The HCF136 protein is essential for assembly of the photosystem II reaction center in *Arabidopsis thaliana*

Henning Plücken[a], Bernd Müller[b], Dina Grohmann[a], Peter Westhoff[a], Lutz A. Eichacker[b,*]

[a] *Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität, Universitätsstrasse 1, 40225 Düsseldorf, Germany*
[b] *Botanisches Institut der Ludwig-Maximilians-Universität, Menzingerstrasse 67, 80638 München, Germany*

Received 15 October 2002; accepted 16 October 2002

First published online 5 November 2002

Edited by Richard Cogdell

Abstract ***Hcf136*** **encodes a hydrophilic protein localized in the lumen of stroma thylakoids. Its mutational inactivation in** ***Arabidopsis thaliana*** **results in a photosystem II (PHII)-less phenotype. Under standard illumination, PSII is not detectable and the amount of photosystem I (PSI) is reduced, which implies that HCF136p may be required for photosystem biogenesis in general. However, at low light, a comparison of mutants with defects in PSII, PSI, and the cytochrome b_6f complex reveals that HCF136p regulates selectively biogenesis of PSII. We demonstrate by in vivo radiolabeling of** ***hcf136*** **that biogenesis of the reaction center (RC) of PSII is blocked. Gel blot analysis and affinity chromatography of solubilized thylakoid membranes suggest that HCF136p associates with a PSII precomplex containing at least D2 and cytochrome b_{559}. We conclude that HCF136p is essential for assembly of the RC of PSII and discuss its function as a chaperone-like assembly factor.**

Key words: Assembly; HCF136p; Photosystem II; High chlorophyll fluorescence (*hcf*); Blue native PAGE

1. Introduction

Photosystem II (PSII) is one of two chlorophyll a (Chl a) binding multisubunit pigment protein complexes of plastid thylakoid membrane in oxygen evolving photosynthetic organisms [1]. During illumination of this complex water is oxidized. Hereby, molecular oxygen and protons are released on the lumenal side and electrons, which are captured by the reaction center (RC) of PSII, are used to reduce plastoquinone on the stromal side of the thylakoid membrane.

Although numerous biochemical [2,3] and mutational approaches in *Synechocystis* and *Clamydomonas* [4,5] have given detailed insights into the structure and function of the PSII complex, much less is known about the biogenesis of the protein complex in the thylakoid membrane [6,7]. In addition to its role in photosynthesis, the membrane is, together with the endoplasmic reticulum and the inner membrane of mitochondria, one of the major protein export membranes of the photosynthetic cell [8–10]. Like the other thylakoid membrane complexes, PSII is a genetic mosaic which consists of plastic and nuclear encoded subunits. Its biogenesis depends on a strictly coordinated gene expression in both the chloroplast stroma and the cytosol and on the import of nuclear encoded subunits into the chloroplast [4]. The coordination of gene expression is guaranteed by a number of different biogenesis factors, which intervene at the different steps of the gene expression cascade, thus building a complex regulatory framework [4]. A further regulatory level is found during transport of imported or plastic expressed subunits into or across the thylakoid membrane and during binding of cofactors like Ca, Fe, and Mn or pigments like chlorophylls and carotenoids [9,11,12]. Finally, assembly of the subunits into a fully functional photosystem structure has to be regulated [13].

Analysis of high Chl fluorescence (*hcf*) photosynthetic mutants of *Arabidopsis thaliana* led to the identification of a mutant *hcf136* and the nuclear encoded protein of prokaryotic origin responsible for the phenotype, the protein HCF136p [14]. In the absence of HCF136p, incorporation of radiolabel could be detected for PSII core complex subunits D1, D2, cytochrome b_{559}, CP47 and CP43 in plastids of mutant seedlings, but no stable accumulation of the proteins was found [14]. It was concluded that HCF136p is necessary for assembly or stability of the PSII subunits.

Since the *hcf136* mutation was also associated with a significant reduction of photosystem I (PSI), we investigated the biogenesis of PII, PSI and the cytochrome b_6f (Cyt b_6f) complex by comparative gel blot analysis of hcf mutants with defined lesions. By 2D Native-/SDS–PAGE separation of in vivo pulse-labeled PSI and II complexes and their assembly intermediates we demonstrate that HCF136p is specifically required for assembly of the PSII RC complex, an assembly intermediate in PSII biogenesis. We provide evidence that HCF136p is associated with a nascent PSII assembly intermediate, suggesting that the protein may function as a chaperone-like assembly factor.

