Video Article

A New Approach for the Comparative Analysis of Multiprotein Complexes Based on ^{15}N Metabolic Labeling and Quantitative Mass Spectrometry

Kerstin Trompelt[1], Janina Steinbeck[1], Mia Terashima[1,2], Michael Hippler[1]

[1]Institute of Plant Biology and Biotechnology, University of Münster

[2]Department of Plant Biology, Carnegie Institution for Science

Correspondence to: Michael Hippler at mhippler@uni-muenster.de

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Abstract

The introduced protocol provides a tool for the analysis of multiprotein complexes in the thylakoid membrane, by revealing insights into complex composition under different conditions. In this protocol the approach is demonstrated by comparing the composition of the protein complex responsible for cyclic electron flow (CEF) in *Chlamydomonas reinhardtii*, isolated from genetically different strains. The procedure comprises the isolation of thylakoid membranes, followed by their separation into multiprotein complexes by sucrose density gradient centrifugation, SDS-PAGE, immunodetection and comparative, quantitative mass spectrometry (MS) based on differential metabolic labeling ($^{14}N/^{15}N$) of the analyzed strains. Detergent solubilized thylakoid membranes are loaded on sucrose density gradients at equal chlorophyll concentration. After ultracentrifugation, the gradients are separated into fractions, which are analyzed by mass-spectrometry based on equal volume. This approach allows the investigation of the composition within the gradient fractions and moreover to analyze the migration behavior of different proteins, especially focusing on ANR1, CAS, and PGRL1. Furthermore, this method is demonstrated by confirming the results with immunoblotting and additionally by supporting the findings from previous studies (the identification and PSI-dependent migration of proteins that were previously described to be part of the CEF-supercomplex such as PGRL1, FNR, and cyt *f*). Notably, this approach is applicable to address a broad range of questions for which this protocol can be adopted and *e.g.* used for comparative analyses of multiprotein complex composition isolated from distinct environmental conditions.

Video Link

The video component of this article can be found at http://www.jove.com/video/51103/

Introduction

Photosynthetic processes in thylakoid membranes of plants and algae can function in a linear and cyclic mode. During linear electron flow (LEF) photosystem I (PSI), photosystem II (PSII) and cytochrome b_6/f ultimately transfer electrons from water to NADP+[1], leading to the generation of NADPH and ATP[2]. In contrast, cyclic electron flow (CEF), which is known to be induced under diverse environmental conditions like state 2 [3] and anaerobic conditions[4], results in the re-reduction of oxidized PSI by injecting electrons back into the electron transport chain. This process can take place either at the stromal side of the cytochrome b_6/f complex[1] or at the plastoquinone pool[5] and generates ATP, but no NADPH[2].

The aim of the presented protocol is to demonstrate a mass spectrometry (MS) based method for the comparative, quantitative analysis of multiprotein complexes in thylakoid membranes of *Chlamydomonas reinhardtii* to gain insight into the composition of these complexes under different conditions (exemplified by comparing genetically different strains). This approach was applied in a publication by Terashima *et al.* in 2012 showing a Ca^{2+}-dependent regulation of CEF in *C. reinhardtii* mediated by a multiprotein complex including the proteins CAS, ANR1, and PGRL1[6]. The procedure will be explained by comparatively analyzing the composition of the CEF-supercomplex in two genetically different strains, thereby taking advantage of labeling one of the two strains with heavy nitrogen (^{15}N). Briefly, the protocol includes the preparation of thylakoid membranes, followed by detergent solubilization and fractionation of photosynthetic complexes in a sucrose density gradient. After fractionation of the gradient, selected fractions of two strains are mixed based on equal volume, separated by SDS-PAGE followed by in-gel digestion and subsequent quantitative MS analysis.

As mentioned above, CEF is induced under different environmental conditions and a publication from 2010 demonstrates the isolation of a functional CEF-supercomplex from state 2 locked cells of *C. reinhardtii*[7], which was performed by separating solubilized thylakoid membranes on a sucrose density gradient during ultracentrifugation. Different from Iwai *et al.*[7], the presented protocol describes the isolation of the CEF-supercomplex from anaerobic grown *C. reinhardtii* cultures by following an alternative procedure. This comprises changes in the thylakoid isolation protocol as well as differences concerning the solubilization step and the separation of protein complexes by ultracentrifugation. In the present protocol, thylakoid membranes are isolated by applying the procedure published by Chua and Bennoun[8], while the buffers used for

thylakoid preparation by Iwai *et al.* contained 25 mM Mes, 0.33 M sucrose, 5 mM $MgCl_2$, 1.5 mM NaCl (pH 6.5) as described[9]. The solubilization was performed with 0.7-0.8% detergent (n-tridecyl-β-D-maltoside) for 30 min on ice in the case of Iwai and coworkers, while the solubilization method described here relies on the use of 0.9% detergent (n-Dodecyl-β-D-maltoside (β-DM)) and is performed for only 20 min on ice. Both groups used 0.8 mg of chlorophyll per ml for the solubilization with the respective detergent. For the separation of photosynthetic complexes from solubilized thylakoid membranes Iwai *et al.* applied sucrose concentrations between 0.1-1.3 M, whereas the authors of this protocol used concentrations ranging from 0.4-1.3 M. The last difference is the centrifugation speed, which is lower compared to the earlier publication.

Solubilization of thylakoid membranes with nonionic detergents followed by sucrose density gradient fractionation has already been applied in numerous studies ranging from the 1980s until today[7, 9-14] and also the application of metabolic labeling of proteins is a widespread method in the proteomics field. The described approach applies the ^{15}N metabolic labeling for one of the two compared strains by culturing it in the presence of heavy nitrogen as sole nitrogen source in the form of ^{15}N NH_4Cl, which is incorporated into all amino acids leading to a mass shift depending on the amino acid sequence of the peptide. When analyzing a mixture of ^{14}N and ^{15}N within one MS run, this mass shift can be used to determine the sample origin for each peptide and relative peptide abundances can be calculated representing relative abundances for the corresponding protein[15].

