Biochimica et Biophysica Acta 1837 (2014) 1548–1552

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Repressible chloroplast gene expression in *Chlamydomonas*: A new tool for the study of the photosynthetic apparatus ☆

Emine Dinc [a], Silvia Ramundo [b], Roberta Croce [a], Jean-David Rochaix [b,*]

[a] Department of Physics and Astronomy, Faculty of Sciences, VU University Amsterdam, 1081 HV Amsterdam, The Netherlands
[b] Departments of Molecular Biology and Plant Biology, University of Geneva, 30, Quai Ernest Ansermet, 1211 Geneva, Switzerland

ARTICLE INFO

Article history:
Received 7 October 2013
Received in revised form 26 November 2013
Accepted 29 November 2013
Available online 12 December 2013

Keywords:
Chloroplast
Gene expression
Photosystem degradation
Chlamydomonas

ABSTRACT

A repressible/inducible chloroplast gene expression system has been used to conditionally inhibit chloroplast protein synthesis in the unicellular alga *Chlamydomonas reinhardtii*. This system allows one to follow the fate of photosystem II and photosystem I and their antennae upon cessation of chloroplast translation. The main results are that the levels of the PSI core proteins decrease at a slower rate than those of PSII. Amongst the light-harvesting complexes, the decrease of CP26 proceeds at the same rate as for the PSII core proteins whereas it is significantly slower for CP29, and for the antenna complexes of PSI this rate is comprised between that of CP26 and CP29. In marked contrast, the components of trimeric LHCII, the major PSII antenna, persist for several days upon inhibition of chloroplast translation. This system offers new possibilities for investigating the biosynthesis and turnover of individual photosynthetic complexes in the thylakoid membranes. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

1. Introduction

The primary reactions of photosynthesis are catalyzed by three major protein–pigment complexes which are serially connected by the photosynthetic electron transport chain. They include photosystem II (PSII), the cytochrome b_6f complex (Cytb_6f) and photosystem I (PSI). Both PSII and PSI are associated with their own light-harvesting complexes LHCII and LHCI, respectively, and catalyze the primary photochemical reactions in which the absorbed light energy is used to induce a stable charge separation across the thylakoid membrane. The donor side of PSII creates a strong oxidant capable of extracting electrons from water with their subsequent transfer along the electron transport chain to plastoquinone, Cytb_6f and PSI. The acceptor side of PSI creates in turn a strong reductant capable of reducing ferredoxin and finally, through ferredoxin-NADP reductase (FNR), $NADP^+$ to NADPH. This electron flow is coupled to proton transfer from the stroma to the lumen side of the thylakoid membrane and the resulting proton motive force is used by the ATP synthase to generate ATP. Finally, both ATP and NADPH act as energy source and reducing power, respectively, for driving CO_2 assimilation by the Calvin–Benson cycle.

The unicellular alga *Chlamydomonas reinhardtii* has emerged as a powerful model system for studying chloroplast function, biogenesis and regulation for several reasons [1]. First, its photosynthetic function is dispensable, provided a source of reduced carbon such as acetate is present in the growth medium. It is thus easy to isolate, maintain and study mutants deficient in photosynthetic activity either in the dark (heterotrophic conditions) or in the light (mixotrophic conditions). Second, this alga is able to synthesize chlorophyll in a light-independent manner and can thus accumulate a fully functional photosynthetic apparatus when grown in the dark. This feature is particularly important as mutants deficient in photosynthesis are often light-sensitive and difficult to study in land plants. Third, transformation of this alga is possible for the nuclear, chloroplast and mitochondrial compartments. Finally, the nuclear, chloroplast and mitochondrial genomes have been sequenced and can easily be screened for the presence of specific genes [2–4].

Chloroplast genomes encode between 100 and 120 genes in green algae and land plants. These genes can be grouped in three major classes. The first includes genes coding for components of the photosynthetic machinery, the second comprises genes involved in chloroplast gene expression such as the subunits of the plastid RNA polymerase and ribosomal proteins, and the third includes genes involved in various chloroplast functions as well as some genes of unknown function. Because homologous recombination occurs in the chloroplast, it is possible to perform site-specific changes of plastid genes of *C. reinhardtii* through chloroplast transformation [5]. Usually the bacterial *aadA* gene conferring resistance to the antibiotics spectinomycin and streptomycin is used for selection of the transformants [6]. In this way either chloroplast

☆ This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: Keys to Produce Clean Energy.