2. Materials and methods

2.1. Growth conditions

Germination of *Arabidopsis* seeds, growth of seedlings and mutant selection was performed as described in [14], except that seedlings were grown with a 12 h photoperiod at a photon flux density of 50–70 μmol m^{-2} s^{-1} ('standard light') or 2–5 μmol m^{-2} s^{-1} ('low light'), respectively.

2.2. Germination of transgenic *A. thaliana hcf136* lines overexpressing a *HCF136–Protein A fusion protein*

The entire HCF136 reading frame was C-terminally fused with a Protein A (ProtA) tag. The ProtA tag is composed of two IgG binding domains of the *Staphylococcus aureus* Protein A gene followed by a spacer and the TEV protease cleavage site to give a total length of 135 amino acids [15]. The HCF136–TEV–Protein A fusion construct

*Corresponding author. Fax: (49)-89-17861 209.
E-mail address: eichacker@lmu.de (L.A. Eichacker).

0014-5793/02/$22.00
PII: S0014-5793(02)03634-7

(hcf136pA) was cloned into the binary Ti vector pPEX001 (B. Reiss, MPIZ Cologne, personal communication) and was introduced into heterozygous *hcf136* plants by *Agrobacterium tumefaciens* mediated transformation using the floral dip method [16]. Three of 24 T2-plants were identified as homozygous for the *hcf136* mutation and were fully complemented by the HCF136–TEV–Protein A fusion gene to the wild-type phenotype.

2.3. Preparation of thylakoid membrane proteins for gel blot analysis

Leaf material of 14 day old seedlings was homogenized in liquid nitrogen. The homogenate was resuspended in 50 mM Tris–HCl, pH 6.8, 10 mM EDTA, 10 mM EGTA, 10 mM dithiothreitol (DTT), 1 mM phenyl methyl sulfonate (PMSF) and 1 mM benzamidine at 4°C for 10 min and centrifuged at 10 000 rpm at 4°C for 15 min. The pellet containing total membranes was resuspended in the same buffer. SDS–PAGE was carried out according to [17] with addition of 6 M urea. For gel blot analysis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes as described in [18]. Membranes were immunodecorated with specific antisera [14] and visualized by the enhanced chemiluminescence method as described by the manufacturer (Amersham-Pharmacia Biotech, Sweden).

2.4. In vivo labeling of thalykoid membrane proteins

Two-week old seedlings of *A. thaliana* were cut at the beginning of the hypocotyls with razor blades and preincubated for 15 min in 200 μg/ml cycloheximide to block cytosolic translation. The in vivo labeling was performed in a 1:1 mixture of ^{35}S methionine (specific activity >1000 Ci/mmol; Amersham-Pharmacia Biotech, Sweden) and 200 μg/ml cycloheximide for 20 min at room temperature and 50 μmol m^{-2} s^{-1} of photon flux density.

2.5. Blue native PAGE separation of thylakoid membrane proteins

About 10–20 *Arabidopsis* leaves were cut into very small slices with a razor blade in 10 mM Tris–HCl pH 6.8, 10 mM $MgCl_2$, 20 mM KCl at 4°C (TMK). Hereby, organelles were released and lysed. Thylakoids were separated from stroma by centrifugation for 5 min at 5000 rpm and 4°C. After washing once in the same buffer, thylakoids equivalent to 100 μg of Chl were resuspended in 60 μl 750 mM (or 5 mM) ε-aminocaproic acid, 50 mM Bis-Tris–HCl pH 7.0, 0.5 mM EDTA and incubated on ice for 5 min. Proteins were solubilized by addition of 5 μl 10% (or 20%) (w/v) *n*-dodecyl-β-D-maltoside (or 26% (v/v) Triton X-100). After incubation on ice for 10 min, samples were centrifuged for 10 min at 10 000 rpm and 4°C. The supernatant was recovered and 5 μl of 5% (w/v) Serva blue G in 750 mM (or 5 mM) ε-aminocaproic acid was added. The protein solution was loaded onto a pre-cooled PAGE system prepared and run in the blue native first and the SDS second dimension [19].