Numerous quantitative proteomics studies on *C. reinhardtii* are available, which compare a defined amount of protein to analyze changes in the proteome between experimental conditions (*e.g.* changes in the proteome due to nutrient[16-19] or light stress[20,21]). Compared to those studies, in the currently presented approach equal volumes of samples are combined and analyzed. This setup allows to study the migration behavior of proteins within the gradient and moreover to analyze the composition of different complexes with respect to the investigated strains.

This method will be explained by mainly concentrating on three proteins: The first candidate is the chloroplast-localized calcium sensor protein CAS, which was shown to be involved in photo-acclimation in *C. reinhardtii*[22]. Calcium is considered to be an important signaling ion for pathways that are activated due to different biotic and abiotic stresses finally leading to changes in gene expression and cell physiology[23] and it was proposed that chloroplasts might contribute to cellular Ca^{2+} signaling via the CAS protein[22,24,25]. The second protein is ANR1 (anaerobic response 1 [6]), a protein that was shown to be induced under anoxic growing conditions in *C. reinhardtii*[26]. Notably, CAS as well as ANR1 were identified as subunits of the CEF-supercomplex and moreover, by using reverse genetic approaches, it was demonstrated that both proteins contribute functionally to CEF *in vivo*[6], supporting their role as functional subunits of this protein complex. The third protein is the thylakoid protein PGR5-Like 1 (PGRL1), which was shown to be involved in CEF in *Chlamydomonas*[4,27] as well as in *Arabidopsis*[5,28] and was also identified in the work of Iwai *et al.*[7]

This approach will be presented by showing the results of two different experiments: wildtype (WT) versus (vs.) a ΔPSI[29] strain, exhibiting a deletion of the *psab* gene, coding for an essential photosystem I subunit, which is also part of the CEF-supercomplex and WT vs. a *pgrl1 knock-out* strain[4]. For each of those experiments the quantitative composition of the CEF-supercomplex between a ^{15}N- and a ^{14}N-labeled strain has been compared.

Protocol

1. Culturing of *Chlamydomonas*

1. The following *C. reinhardtii* strains were used in the present study: WT cc124, WT *cw15-arg7* (cell-wall deficient and arginine auxotroph), a ΔPSI mutant strain[29] and a *pgrl1 knock-out* strain[4].
2. All strains were grown in Tris-Acetate-Phosphate (TAP)-medium[30], at 25 °C with a continuous light intensity of 20-50 μE/m^2 sec and shaking at 120 rpm. The culture of ΔPSI should be wrapped with some tissue paper for light exposure of <5 μE/m^2 sec.
3. The cultures of the labeled strains (ΔPSI and WT cc124, respectively) contained 7.5 mM ^{15}N NH_4Cl, whereas the cultures for the nonlabeled strains (WT *cw15-arg7* and *pgrl1*, respectively) contained 7.5 mM ^{14}N NH_4Cl. Cells have to grow for at least four generations to achieve full labeling and must be kept at exponential growth phase.
4. On the day before starting the experiment count and dilute cells to a density of 1 x 10^6 cells/ml in a volume of at least 750 ml/strain.

Please note that two types of discontinuous sucrose density gradients are described in the following protocol. The **photosystem sucrose density gradients** according to Takahashi *et al.*[9] are used to separate the different photosynthetic protein complexes from isolated and solubilized thylakoids during over-night centrifugation and have to be prepared the day before (see Protocol 2) and the **thylakoid sucrose density gradients**[8] are applied in the thylakoid isolation procedure (see Protocol 3).

2. Preparation of Photosystem Sucrose Density Gradients

1. For pouring the gradients three stock solutions are needed:
    1. 2 M Sucrose
    2. 10% β-DM in H_2O
    3. 0.5 M Tricine, pH 8.0 (NaOH)
2. Using these stocks, prepare the following solutions:

Concentration:	1.3 M	1.0 M	0.85 M	0.7 M	0.65 M	0.4 M
2 M Sucrose	13 ml	10 ml	8.5 ml	7 ml	6.5 ml	4 ml
10% β-DM	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
0.5 M Tricine	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl
H_2O	6.7 ml	9.7 ml	11.2 ml	12.7 ml	13.2 ml	15.7 ml

3. Use 14 mm x 89 mm centrifuge tubes and pour the gradients very slowly, starting with the highest sucrose density solution to lower density solution.
4. Pour only 1 ml each for the 1.3 and 1.0 M solutions and 2 ml for the rest of the solutions.
5. Leave gradients overnight in the cold room.

3. Anaerobic Induction and Isolation of Thylakoid Membranes[8]

1. Before starting with the isolation of thylakoid membranes, induce anaerobic conditions by bubbling with argon for 4 hr. The bubbling can be performed through a glass pipette in culturing flasks with constant mixing of the culture with a magnetic stir bar.
2. Start with the isolation of thylakoid membranes by pelleting the cells for 5 min at 2,500 x g, resuspend in H1 buffer (0.3 M Sucrose, 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$).

   (Please note that when starting with the isolation of thylakoid membranes samples should be kept on ice to avoid protein degradation, all centrifugation steps are carried out at 4 °C and working with gloves is strongly recommended to avoid keratin contamination.)
3. Pellet cells for 5 min at 2,500 x g, resuspend in H1 buffer.
4. Break cells with two passages through a nebulizer with a nitrogen pressure of 1,500 hPa (for strains without cell wall do only one passage).
5. Spin down cells for 7 min at 2,500 x g.