* Corresponding author. Tel.: +41 22 379 6187.
E-mail address: Jean-David.Rochaix@unige.ch (J.-D. Rochaix).

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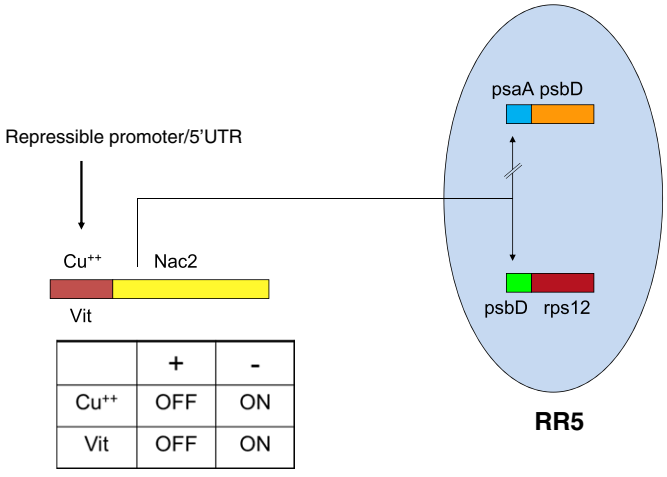
http://dx.doi.org/10.1016/j.bbabio.2013.11.020

gene disruptions or site-specific mutagenesis can be achieved. However this is only possible if the genetic changes do not compromise cell viability. Chloroplast genomes are polyploid and in the particular case of *C. reinhardtii* there are 80 copies of the chloroplast genome per chloroplast or cell as this alga contains a single chloroplast. If all copies of a particular chloroplast gene can be disrupted, i.e. if a homoplasmic state is achieved, under hetero- or mixo-trophic growth conditions, this indicates that this gene does not have an essential function. This applies in particular for genes involved in photosynthesis. If however the gene one attempts to inactivate has an essential function it is not possible to achieve homoplasmicity and, as long as the selection for antibiotic resistance is maintained, a heteroplasmic state persists with a mixed population of intact and disrupted genes.

The photosynthetic complexes have a dual genetic origin as their subunits are encoded both by the chloroplast and nuclear genomes. Their assembly thus depends on a concerted interplay between these two genetic systems. Genetic analysis of the biogenesis of the photosynthetic apparatus in the green alga *C. reinhardtii* and the land plants Arabidopsis and maize revealed a large number of nucleus-encoded factors which act as chloroplast trans-acting activators of chloroplast gene expression [7]. They are mostly involved in different post-transcriptional steps of chloroplast gene expression including RNA processing, RNA stability, splicing and translation. A remarkable feature is that several of these factors act in a gene-specific manner indicating that altogether hundreds of factors of this sort must be acting in the plastids as activators of gene expression. Another important point is that many of these factors interact specifically with the 5′untranslated region (5′UTR) of their plastid target gene. It is therefore possible to make the expression of a reporter gene dependent on one of these factors by fusing the reporter gene to the 5′UTR of the corresponding target gene (Fig. 1). We have exploited these unique features of plastid gene expression for developing a robust inducible and repressible chloroplast gene expression system in *C. reinhardtii* [8,9]. This system opens new interesting possibilities for the study of both basic and applied aspects of chloroplast biology and photosynthesis.

Because no tight repressible chloroplast promoter is available in *C reinhardtii*, the following strategy was used. We took advantage of our earlier studies on the nuclear gene Nac2 which is specifically required for the accumulation of the chloroplast *psbD* mRNA [10]. The Nac2 protein interacts with the *psbD* 5′UTR and thereby stabilizes the *psbD* mRNA. In the absence of Nac2 this mRNA is specifically degraded but all other plastid RNAs accumulate normally. To obtain repressible chloroplast gene expression, the Nac2 gene was fused to a repressible/inducible promoter, either the Cyc6 promoter of cytochrome *c6*, which is repressed by copper and expressed in its absence [9] or the MetE promoter and the Thi4 riboswitch, which are repressed in the presence of vitamins B12 and thiamine, respectively [8,11,12] (Fig. 1). Any chloroplast gene can be used as target gene, provided it is fused to the *psbD* 5′UTR, the target of Nac2. This system offers many possibilities for investigating problems related to the function and regulation of chloroplasts. First it allows one to progressively deplete essential proteins from the chloroplast and to study the effect of this depletion. This approach is particularly powerful for essential plastid genes of unknown function. Second, it allows one to remove specifically and in a reversible way any of the major photosynthetic complexes and to examine its biosynthesis in a preformed thylakoid membrane as well as the effect caused by its depletion on cellular metabolism. As an example, by depleting PSII, cells enter into an anaerobic state because of consumption of oxygen by respiration, a state which induces the production of hydrogen, at least transiently [9]. Third, this system allows one to transiently express foreign proteins of biotechnological interest which are toxic to the cells.