2.6. Affinity chromatography using a Protein-A–TEV–hcf136p protein construct

For affinity purification of proteins binding to a Protein-A–TEV–hcf136p protein construct (hcf136pA), a chromatography column (PolyPrep, Bio-Rad) was filled with 200 μl IgG-Sepharose (IgG-Sepharose 6, fast flow; Amersham Bioscience). The IgG-Sepharose matrix was conditioned as described by the manufacturer and finally washed with TMK-T [Tris–HCl, pH 6.8, 10 mM; $MgCl_2$, 10 mM; KCl, 20 mM; Triton X-100, 0.08% (v/v)]. Thylakoid membrane proteins were radiolabeled as described in Section 2.4 and membranes were concentrated by centrifugation in an Eppendorf microcentrifuge (5000 rpm, 5 min, 4°C). Membrane proteins were solubilized for 5 min in 1% (v/v) Triton X-100 on ice and protein aggregates were removed by centrifugation (10 000 rpm, 10 min, 4°C). The supernatant was diluted with 1 ml TMK buffer and radiolabel equivalent to an amount of 5×10^5 cpm was loaded onto the column. The column was placed on a shaker (REAX 2, Heidolph) and incubated for 2 h at 4°C. Thereafter, the column was loaded with 5 ml TMN-T buffer [Tris–HCl, pH 6.8, 10 mM; $MgCl_2$, 10 mM; NaCl, 25 mM; Triton X-100, 0.08% (v/v)] and the flow-through was discarded. Proteins bound by the column were eluted in a volume of 1 ml within 30 s by an acid pH shift (Glycin/HCl, pH 3.0, 100 mM; NaCl, 150 mM; Triton X-100, 0.08%) and the flow-through was neutralized directly in 2 M Tris–HCl, pH 8.0). Alternatively, proteins were released by incubation of the column in a cleavage buffer (Tris–HCl, pH 8.0, 10 mM; EDTA, 0.5 mM; NaCl, 150 mM; DTT, 1 mM; Triton X-100, 0.08%) containing 20 U recombinant TEV-Protease (Invitrogen) for 2 h, at RT. Protein extracts were precipitated in 10% (v/v) trichloracetic acid (TCA) for 30 min on ice and were concentrated by centrifugation (10 000 rpm, 4°C). Precipitates were washed in 80% (v/v) acetone and proteins prepared for SDS–PAGE as described in Section 2.3.

3. Results

3.1. HCF136 is a PSII mutant

In the *A. thaliana* mutant *hcf136*, a depletion of PSI and PSII subunits was reported [14]. To differentiate whether PSII or PSI were directly affected by the loss of the HCF136 protein (HCF136p), we compared the *hcf136* mutant to the *Arabidopsis* wild type (WT) and characteristic PSII, Cyt b_6f and PSI mutants. The level of protein subunits characteristic for PSII, the Cyt b_6f, and PSI was investigated under standard and low-light growth conditions.

As reference for mutations affecting PSII we used *hcf173*, *hcf154* and *hcf107* [20]. In comparison to the WT, these mutants show reduced levels of PSI (psaA/Bp, PsaDp) and PSII subunits (CP47, CP43, D2, D2, PsbOp, psbEp). In addition, *hcf* mutants with defined defects in the Cyt b_6f complex (*hcf164*) and PSI (*hcf145*) were included in the comparison. *Hcf164* lacks the Cyt b_6f complex, most likely due to a defect in cytochrome f maturation [21]. *Hcf145* does not accumulate the *psaA/B* mRNA which encodes the P700 Chl a-apoproteins (psaA/psaB) of PSI RC (J. Meurer, personal communication) and is consequently depleted of PSI (Fig. 1A, psaA/Bp and PsaDp).

Since we suspected that the photosynthetic photon flux might influence the level of photosynthetic electron transport complexes within the chloroplast, we compared the amount of protein subunits after seedlings were grown under standard photosynthetic (50–70 μmol m^{-2} s^{-1}) or under low light fluxes (2–5 μmol m^{-2} s^{-1}).

Under standard light intensities, the levels of PSII subunits were drastically reduced in the PSII mutants, whereas, in the PSI and Cyt b_6f mutants, protein levels were comparable to those in the WT. The level of the Cyt b_6f subunit petDp remained unchanged except for the Cyt b_6f mutant *hcf164* itself. *Hcf164* and all four PSII mutants *hcf136*, *hcf107*, *hcf173*, and *hcf154* showed an about eight-fold decrease in the level of PSI subunits psaA/Bp and PsaDp when compared to the WT (Fig. 1A). Hence, reduced levels of PSI proteins are observed in the Cyt b_6f and all PSII mutants. We therefore conclude that a decrease in PSI levels is not specific for PSII mutants.