   (After this step the supernatant should be light green, if not, the cell breakage was not successful.)
6. Resuspend cells in H2 buffer (0.3 M Sucrose, 5 mM HEPES (pH 7.5), 10 mM EDTA (pH 8.0)) and pellet cells for 10 min at 32,800 x g.
7. Resuspend cells in H3 buffer (1.8 M Sucrose, 5 mM HEPES (pH 7.5), 10 mM EDTA (pH 8.0)) and homogenize pellet with a potter (no green particles should remain at the end of this working step).
8. Prepare **thylakoid sucrose density gradients** in tubes (25 mm x 89 mm) by starting at the bottom with a layer of 12 ml H3 buffer with cells, pour a middle layer of 12 ml H4 buffer (1.3 M Sucrose, 5 mM HEPES (pH 7.5), 10 mM EDTA (pH 8.0)) and on top 12 ml H5 buffer (0.5 M Sucrose, 5 mM HEPES (pH 7.5), 10 mM EDTA (pH 8.0)). Be careful when pouring the gradients and avoid mixing of the three layers.
9. Balance with H5 buffer and centrifuge thylakoid sucrose density gradients for 1 hr at 70,700 x g.
10. Remove thylakoid bands from the thylakoid sucrose density gradients and dilute them with an appropriate volume H6 buffer (5 mM HEPES (pH 7.5) 10 mM EDTA (pH 8.0)).
11. Spin down cells at 37,900 x g for 20 min. If the pellet is not tight, more H6 buffer is required for this centrifugation step.
12. Resuspend thylakoids in a small volume H6 buffer and proceed with determination of the chlorophyll concentration.

4. Determination of Chlorophyll Concentration[31]

1. The determination of the chlorophyll amount is performed with 80% acetone.
2. Mix 995 µl 80% acetone and 5 µl of thylakoids in H6 buffer (dilution 1:200).
3. Vortex for several seconds until no green particles remain and then spin down at 14.1 x g for 5 min.
4. Take the supernatant and measure the extinction at 663.6 and 646.6 nm and 750 nm, respectively.
5. Calculate the chlorophyll amount using the following formulas:
   1. $C_{Chl\ a}$ [mg/ml] = (0.01225 * $E_{663.6}$ – 0.00255 * $E_{646,6}$) * dilution factor
   2. $C_{Chl\ b}$ [mg/ml] = (0.02031 * $E_{646.6}$ – 0.00491 * $E_{663.6}$) * dilution factor
   3. C_{Chl} [mg/ml] = $C_{Chl\ a}$ + $C_{Chl\ b}$

5. Loading of Photosystem Sucrose Density Gradients

1. Calculate the amounts of thylakoids, β-DM and H6 buffer that are needed for each gradient:
   1. Each gradient should be loaded with a total volume of 700 µl with 0.8 mg/ml chlorophyll in 0.9% β-DM (dissolve β-DM in H_2O), fill the remaining volume with H6 buffer.
2. For solubilization prepare different aliquots with thylakoids, β-DM and H6 buffer for each gradient.
3. Leave samples for 20 min on ice with regularly mixing (inverting) every few minutes (solubilization step).
4. Centrifuge at maximum speed (14,000 x g) for 10 min at 4 °C.

   (After centrifugation, the pellet should be small and whitish while the supernatant is dark-green.)
5. Load supernatant on the gradients.
6. Balance with H6 buffer and spin using ultracentrifuge at 134,470 x g overnight (14 hr) at 4 °C.

(Please note that the successful separation of photosynthetic complexes using photosystem sucrose density gradients as presented in this protocol is only achieved when using freshly isolated thylakoids, since a prediction for solubilization and complex separation is difficult to make when working with thylakoid membranes that have been frozen before.)

6. Fractionation of Photosystem Sucrose Density Gradients

1. Take pictures of the gradients.
2. Puncture a hole at the bottom of the tube using a needle and fractionate gradients in 500 µl tubes. Alternatively, the fractionation can also be performed by using a 96-well plate (microplate).
3. When using a microplate, determine absorbance of the different gradient fractions at 675 nm.

(At this step samples can be stored at -80 °C for a few weeks.)

7. SDS-PAGE and Immunodetection

(Please note that only for the comparison of WT vs. ΔPSI a western blot analysis has been performed to select the fractions for subsequent MS-analysis. For the experiment WT vs. *pgrl1* fractions were chosen by means of the absorbance in the different fractions.)

1. Separate 30 µl of each fraction from WT and ΔPSI gradient on a 13% SDS polyacrylamide gel[32].
2. Perform western blot analysis[33] using the following antibodies: ANR1 (TEF7)[26], PGRL1[34], CAS[6], the PSI subunit PSAD[32] and the PSII core subunit D1. Use all antibodies with a dilution of 1:1,000 (for enhanced chemiluminescence detection technique), except for the D1 antibody, which should be used with a dilution of 1:10,000.
3. Based on the results of the immunodetection, take the peak fractions of ANR1, PGRL1 and PSAD for WT and ΔPSI (here fractions 6 and 13, respectively), mix 30 µl of fraction 6 from WT and ΔPSI and do the same with fraction 13 from both preparations and separate them again on a 13% SDS polyacrylamide gel.
4. For the quantitative comparison of WT vs. *pgrl1* take the CEF-supercomplex fractions as determined by measuring the absorbance in the different gradient fractions and mix 30 µl of both samples followed by separation on a 13% SDS polyacrylamide gel.
5. Stain the protein bands with Coomassie solution (85% phosphorous acid, 757 mM ammonium sulfate, 1.2% Coomassie brilliant blue, 20% methanol) for 2 hr at room temperature or over-night at 4 °C.
6. To remove unspecific staining, wash several times with ddH_2O.
7. Cut the lanes into 1 mm gel pieces and proceed with the in-gel digestion.

(Alternatively, samples can be dried a few minutes in a vacuum centrifuge and stored for a few weeks before continuing with the in-gel digestion.)

8. In-gel Digestion (Modified from Shevchenko *et al.*[35])

Please note to prepare all buffers and solutions shortly before use and take glass bottles for buffers containing acetonitrile (ACN).

CAUTION! Working with ACN might be harmful; more information can be downloaded from: http://www.sciencelab.com/msds.php?msdsId=9927335 (2013).

1. Wash gel pieces with >10 volumes of ddH_2O (~200 µl) for 30 sec.
2. Incubate the gel pieces with 300 µl of 25 mM NH_4HCO_3 for 15 min with shaking, remove liquid.
3. Incubate the gel pieces with 300 µl of 25 mM NH_4HCO_3 in 50% ACN (prepare by mixing ACN and 50 mM NH_4HCO_3 at a ratio of 1:1) for 15 min with shaking, remove liquid.
4. If the gel pieces are still blue, repeat the NH_4HCO_3 and NH_4HCO_3 / ACN washes until most of the Coomassie is removed.