	+	-
Cu++	OFF	ON
Vit	OFF	ON

Fig. 1. Repressible chloroplast gene expression system. Scheme of the repressible chloroplast gene expression system. The nuclear Nac2 gene is fused either to the Cyc6 promoter which is repressed by copper or the MetE promoter and Thi4 riboswitch which are repressed by vitamin B12 and thiamine, respectively. The target of Nac2 is the *psbD* 5′UTR. In this particular case it was fused to the coding sequence of *rps12* making the expression of this gene dependent on Nac2 (strain RR5). The coding sequence of *psbD* was fused to the *psaA* 5′UTR to render *psbD* expression independent of Nac2 [8].

Here we describe and discuss this new approach and present one example in which the system can be used to follow the decline of the proteins of PSII and PSI and their associated light-harvesting complexes when chloroplast protein synthesis ceases. Although, protein turnover is normally studied through pulse and pulse-chase experiments with labeled methionine [13] or by using antibiotics which specifically inhibit translation [14], these methods are not suitable for the study of the light-harvesting complexes in *Chlamydomonas* for several reasons. First, protein labeling of *Chlamydomonas* with amino acids is only possible with labeled arginine but not with methionine although cells can be labeled with S35-sulfate or C14-acetate [1]. Second the very low turnover of the light harvesting systems makes the interpretation of pulse labeling and pulse-chase experiments difficult. Third, secondary effects of antibiotics cannot be excluded especially if they are used for long periods. Because repression of chloroplast gene expression occurs gradually in this system, it allows one to extend the time scale which is particularly useful when processes that occur sequentially in time are studied. We have taken advantage of our newly developed repressible chloroplast gene expression system for monitoring the fate of the core PSII and PSI subunits and of individual components of their light-harvesting complexes upon inhibition of chloroplast protein synthesis.

2. Material and methods

2.1. Strains and cell growth

The RR5 strain containing the Nac2 gene fused to the MetE promoter and Thi4A riboswitch [8] was grown in TAP medium [15] under continuous light (60 μmol photons m^{-2} s^{-1}). Addition of vitamins B12 and thiamine-HCl for Nac2 repression was done as described [8].

2.2. Determination of Chl content

The chlorophyll concentration was determined as described previously [16]. The values are averages ± SD of four measurements.

2.3. Fluorescence measurements

Maximum quantum efficiency of PSII (Fv/Fm) was measured with a chlorophyll fluorescence & P700 photosynthesis analyzer (Dual-PAM 100; Walz Instruments). Prior to each measurement, cells were dark-adapted for ~1 to 5 min.

2.4. 77 K-measurements

Low temperature (77 K) fluorescence emission spectra were recorded using a Fluorolog 3.22 spectrofluorometer (JobinYvon-Spex). For 77 K measurements a home built liquid nitrogen cooled device was used. The excitation wavelengths were 440 nm, 475 nm and 500 nm and emission was detected in the 640–750 nm range. Excitation and emission slit widths were set to 3 nm. All fluorescence spectra were measured at OD 0.05 at the maximum of the Qy absorption band. Samples were in 60% glycerol (w/v), 10 mM Hepes (pH 7.5).

2.5. Thylakoid preparations

Cells were harvested by centrifugation (2200 *g*, 5 min, 4 °C) at different times after addition of vitamins. Thylakoid membranes were prepared and separated on a discontinuous gradient (71,000 *g*, 1 h, 4 °C) in a SW41 swinging bucket rotor as described [17] with a few modifications.