In low light, the levels of all protein subunits from both photosystems and from the Cyt b_6f complex were reduced in WT plants as well as in the PSI mutant relative to the standard light conditions (Fig. 1B). Although the level of PSII subunits in the PSII mutants and in *hcf136* was lower than in the WT, an increase relative to standard light conditions was detectable. In the PSI and PSII mutants, the amount of petD remained at a constant value relative to the WT protein subunit. However, the amount of psaA/B and psaD increased by a factor of four in the PSII mutants relative to the WT. This suggested that in PSII mutants, PSI was light-sensitive, whereby the level of PSI subunits was reduced under standard light. Interestingly, the same effect could be observed when the Cyt b_6f mutant *hcf164* was investigated. We conclude that *hcf* mutations affecting PSII or Cyt b_6f subunits influenced the steady-state level of PSI subunits. In contrast, *hcf* mutations

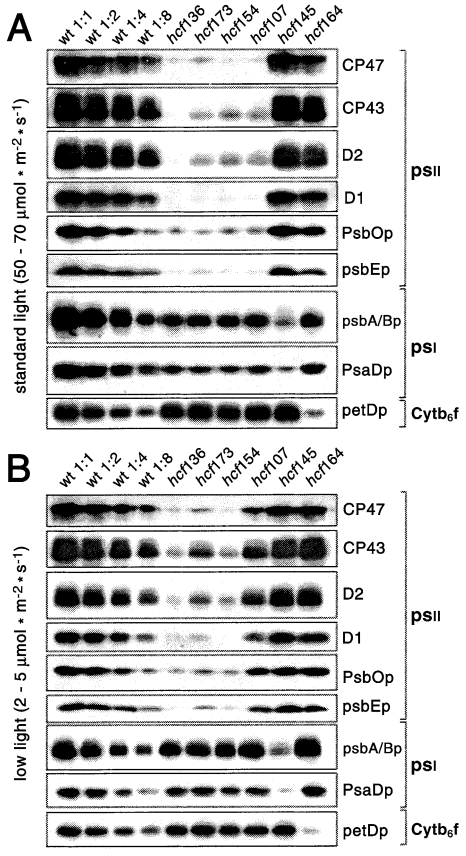

Fig. 1. Gel blot analysis of photosystem proteins from *Arabidopsis* WT and hcf mutants. Steady-state levels of protein subunits from PSI and II core complexes and of the Cyt b_6f complex were isolated from WT plants and from the *hcf* mutants *hcf136*, *hcf173*, *hcf154*, *hcf107*, *hcf145* and *hcf164* after growth at standard (50–70 µmol m^{-2} s^{-1}) (A) or low light intensity (2–5 µmol m^{-2} s^{-1}) (B). Protein subunits were analyzed on gel blots with antibodies directed against PSI: psaA/Bp, PsaDp; PSII: CP47, CP43, D2, D1, PsbOp, psbEp; and $Cytb_6f$: petDp. A standard equal amount of protein (15 µg) or a dilution of the standard was loaded for SDS–PAGE of WT and *hcf* mutant.

affecting PSI subunits did not influence the steady-state level of PSII or Cyt b_6f subunits (Fig. 1A,B).

The reduced amounts of PSI which had been described in *hcf136* under standard light conditions [14] can therefore not be explained by a direct effect of HCF136p on PSI. Hence, the data indicated that the reduction in PSI levels was due to a secondary effect of the mutation, indicating that *hcf136* is a PSII mutant.