(Although the bulk of Coomassie staining should be removed, it is not necessary to destain the gel pieces completely.)
5. Add 100 µl of ACN to dehydrate the gel pieces for 5 min. The gel pieces should shrink and look completely white.
6. Remove as much ACN as possible and proceed with trypsin digestion. Alternatively, samples can be stored at -20 °C for a few weeks.
7. Add 10-20 µl per band of 20 ng/µl trypsin in 10% ACN / 25 mM NH_4HCO_3 (freshly diluted), keep samples on ice.
8. After 30 min, check if all solution was absorbed and add more trypsin buffer, if necessary. Gel pieces should be completely covered with trypsin buffer. Keep samples on ice.
9. Leave gel pieces for another 90 min on ice to saturate them with trypsin.

(Critical step: the yield of tryptic peptides increases considerably with increasing incubation times on ice, probably because of slow diffusion of the enzyme into the polyacrylamide matrix. It is necessary to have a small excess of solution covering the gel pieces to avoid that the pieces fall dry during the overnight incubation.)
10. Digest at 37 °C for 4-6 hr. For experiments requiring maximal peptide recovery, digest overnight.
11. After digestion spin down condensed buffer.
12. Sonicate tubes for 5 min to elute peptides and centrifuge again.
13. Collect supernatant and transfer it to a new tube.
14. Perform peptide extraction with 80 µl of 30% ACN / 1% formic acid (FA) in a sonic bath for 15 min.
15. Perform extraction with 80 µl of 30% ACN / 1% FA in a sonic bath for 15 min.
16. Perform extraction with 80 µl of 70% ACN / 1% FA in a sonic bath for 15 min.
17. Centrifuge again and pool supernatant with the prior eluate.

(FA serves to inactivate trypsin and increase peptide solubility.)
18. Dry peptide solution completely in a vacuum centrifuge.

(At this point samples can be stored for a few weeks in -20 °C before proceeding with MS-analysis.)
19. Resuspend peptides in 6 µl MS buffer (5% ACN, 0.1 v/v FA, water) and sonicate for 2-5 min.
20. Centrifuge samples at maximum speed for 5 min to remove particulate material.
21. Use 4 µl of supernatant for mass spectrometric analysis.

9. MS-Data Analysis with "Proteomatic"

The data analysis was performed with the open-source software "Proteomatic" (which can be downloaded at http://www.proteomatic.org/), a platform that allows the generation and completion of MS/MS data evaluation pipelines, by using free and commercial software[36]. Briefly, the settings are described below and more detailed information can be found in[6,26].

1. Identification and Quantification of MS-data

Identification and quantification of proteins from the experiments WT vs. ΔPSI were done with OMSSA (version 2.1.4[37]) and qTrace[26], respectively, as described[6,26]. For the MS-data analysis from the experiment WT vs. *pgrl1* the following updated pipeline was used:

1. In addition to OMSSA[37], proteins were also identified with X! Tandem (version 2013.02.01[38]). Both algorithms were applied by searching against a database generated by combining the JGI *Chlamydomonas* gene model database version 4.3 with the AUGUSTUS database version 10.2 as well as against a joined database of the NCBI databases BK000554.2 and NC_001638.1.
2. MS^2 identification of peptides was performed by using a target-decoy approach[39]. A decoy for each protein was created by randomly shuffling tryptic peptides while retaining the redundancy of nonproteotypic peptides.
3. Statistical validation of the peptides was performed with the software qvality (version 0.3.3[40]) using a posterior error probability (PEP) threshold of less than 0.01 and results were filtered with a precursor mass tolerance of 5 ppm.
4. Peptides from OMSSA and X! Tandem runs were joined and quantified with qTrace[26] applying the following filter steps: require MS^2 identification, add protein names / group information and require both sister peptides.

To investigate whether protein ratios were significantly different towards each other in the mixed CEF-supercomplex fractions from WT and *pgrl1*, statistical analysis was performed applying the software SPSS (version 21). The subunits of the cytochrome b_6/f complex were tested against each other as well as the proteins FNR, FTSH2 and HCF136 against the PSI subunits. The peptide ratios of each scan count per protein of all four replicates were tested for normal distribution with the Shapiro-Wilks test. Since the peptide populations were not normally distributed ($p<0.001$), nonparametric statistics were performed. First, the Kruskal-Wallis test was applied assessing a significant divergence among groups. Only if this test predicted significant differences between groups, further analysis was performed to predict significant differences between two independent groups with the Mann-Whitney-U Test.

Representative Results

The introduced quantitative proteomics approach aims to characterize the composition of multiprotein complexes in thylakoid membranes demonstrated by the comparative analysis of CEF-supercomplex components in genetically different *C. reinhardtii* strains. The described method has successfully been applied by Terashima *et al.*[6] and comprises the isolation of thylakoid membranes from anaerobic grown cultures, followed by detergent solubilization. Subsequently, samples are loaded onto a sucrose density gradient based on equal chlorophyll amount and complexes are fractionated by ultracentrifugation. For the quantitative MS-analysis equal volumes of two gradient fractions from different strains are mixed and comparatively analyzed (**Figure 1**). The presented results include the comparison of the CEF-supercomplex fraction between WT *cw15-arg7* and ΔPSI as well as WT cc124 and *pgrl1*, respectively.

For the comparison of CEF-supercomplex components between WT and the ΔPSI strain fraction 6, harboring the CEF-supercomplex in the WT and fraction 13, the peak fraction of ANR1 in the ΔPSI strain, as revealed by immunoblot analysis (**Figures 2A** and **2B**) were chosen[6]. **Figure 3** depicts the relative protein ratios as WT / ΔPSI ($^{14}N/^{15}N$) for several PSI core and LHCI proteins (light harvesting proteins of PSI also named as Lhca proteins) as well as for proteins assigned with the CEF-supercomplex. As expected, PSI core proteins are only found in WT samples demonstrated by infinity ratios in both fractions. Lhca2 and Lhca9 are enriched in the WT, while Lhca4 -6 and -8 show a different regulation in both fractions. It is important to note that LHCI proteins are synthesized and accumulated normally in the ΔPSI mutant[41]. The polypeptide comparison of the PSI-LHCI complex from *C. reinhardtii* WT with the LHCI complex of the ΔPSI mutant revealed that the majority of Lhca2, -3 and -9 seems to be only weakly bound to LHCI and moreover that the presence of the PSI core is necessary for a stable binding. In contrast, Lhca1, -4, -6, -7, and -8 form a complex independent of the assembly of PSI[14], which could explain the different regulation of Lhca4, -6 and -8 in the present experiment.