2.6. Protein extraction and immunoblot analysis

Total protein extraction was performed as described [8]. For immunoblot analysis, total protein extracts were separated by SDS-PAGE (acrylamide [40%] 29:1; Biorad) [18] and transferred to Protran 0.45-mm nitrocellulose membrane (Schleicher and Schuell) with a wet transfer cell. Membranes were blocked in phosphate-buffered saline solution containing 5% non-fat dry milk and 0.1% Tween 20 (PBS-T). For primary antibodies, dilutions in PBS-T containing 5% nonfat milk were prepared as follows: D2 (1:5000), D1 (1:5000), PsaA (1:1000), PsaD (1:1000), PsaC (1:1000), CP47 (2:1500), CP43 (1:2500), CP26 (1:3000), CP29 (1:10,000) (Agrisera), Rps12 and Rpl37 antibodies (1:10,000 dilution). Incubation was performed for 1 h at room temperature. Subsequently, the membrane was washed three times for 10 to 15 min in PBS-T containing 1 or 5% nonfat milk. For secondary antibodies, the membrane was incubated for 1 h at room temperature using goat anti-rabbit IgG (H + L), horseradish peroxidase conjugate (Agrisera) in PBS-T containing 1 or 5% nonfat milk at a final antibody dilution of 1:5000. Membranes were incubated for 5 min in ECL Plus horseradish peroxidase substrate (GE Healthcare Bio-Sciences), and chemiluminescence was detected using Image Quant LAS 4000 imaging system. Band intensities were analyzed by GelPro31 software. In each blot cytoplasmic Rpl37 was used as loading control.

3. Results and discussion

Although Nac2 expression can be controlled through the copper-repressible Cyc6 promoter [9], its use requires acid-washed glassware and pure chemicals as trace amounts of copper are sufficient to repress Cyc6 [19]. It is therefore easier to use the vitamin-repressible MetE–Thi4 system which does not require special precautions for the preparation of the growth media [11,12]. A mutant deficient in Nac2 protein was transformed with a construct in which Nac2 gene expression is driven by the MetE promoter and Thi4 riboswitch (Fig. 1) [8]. The transformants obtained accumulate normal levels of *psbD* mRNA and PSII reaction center D2 protein and PSII when grown in vitamin-depleted medium but loose this complex when grown in vitamin-replete medium. In the presence of vitamins, Nac2 expression is abolished and consequently no D2 protein and PSII complex accumulates. In order to use this system for any chloroplast gene and to maintain photosynthetic activity in the presence of vitamins, it was first necessary to render *psbD* expression independent of Nac2 by replacing its 5′UTR with that of *psaA*, a chloroplast gene encoding the PsaA reaction center protein of PSI [8] (Fig. 1A). The target gene used in this study was the ribosomal protein gene *rps12* which is part of the ribosome decoding center and essential for chloroplast protein synthesis. The 5′UTR of this gene was replaced by that of *psbD* resulting in the strain RR5. Upon addition of vitamins to this strain, the level of rps12 mRNA and protein gradually decreased and, in turn, chloroplast translation activity was inhibited. During this time course cells became paler due to the progressive decrease in the amount of chlorophyll (Fig. 2A, Table 1). After 96 h of vitamin treatment cell growth ceased and the Fv/Fm ratio which provides a measure of the maximum PSII photosynthetic yield also declined drastically (Fig. 2B, C) with a similar kinetics as reported previously [8]. Moreover the low temperature fluorescence emission spectra were significantly altered upon vitamin treatment. In particular there is a decrease in the PSII emission peak (686 nm) in comparison to the PSI peak (>700 nm) and the PSI emission is shifted 8 nm to the blue (Fig. 3). These results together with the lower Chl a/b