3.2. The HCF136 gene product is essential for assembly of PSII RC complexes

Previous in vivo labeling and gel blot experiments of *hcf136* seedlings had shown that the PSII core subunits D1, D2, CP43, CP47 and cytochrome b_{559} (Cyt b_{559}) were synthesized as in WT, but did not accumulate. We therefore investigated in detail in *hcf136* at which point during the assembly of PSII HCF136p is required. Chloroplast proteins were radiolabeled in vivo by infiltration of leaves with [^{35}S]methionine, thylakoid membranes were isolated from in vivo labeled seedlings and assembly of protein subunits was analyzed by blue native polyacrylamide gel electrophoresis (BN–PAGE) according to molecular mass [22] (Fig. 2). Cycloheximide was added to block radiolabel incorporation by cytoplasmic protein synthesis. For SDS–PAGE, subunits contained within the complexes were released and were separated. In this second dimension, plastome-encoded subunits of PSII, i.e. CP47 (PsbB), CP43 (PsbBC), D2 (PsbD) and D1 (PsbA), and of PSI, (PsaA and PsaB) were found to be radiolabeled. By BN–PAGE, several PSII complexes can be distinguished. Four major complexes of increasing molecular mass were resolved and identified by gel blot analysis and mass spectrometry as RC, RC-CP47 (RC47), monomeric (CCI) and dimeric (CCII) RC core complexes (Fig. 2A).

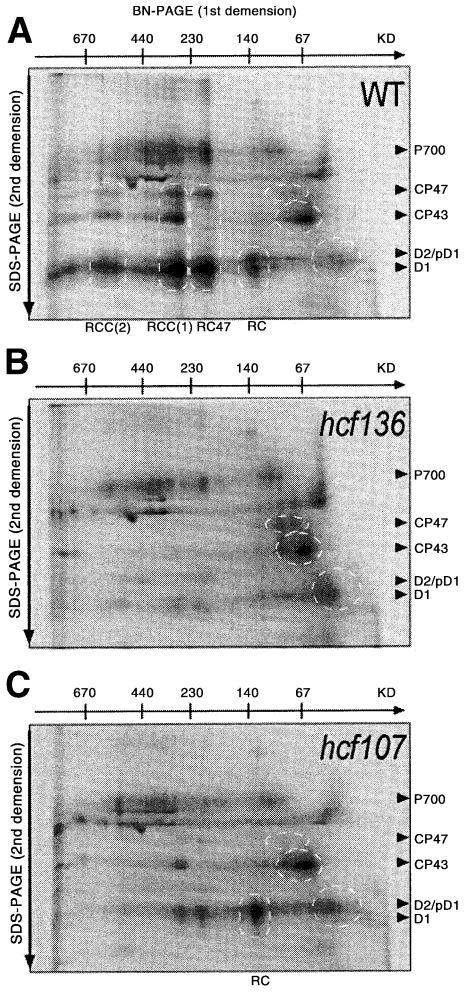

Fig. 2. Autoradiogram of radiolabeled thylakoid membrane proteins after separation by two-dimensional Native–/SDS–PAGE. Protein complexes of WT and hcf mutants (*hcf136*, *hcf107*) were separated by a 2D Native–/SDS–PAGE setup. In Native–PAGE (BN–PAGE), protein complexes were loaded with negatively charged Coomassie and separated according to molecular mass (molecular mass of marker proteins is given at the top of the figure). In denaturing SDS–PAGE, protein subunits of preseparated complexes were released by SDS and proteins separated according to molecular mass orthogonal to the first electrical field gradient (arrow at the left hand side of figure indicates direction of protein migration). Protein subunits P700 of PSI and CP47, CP43, D2, pD1, and D1 of PSII were identified by mass spectrometry and gel blot analysis. For PSII, accumulation of radiolabeled protein subunits was identified in dimeric core complex, CCII; in monomeric core complex, CCI; in a RC complex containing CP47, RC47; and in a RC complex, RC; in thylakoids isolated from wild-type (A, WT) and mutant (B, *hcf136*; and C *hcf107*) after radiolabeling in vivo (see Section 2).

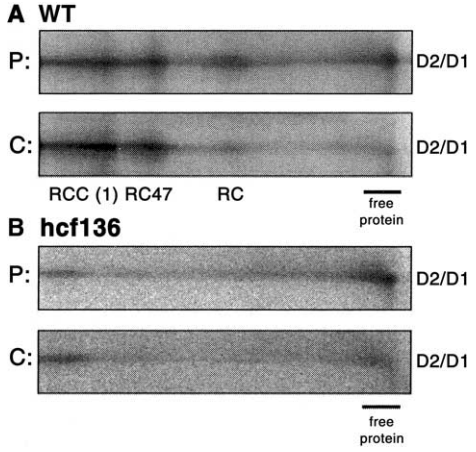

Fig. 3. Autoradiogram of D1 in PSII complexes after in vivo pulse/chase radiolabeling. *Arabidopsis* WT and *hcf136* mutant seedlings were infiltrated with [^{35}S]methionine for 60 min (Pulse). Thereafter, [^{35}S]methionine was replaced by a solution containing 10 mM L-methionine and seedlings were infiltrated for an additional 60 min (Chase). Thylakoid membranes were prepared from seedlings and protein complexes were separated by BN–PAGE as described in Section 2.