For proteins to be assigned with the CEF-supercomplex, it can be distinguished between those that are more abundant in WT fraction 6 and fraction 13 of the ΔPSI mutant, showing a strong PSI-dependent migration displayed by ratios higher than one for fraction 6 and ratios lower than one or not detected in fraction 13 (Lhcbm5, PGRL1, ANR1, CAS, PFD, HCF136 and cyt *f*) and those showing not such a strong, but nevertheless a PSI-dependent localization (FNR, FTSH1, FTSH2, and ATPC).

With this experiment Terashima *et al.*[6] demonstrated a PSI-dependent migration behavior for ANR1 and CAS (**Figures 2A-D** and **Figure 3**), thereby revealing them as novel proteins of the CEF-supercomplex , previously not identified[7]. The findings of the MS-analysis were also confirmed by immunodetection and supported by the application of reverse genetics to confirm a functional role of these proteins for CEF *in vivo*[6]. Moreover, this comparative MS-approach strongly supports the results from previous studies, by confirming proteins that have already

been demonstrated to be part of the CEF-supercomplex such as PGRL1, cyt *f* and FNR[7], as well as Lhcbm5[7,9] to show a PSI-dependent localization in the sucrose density gradient, which is comparable to ANR1 and CAS.

The comparison of fraction 6 and 13 of *anr1* and ΔPSI using the same approach (see Figure S8[6]) demonstrated a similar composition of the CEF-supercomplex in *anr1* and the same trend for the proteins ANR1, CAS and PGRL1 as revealed in the experiment WT vs. ΔPSI. Notably, although the protein ratio of ANR1 in fraction 6 (*anr1* vs. ΔPSI) is comparable to the ratio of the WT vs. ΔPSI experiment, the amounts of CAS and PGRL1 seem to be higher in the former experiment and might be a result of compensating for the down-regulation of ANR1[6].

Besides, the enrichment of ANR1 and CAS in the CEF-supercomplex fraction one could also deduce the PSI-dependent migration of several other proteins from this experiment. These are two ATP-dependent metalloproteases FTSH1 and FTSH2 that play a fundamental role in the degradation of the PSII reaction center protein D1 in chloroplasts[42,43], a prefoldin-domain containing protein, the HCF136, which is a crucial factor for the stability or assembly of PSII[44] and the γ subunit of the ATPase. Whereas for the later one, the ratios between fraction 6 and 13 do not show such a clear difference, further experiments have to be carried out to verify whether the other proteins might also be candidates of the CEF-supercomplex.

As a proof of concept experiment for this comparative approach, we analyzed the CEF-supercomplex composition in two strains, which both have PSI, allowing the formation of this complex. The respective fractions were selected based on the absorbance measurements of the fractionated gradients. **Figures 4A** and **4B** show the results for the quantitative comparison between WT and a *pgrl1 knock-out* strain. It should be pointed out that ANR1 and CAS are not remarkably changed between WT and *pgrl1*. Interestingly, HCF136 seems to be enriched in the WT, while FTSH2 seems to be enriched in *pgrl1*. A statistically significant difference for both aforementioned proteins to all PSI-subunits (including the LHCI proteins) could be confirmed.

Moreover, the cytochrome b_6/f complex subunits cyt *f* and PETO were shown to be significantly different to cyt b_6, cyt b_6/f IV and PETC, respectively. Notably, cyt *f* seems to be up-regulated in *pgrl1*, while cyt b_6 and cyt b_6/f IV are slightly down-regulated, although the regulation of cyt *f* synthesis probably involves cyt b_6 and cyt b_6/f IV[45]. The nuclear-encoded PETC, which is also known as Rieske protein shows a similar regulation as cyt b_6 and cyt b_6/f IV (*i.e.* a down-regulation in *pgrl1*). This is striking as a reduction or absence of the Rieske protein still lead to the accumulation of the other cyt b_6/f subunits to ~60% of WT level in *C. reinhardtii*[46]. Additionally, the up-regulation of PETO in *pgrl1* is important to note, since this loosely bound subunit was shown to accumulate to reduced levels in *C. reinhardtii* mutants lacking either cyt b_6, cyt b_6/f IV or PETC[47], which are less abundant in *pgrl1* compared to the WT in the present experiment.