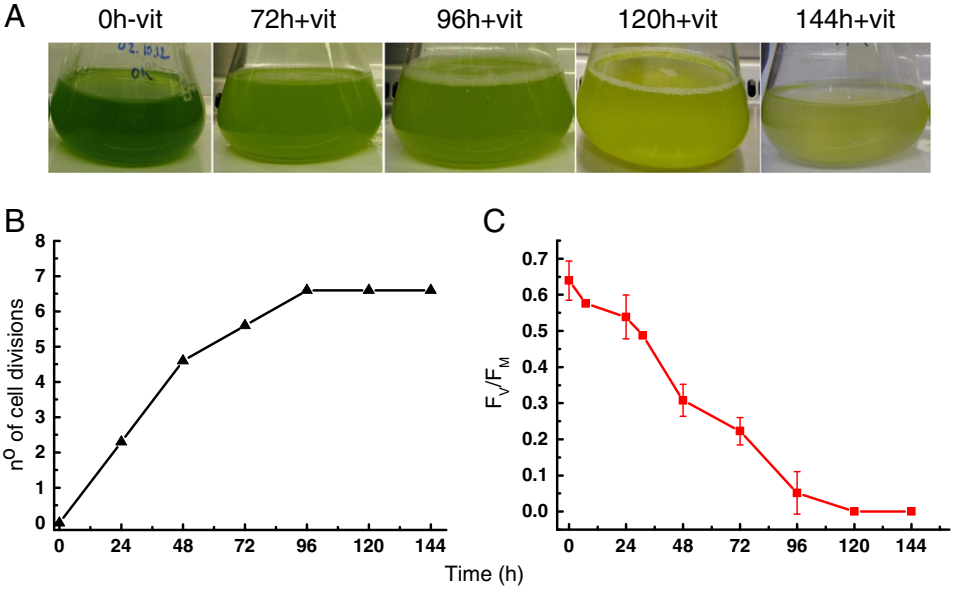


Fig. 2. Growth patterns upon repression of chloroplast translation in RR5. Panel A: Cell cultures of RR5 were grown under 60 μmol photons m^{-2} s^{-1} in the absence or presence of vitamins for the times indicated. Panel B: Cell concentration was measured at different times after addition of vitamins. Panel C: Fv/Fm was measured in cells grown in the presence of vitamins as indicated.

Table 1
Chlorophyll content at different times after addition of vitamins.

Time (h)	Chl a (μg/μl)	Chl b (μg/μl)	Chl a + b (μg/μl)	Chl a/b
0	11.04 ± 0.45	4.68 ± 0.11	15.72 ± 0.37	2.36 ± 0.14
24	5.03 ± 0.15	1.92 ± 0.16	6.96 ± 0.23	2.62 ± 0.2
48	3.75 ± 0.05	1.80 ± 0.06	5.55 ± 0.11	2.08 ± 0.05
72	1.61 ± 0.01	0.85 ± 0.06	2.45 ± 0.014	1.90 ± 0.04
96	1.13 ± 0.02	0.61 ± 0.01	1.74 ± 0.03	1.84 ± 0.004
120	0.67 ± 0.01	0.34 ± 0.007	1.01 ± 0.02	1.95 ± 0.003
144	0.62 ± 0.02	0.32 ± 0.008	0.94 ± 0.03	1.89 ± 0.009

ratio observed after 48 h of vitamin treatment (Table 1) suggest that at this stage the membrane contains mainly light-harvesting complexes from PSII and PSI.

To test the effect of the inhibition of chloroplast translation on the organization of the thylakoid membranes, these membranes were purified at different stages of the vitamin treatment. At time zero a very intense green band containing the thylakoid membrane was visible in the discontinuous sucrose density gradient. However, already after 48 h of vitamin treatment the intensity of this band was strongly reduced and after 72 h the band had almost disappeared. At longer times only the green upper band containing smaller membrane fragments was present (Fig. 4). Next, we analyzed the protein composition of the thylakoid membranes. Immunoblot analysis of total cell extracts with a battery of antibodies directed against the major subunits of PSII and PSI and some of their light-harvesting complex proteins revealed drastic changes in protein levels (Fig. 5). As expected the chloroplast-encoded PSII proteins were the first to decrease with apparent half-lifes comprised between 16 and 25 h. These half-lifes are apparent because inhibition of translation occurs only gradually with the decline of Rps12 which in turn depends on the decrease of Nac2. Nevertheless they provide a measure of the relative stabilities of the photosystem subunits. We showed previously that the mRNAs of the photosystem core proteins remain stable during vitamin treatment [8]. Amongst these components D1 had the shortest half-life, in agreement with its very fast turnover [20]. It was the first to disappear, followed by CP43, D2 and CP47 with half-lifes of 19, 22 and −25 h, respectively. These results are in agreement with a study in the cyanobacterium *Synechocystis* sp. *PCC 6803* [21,22] which showed that the CP47/D2 complex protects D2 from degradation at least transiently and it is also consistent with the faster degradation of CP43 observed in our study.

The PSI subunits were more stable. Surprisingly, PsaA decreased at a slower rate than PsaC and PsaD (Fig. 5, Table 2, Supplementary Fig. 1).

