modifying compounds exerted strong negative effects on the reassembly of CP43 [25]. Clearly, CP43 is a very dynamic component in PS II with dissociation and reassociation constantly occurring during the damage–repair cycle of PS II centers in vivo.

The D1 protein is synthesized as a precursor with a C-terminal extension of 9–16 amino acid residues [67,68], and only subsequently processed to mature form by a lumenal protease CtpA [67,69,70]. C-terminal processing is critical for several posttranslational assembly steps of PS II, such as ligation of the manganese cluster and reassociation of the oxygen-evolving complex. *Scenedesmus obliquus* mutant deficient in the C-terminal processing of pD1 is not capable of water oxidation due to the absence of the manganese complex [71–73]. In *Synechocystis* mutant defective in desaturation of 18:1 fatty acid of the thylakoid membrane the processing of pD1 was strongly hampered when temperature was lowered [74]. In these centers no PS II activity could be restored though the newly synthesized D1 precursor was found to assemble into the PS II complex. Incorporation of newly synthesized D1 protein into PS II complexes, analyzed by sucrose gradient fractionation, revealed a distinct correlation between the C-terminal processing of pD1 and the reassembly of CP43 into the PS II intact core complex. CP43 was found to stably assemble only in those PS II complexes where the newly synthesized D1 protein was present in its processed form [25]. From a functional point of view, such coordination of the assembly and processing steps could guarantee the correct timing for ligation of the manganese cluster, thereby protecting the sensitive reactivation of water splitting of PS II from photoinactivation [75].

During PS II repair, the pigments have to be re-ligated to the newly synthesized D1 protein. In in organello translation system, the synthesis of the full-length D1 protein and incorporation into PS II were not affected by the presence of chlorophyll synthesis inhibitor gabaculin or carotenoid synthesis inhibitor norflurazon [25]. Therefore, in the experimental system where only one turnover cycle of the D1 protein was studied, the existing pools of chlorophyll and carotenoid, or their intermediates, are available to stabilize the newly synthesized D1 protein. Newly synthesized pigments are, however, essential in intact leaves or algae cells. This was evidenced by great reduction in functional PS II centers when chlorophyll or carotenoid synthesis inhibitors were applied to the plant or to *C. reinhardtii* cells in vivo [76–78].

Synthesis of D1 and assembly into PS II are likely to require additional factors assisting this complex and multi-step process in chloroplasts. One of such auxiliary proteins identified so far is the chloroplast-targeted heat shock protein 70 (Hsp70) [79]. Underexpression of the chloroplast-localized Hsp70 protein caused an increased sensitivity of PS II to high light, whereas overexpression of Hsp70 protein had a protective effect. Protection occurred by enhancing the repair of PS II and it is thus reasonable to assume that Hsp70 plays a role as a molecular chaperone in the assembly and repair of PS II. Another 'assembly factor', Hcf136, has been identified in *Arabidopsis* by screening of the high chlorophyll fluorescence mutants [80]. The mutant lacks both the PS II core proteins and the oxygen-evolving complex. Protein labeling studies showed that the chloroplast-encoded PS II core proteins are synthesized, but unstable. The Hcf136 protein, with a typical bipartite structure of the transit peptide, has been localized to the thylakoid lumen where it accumulates in dark-grown wild-type seedlings, before accumulation of PS II core proteins [80]. Hcf136 probably functions as a stability and/or assembly factor for PS II. It is highly likely that with the assignment of the function for the up to 2500 chloroplast-targeted proteins, as deduced from the *Arabidopsis* genome [81], a number of novel proteins with special assisting functions in the biosynthesis and assembly of PS II will be discovered.

6. Concluding remarks

Biogenesis of chloroplast membrane protein complexes requires a coordinated synthesis and assembly of both the chloroplast- and nuclear-encoded proteins. Recent results obtained on D1 protein biogenesis suggest that the targeting and translocation of chloroplast- and nuclear-encoded proteins into the thylakoid membrane might converge at various steps. The development of chloroplast homologous S30 translation system [47] has made in vitro studies feasible on targeting and insertion of chloroplast-encoded proteins, and will be invaluable for further unraveling the detailed mechanisms for these processes. Synthesis, membrane insertion and assembly processes are also likely to require the participation of many still unknown auxiliary proteins, which are mostly nuclear-encoded. Moreover a strict quality control at various levels is required to avoid accumulation of non-functional proteins in the thylakoid membrane. The global gene expression and proteome analysis of chloroplast-targeted proteins, together with reverse and forward genetics, will undoubtedly disclose new components involved in regulation of thylakoid protein synthesis, and their successful assembly into multiprotein membrane complexes, such as PS II.

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