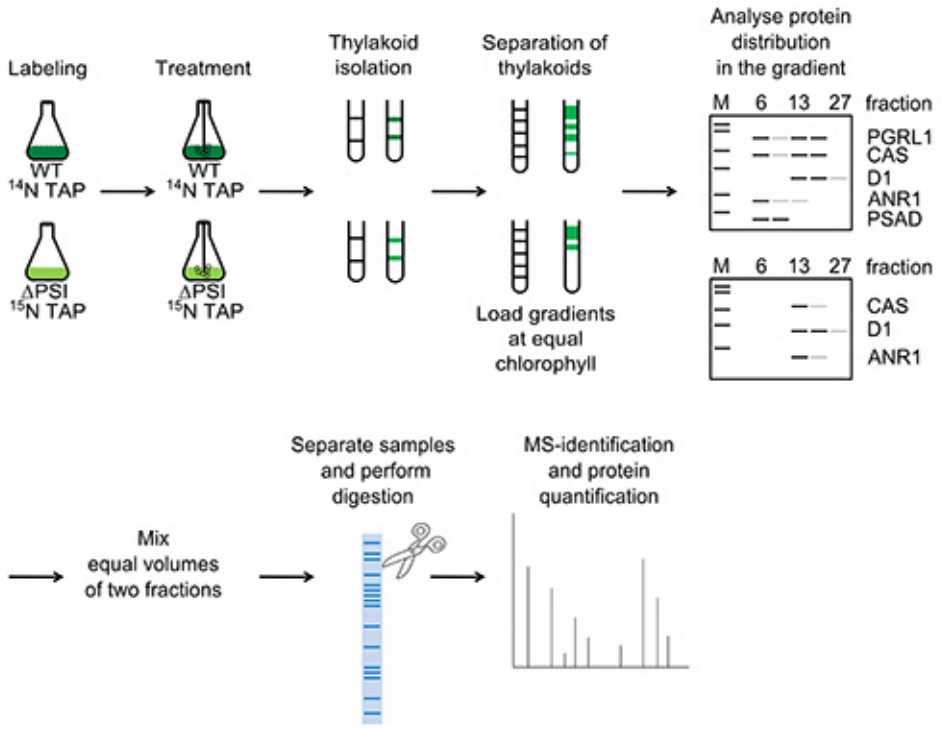

Figure 1. Experimental workflow as depicted for the WT and ΔPSI. Cells are metabolically labeled for at least four generations, anaerobic conditions are induced by argon bubbling for 4 hr and thylakoid membranes are isolated through gradient separation. The isolated membranes are solubilized using β-DM, loaded onto a sucrose density gradient (for both strains equal amounts of chlorophyll are applied in this step) and centrifuged overnight. The gradient fractions are collected and analyzed by SDS-PAGE as well as immunodetection to determine distribution of proteins within the gradient. To identify proteins with a PSI-dependent migration, equal volumes of the WT CEF-supercomplex fraction (fraction number 6) and the corresponding ^{15}N-labeled fraction from the ΔPSI strain were mixed and comparatively analyzed. The same was done with the peak fraction of ANR1 in the ΔPSI strain (fraction number 13). For the comparative, quantitative MS-approach, the mixed protein samples were separated by SDS-PAGE, protein bands were stained with Coomassie blue solution, followed by in-gel digestion prior to MS-analysis. Click here to view larger image.

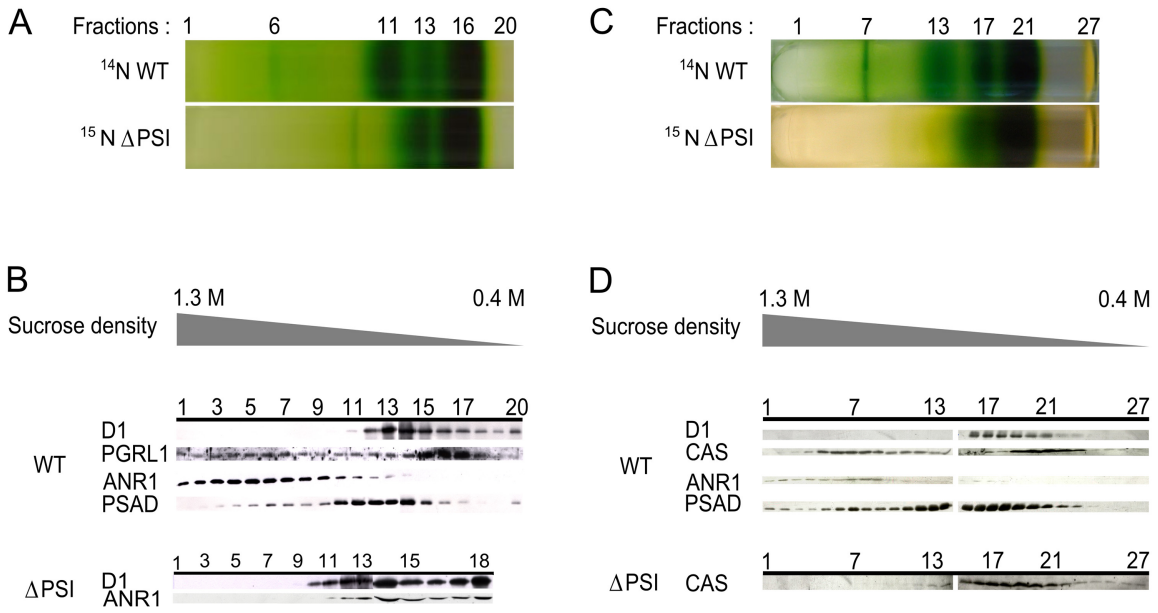

Figure 2. ANR1 and CAS migrate to the lower-density regions in the ΔPSI strain. A: Sucrose density gradients of WT and ΔPSI thylakoids isolated from anaerobic conditions were separated into 20 fractions. **B**: Immunodetection of ANR1 in the 20 fractions of the gradient from WT and ΔPSI. While this protein is localized to the higher-density region in the WT (fraction 6) it is up-shifted to the lower-density regions in the ΔPSI strain (fraction 13). **C**: Sucrose density gradients of WT and ΔPSI thylakoids isolated from anaerobic conditions were separated into 27 fractions. **D**: Immunodetection of CAS in the 27 fractions of the gradient from WT and ΔPSI. While this protein is localized to the higher-density region in the WT (peaking around fraction 7) it is up-shifted to the lower-density regions in the ΔPSI strain (peaking around fraction 19). (This figure has been modified from Terashima *et al.*[6]). Click here to view larger image.