In WT plants, most of the [^{35}S]methionine label was incorporated into protein subunits CP47, CP43, D2 and D1 of the RC47 and monomeric CCI complex. Less label was incorporated into the dimeric CCII and the RC complex. A small amount of label could also be detected in unassembled D1, D2 and CP47, whereas the levels of unassembled CP43 were clearly higher (Fig. 2A). It follows that under the experimental conditions chosen mature PSII complexes can assemble, starting from de novo synthesized proteins. With respect to PSI, most of the radiolabeled P700 protein was found in PSI complexes of about 550 and 400 kDa, indicating that under the experimental conditions chosen, mature PSI-LHCI and PSI RC complexes assemble as well.

In vivo incorporation experiments with *hcf136* mutants led to strikingly contrasting results with respect to PSII, but not in the case of PSI (Fig. 2B). Both large PSI complexes could be detected, suggesting that the PSI assembly pathway operates in *hcf136* as in the WT. In contrast, hardly any radiolabeled proteins were found at the position of CCII, CCI, RC47, and the RC complex (Fig. 2B, *hcf136*; dashed circles), suggesting that these PSII complexes did not assemble in the *hcf136* mutant background. In line with this conclusion, large amounts of unassembled radiolabeled CP47, CP43, D2 and D1 proteins accumulated during pulse labeling.

To confirm these findings, the assembly of PSII complexes was analyzed in *hcf107*. As mentioned above, *hcf107* mutants do not translate PSII-H and CP47 efficiently, but assemble PSI [20]. In this mutant, only the RC complex accumulated (Fig. 2C). Formation of the RC47 complex and of CCI was drastically impaired, and CCII was almost undetectable. We therefore concluded that assembly of the RC complex was blocked in the *hcf136* mutant and preceded that of RC47 and CCI in the WT. From the results concerning the *hcf107* mutant, we concluded that CP47 and PSII-H are not required for assembly of the RC complex, but for RC47 formation. Results indicated that the RC complex is an early assembly intermediate for assembly of PSII-H and CP47.

3.3. The RC of PSII is an assembly intermediate

The findings described above suggested that the RC complex could be an assembly intermediate of PSII and consequently it should be possible to chase radiolabeled PSII from the RC into RC47 and CCI complexes in WT seedlings. We therefore selected the radiolabeled D1 protein as molecular mass marker to investigate whether distribution and radiolabel intensity of the protein changed during an assembly kinetic in WT and *hcf136* seedlings. Radiolabel which accumulated in free D1 and the RC complexes during pulse-labeling with [^{35}S]methionine could be chased into RC47 and CCI complexes when WT seedlings were infiltrated with non-radiolabeled L-methionine (Fig. 3). In contrast, in *hcf136*, radiolabeled free D1 protein was lost during the chase; however, some radiolabel accumulated in a 290 kDa band (Fig. 3A,B). Since no other radiolabeled proteins besides D1 were found in this fraction, we speculate that the 290 kDa complex arises due to aggregation of non-assembled D1 proteins. Taken together, the pulse-chase experiments reinforced that HCF136p is specifically and essentially required for assembly of the PSII RC complex. Furthermore, the data demonstrated that the RC itself is an intermediate for RC47 assembly.

3.4. The HCF136 protein comigrates with PSII complexes

The data presented here led to the conclusion that HCF136p is essential for assembly of RCs and may function as a chaperone-like protein. As an assembly chaperone,