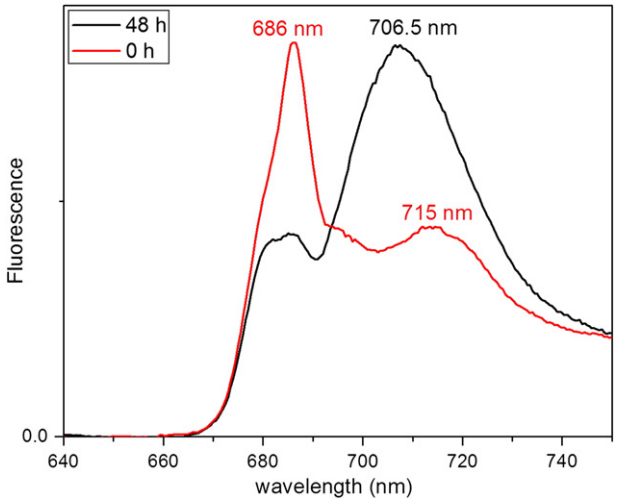


Fig. 3. Low temperature (77 K) fluorescence emission spectra of RR5 cells in the absence of vitamins (red) and after 48 h of vitamin treatment (black) upon excitation at 440 nm. The spectra are normalized to the maximum.

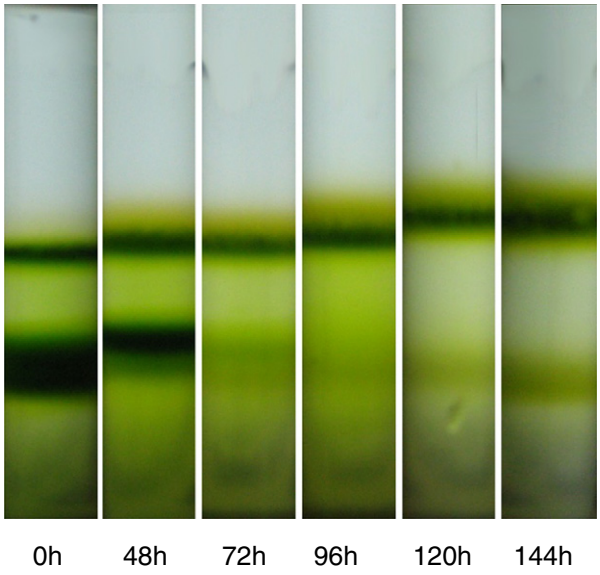


Fig. 4. Purification of thylakoid membranes by sucrose density gradient centrifugation at different times after addition of vitamins.

One possible reason is that PsaD mRNA decreases whereas *psaA* mRNA remains stable upon repression of chloroplast translation [8]. The decrease of the PSII core proteins relative to each other is very similar to previous observations in cyanobacteria and higher plants [21–23]. However this is not the case for the PSI subunits. Whereas the difference in half-lifes between PSI and PSII core subunits is 30–70 h in cyanobacteria [21,22], here we observed smaller differences in the range of 0–15 h.

A large variation in apparent half-life was observed for the nucleus-encoded proteins of LHCII and LHCI. The decrease of these proteins could be mediated through different mechanisms. It is well established that inhibition of plastid protein synthesis in land plants induces retrograde signaling with an inhibitory effect on the expression of nuclear

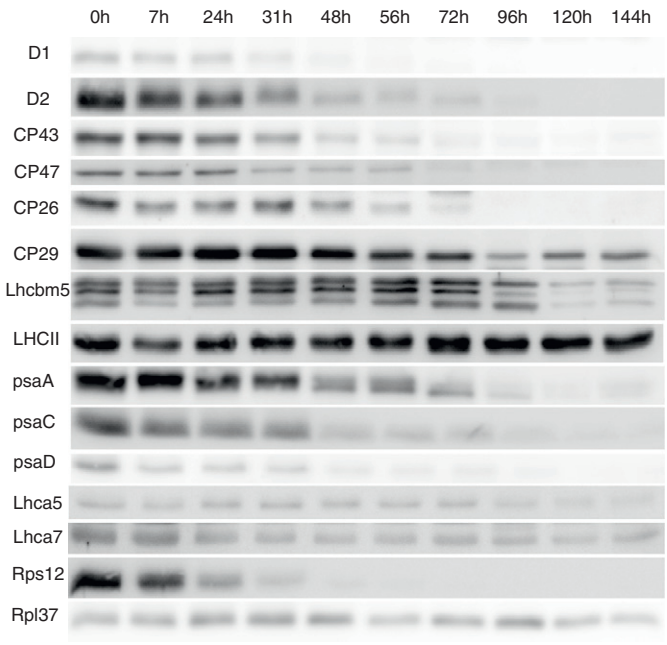


Fig. 5. Immunoblot analysis of PSI, PSII core and antenna proteins in RR5. Protein levels were determined using antibodies against the indicated proteins at different time points after addition of the vitamins. Rpl37 was used as loading control in each blot; 10 μg of total protein was loaded in each well.