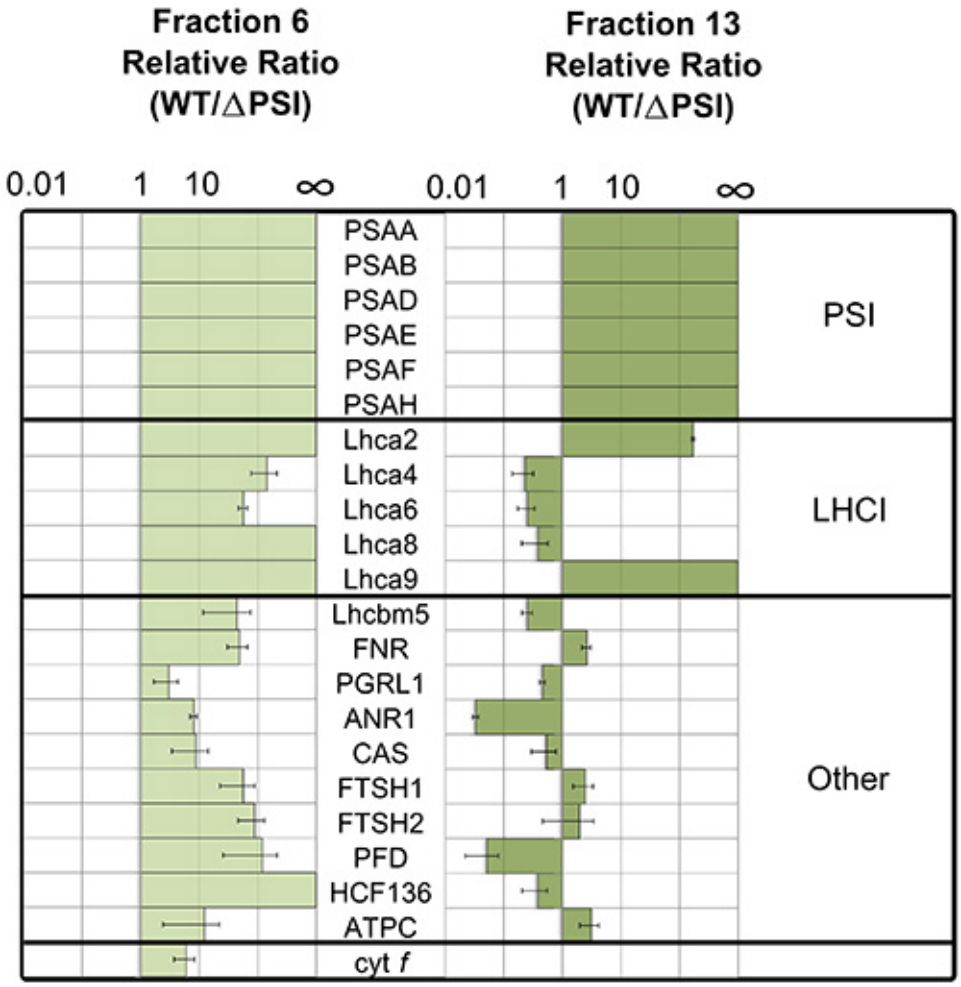

Figure 3. Relative protein ratios determined in fraction 6 and 13 from WT and ΔPSI by quantitative mass spectrometry. Fractions 6 and 13 from WT have been compared with the respective fractions from the ^{15}N-labeled ΔPSI strain. The relative protein ratios that represent two biological replicates and three MS-runs are depicted as WT / ΔPSI (^{14}N/^{15}N) for both analyzed fractions with the exception of cyt *f*, which was only identified in fraction 6. This comparative quantification reveals that ANR1 and CAS show a PSI-dependent migration in the sucrose density gradient (this figure has been modified from Terashima *et al.*[6]). Click here to view larger image.

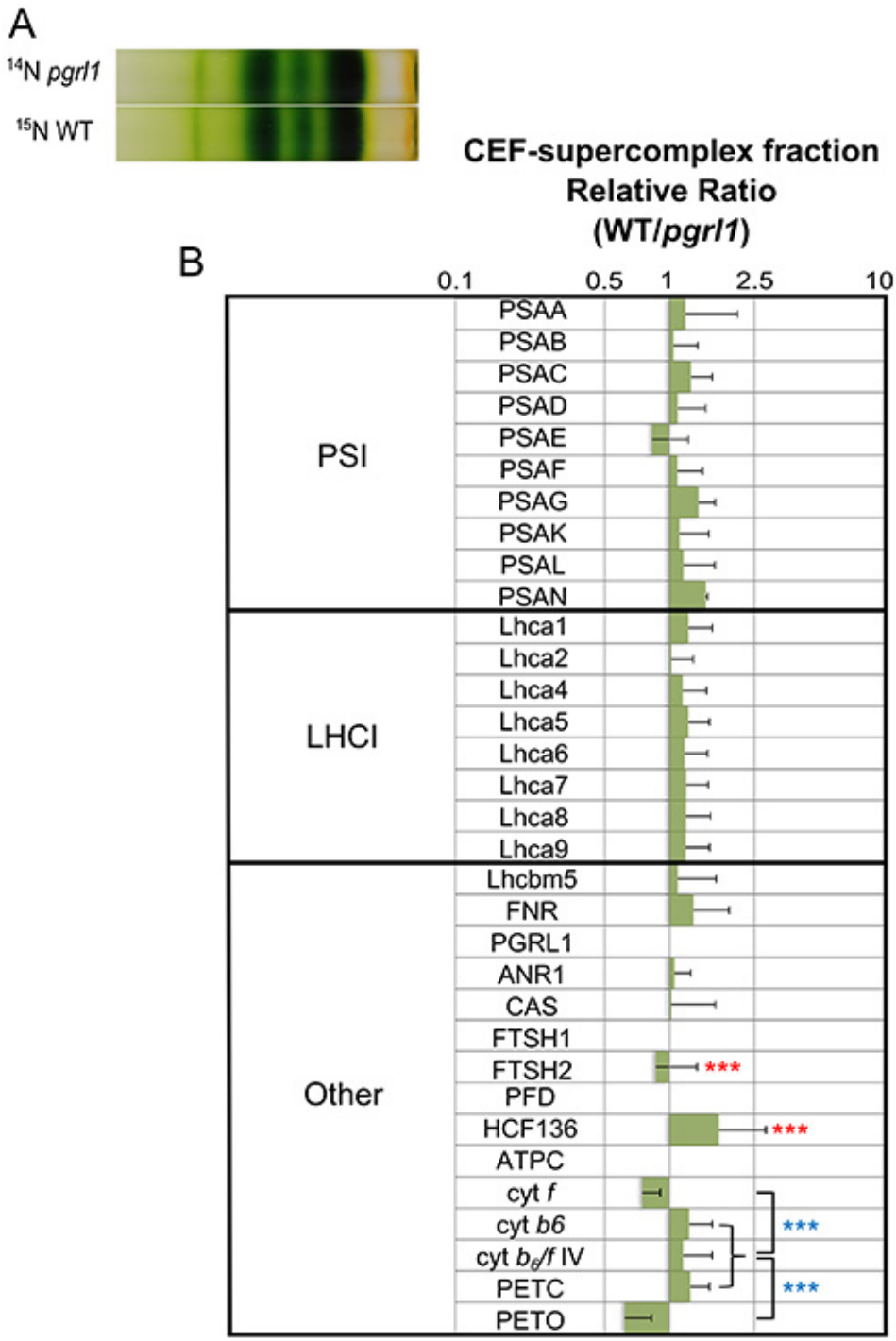

Figure 4. Relative protein ratios determined in the CEF-supercomplex fraction of WT and *pgrl1* by quantitative mass spectrometry. **A**: Sucrose density gradients of WT and *pgrl1* thylakoids isolated from anaerobic conditions. **B**: Relative protein ratios between the CEF-supercomplex fraction stemming from four different replicates depicted as WT / *pgrl1* ($^{14}N/^{15}N$). In this experiment ANR1 and CAS are not remarkably changed, while FTSH2 and HCF136 exhibit significant differences compared to all PSI-subunits ($p \leq 0.001$ as indicated by *** in red; HCF136: 1900 < U < 58127; FTSH2: 2117 < U < 164197). Additionally, the cyt b_6/f complex subunits cyt *f* and PETO were shown to be significantly different from cyt b_6, cyt b_6/f IV and PETC, respectively ($p \leq 0.001$ as indicated by *** in blue; cyt *f*: 2684 < U < 11535; PETO: 4700 < U < 23663). Click here to view larger image.

Discussion

Different quantitative proteomic studies using stable isotope labeling have been published in the last years. In these experiments usually two different samples are compared, of which one sample is labeled with a stable isotope. Thereafter proteins or peptides from the two samples are

combined in an equal ratio and further processed together[48]. Such studies often intend to compare defined isolated cellular compartments (*e.g.* chloroplasts, mitochondria, or thylakoid membranes) exposed to different stress conditions[26,34,49] to investigate up- or down-regulation of specific proteins. The described comparative, quantitative proteomic approach aims to analyze the composition of multiprotein complexes in the thylakoid membrane and, in contrast to the aforementioned studies, is based on mixing the same volume of samples after sucrose density separation of thylakoid membranes loaded at equal chlorophyll concentration before ultracentrifugation. This method was effectively applied by Terashima *et al.*[6] who comparatively analyzed the composition of the CEF-supercomplex isolated from a WT and a ΔPSI strain to identify proteins migrating PSI-dependently in a sucrose density gradient.

The achievement of this strategy is proven by the fact that the MS-results are further supported by immunodetection. Additionally, Terashima *et al.* could confirm the findings from previous studies (*i.e.* the identification and PSI-dependent migration of proteins already described to be part of the CEF-supercomplex, such as PGRL1, FNR, and cyt *f* [7]). The identification of ANR1 and CAS as novel components of the CEF-machinery[6] reveals this approach as a very powerful and efficient tool. Notably, the isolated CEF-supercomplex exhibited *in vitro* activity, thereby demonstrating the successful purification of a functional multiprotein complex[6]. The applicability of this approach also for two strains of which both form a CEF-supercomplex is demonstrated by the comparison between WT and *pgrl1*.

In general, this method can be used to survey complex compositions in different strains or conditions as revealed by the identification of previously unidentified proteins that are copurified with the CEF-supercomplex fraction (*i.e.* the enrichment of FTSH1, FTSH2, PFD, and HCF136 in the CEF-supercomplex fraction). Whether these proteins are possible new candidates of this complex and to improve our understanding for the different regulation of FTHS2, HCF136, as well as for the cyt b_6/f subunits as revealed by the comparison of WT and *pgrl1*, further experiments are necessary.

Besides the described advantages of this approach, there are several critical steps that need to be carefully considered when working with this protocol: The breaking of cells with the nebulizer is the first important step. If the opening of cells is not carried out in a proper way, the yield of isolated thylakoids will be rather low and might result in hardly any detection of the CEF-supercomplex after over-night centrifugation. The successful disruption of cells can be inferred from the light green color of the supernatant after centrifugation. In contrast, if the pressure during cell disruption is too high, parts of the supercomplex might disintegrate and important cofactors might be lost. Another step influencing the yield of thylakoids is the resuspension of cells with a potter. This has to be undertaken carefully and at the end of this step only few green particles should remain. Furthermore, for the preparation of the sucrose density gradients the day before as well as for the solubilization of membrane complexes, it is very important to precisely follow this protocol in terms of the β-DM concentration, since this is crucial to obtain fully solubilized thylakoids[50] and therefore also for purification of the CEF-supercomplex. The solubilization step has been successfully performed if a small, whitish pellet is observed after the subsequent centrifugation step. Another critical point that should be mentioned here is the time of over-night ultracentrifugation. The sucrose density gradients should be centrifuged for at least twelve hours to achieve full separation of the photosynthetic complexes.

Even though the protocol is demonstrated using *C. reinhardtii* for the comparative analysis of the CEF-supercomplex composition in two genetically different strains, it is easily adaptable also to a broad range of other questions, such as the comparative analyses of multiprotein complex composition isolated from distinct environmental / experimental conditions or by using other types of fractionation. The only requirements of this approach are that the model organism can be cultivated in cell culture, is able to use ammonium as nitrogen source and protein complexes can be separated by sucrose density gradients, demonstrating a wide application of this method. For future application of this approach SDS-PAGE fractionation of the mixed $^{14}N/^{15}N$ samples and subsequent in-gel digestion could be replaced by applying the filter-aided sample preparation (FASP) method. This method includes the application of strong detergents for solubilization aiming to “clean up” the proteome before digestion and to obtain purified peptides, preventing the disadvantages of the in-gel digestion approach that can inhibit peptide recovery, although one should consider that in-gel digestion is described to be robust to contaminations that might interfere with the digestion[51].

Disclosures

The authors declare no competing financial interest.

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