Table 2
Apparent half-lifes of PSII and PSI proteins.

Protein	Complex	Genetic origin	Apparent half-time (hours)
D1	PSII	Chloroplast	16.6 ± 1.7
D2	PSII	Chloroplast	21.6 ± 2.1
CP43	PSII	Chloroplast	18.6 ± 0.9
CP47	PSII	Chloroplast	25 ± 4.4
CP26	PSII	Nucleus	23.2 ± 2.7
CP29	PSII	Nucleus	48.4 ± 5.5
Lhcbm5	PSII	Nucleus	85.6 ± 22.8
LHCII	PSII	Nucleus	>144
PsaA	PSI	Chloroplast	30.4 ± 2.6
PsaC	PSI	Chloroplast	23.9 ± 3
PsaD	PSI	Chloroplast	17.3 ± 1
Lhca5	PSI	Nucleus	33.3 ± 5.6
Lhca7	PSI	Nucleus	33.7 ± 10.4
Rps12	Reference		6.8 ± 0.3

genes involved in photosynthesis [24–26]. A similar retrograde signaling pathway also operates in *C. reinhardtii* and affects a restricted set of proteins including those of LHCII [8]. However it is also possible that the decrease of the PSII core affects the stability of some LHCII proteins by making them more susceptible to proteolysis. In our study CP26 (Lhcb5) disappeared first with a half-life similar to that of D2 and CP47. The half-life of CP29 was twice that of CP26. LhcbM5 had a half-life of ca. 85 h indicating that it persists in the membrane long after the arrest of cell growth. Finally, although inhibition of chloroplast translation can negatively impact the expression of the LhcbM1 mRNA [8], the level of LhcbM1 did not decrease during the time of the experiment, indicating that this subunit, which is one of the main components of LHCII in *C. reinhardtii* [27] has an extremely long lifetime. To check the presence of Lhca proteins we used antibodies against Lhca5 and Lhca7 that were previously shown to be part of the outer (Lhca 5) and inner (Lhca7) belt of PSI [28,29]. Their half-lifes are around 33 h (Fig. 5, Table 2, Supplementary Fig. 1). Thus, as indicated by the fluorescence spectra, after 48 h of vitamin treatment the membrane contains mainly Lhcb and Lhca complexes whereas the levels of the PSII and PSI core complexes are strongly decreased.

4. Conclusions

Here we have shown that reversible inhibition of chloroplast translation in the RR5 strain through addition of vitamins to the growth medium can be used to study the relative stability of photosynthetic complexes in the membrane. We observed a slower decrease of the subunits of the PSI core as compared to those of PSII. In general the relative stability of the chloroplast-encoded subunits in the thylakoid membrane is in agreement with previous studies in spinach and in the cyanobacterium *Synechocystis* sp. *PCC 6803* [21,22]. In addition, we also followed the fate of the light-harvesting complexes upon inhibition of chloroplast translation. Pulse-chase experiments indicate that the de novo synthesis of these proteins is rather low [13]. Thus it should have a negligible effect on the estimation of the stability of the antenna complexes of our study. Interestingly, there are large variations in the half-lifes of the individual complexes. Amongst the antenna proteins of PSII, CP26 decreased as fast as the PSII core components upon repression of chloroplast protein synthesis whereas CP29 decline was significantly slower and LHCII persisted even after 144 h of vitamin treatment. This system also offers interesting possibilities to examine the assembly of photosynthetic complexes by de-repressing chloroplast translation in cells which have been depleted of their photosynthetic complexes by prior treatment with vitamins.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.11.020.

Acknowledgements

Part of this work was supported by grant 31003A_133089/1 from the Swiss National Foundation to J.D.R. The work in Amsterdam was supported by the ERC consolidator grant 281341 (ASAP) to R.C.

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