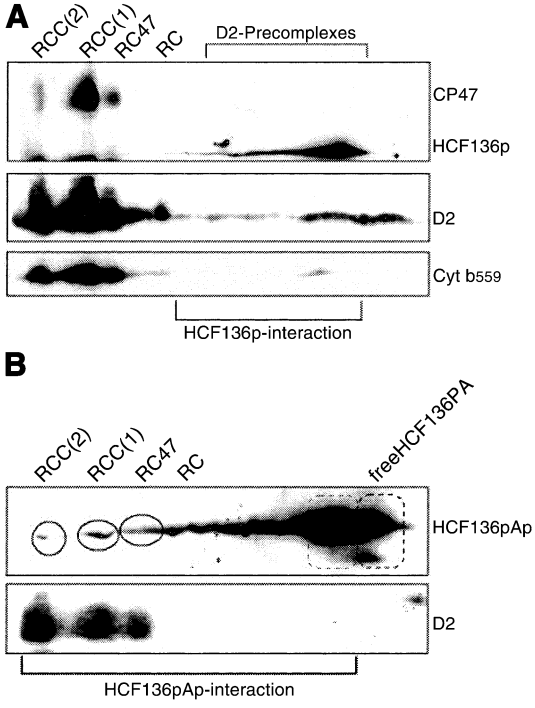

Fig. 4. Gel blot analysis of protein subunits separated by 2D Native–/SDS–PAGE. Thylakoid membrane protein complexes from WT plants were separated by 2D PAGE. In the first dimension an acrylamide/bisacrylamide gradient of 8–16% (w/v) was employed. After SDS–PAGE, protein subunits were detected by gel blot analysis on PVDF membranes. Blots were incubated with antisera against CP47, HCF136p, D2, and Cyt b_{559} (A), and against D2, and transgenic HCF136-Protein A (HCF136PAp) (B). Localization of PSII complexes is given at the top of the figure, the molecular mass range of HCF136p distribution is given below the figure (HCF136p interaction).

HCF136p should transiently interact with one of the RC subunits. To collect evidence for this assumption, the molecular mass of HCF136p and PSII subunits D2 and Cyt b_{559} was measured by BN–PAGE.

Gel blot analysis with antisera directed against PSII subunits CP47, D1, D2 and Cyt b_{559} identified PSII complexes RC, RC47, CC(I) and CC(II) in WT thylakoid membrane preparations (Fig. 4). D2 was detected as free protein and in a D2 precomplex described earlier to contain Cyt b_{559} [12] (Fig. 4A). Most of 43 kDa protein HCF136p was localized at a molecular mass around 70 kDa overlapping this D2 precomplex. However, a small portion of HCF136p was detected at higher molecular masses reaching up to the 200 kDa RC47 complex (Fig. 4A). A similar type of protein streaking was also found with antibodies directed against the D2 protein.

In order to increase the sensitivity for detection of the HCF136p in the gel blot experiments, we subsequently analyzed transgenic lines of *A. thaliana* with a HCF136–Protein A fusion construct (HCF136pPA). In mutant *hcf136* plants that were functionally complemented, detection of HCF136pPA was achieved with increased sensitivity due to the high affinity for antibodies directed against the Protein A domain (Fig. 4A,B). In comparison to gel blot analysis of HCF136p, streaking of HCF136pA was localized up to the dimeric CCII complex. Pronounced streaking of proteins HCF136p and D2 may reflect a protein interaction between the lumenal HCF136 protein and the membrane integral D2 protein during assembly of the RC. The small proportion of HCF136p that colocalized specifically to PSII core complexes RCC(1) and RCC(2) suggests that HCF136p is selectively involved in assembly of PSII subunits.

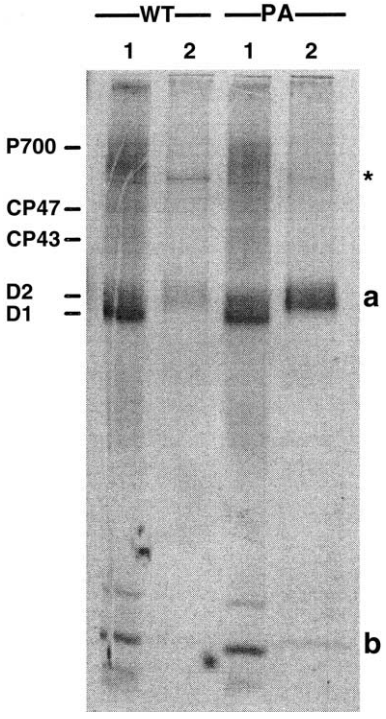

Fig. 5. Affinity purification of radiolabeled proteins interacting with hcf136pA. Thylakoid membrane proteins were radiolabeled in vivo in WT *A. thaliana* (WT) or in the *hcf136* mutant complemented by the hcf136pA fusion gene (PA) for 30 min. A protein amount equivalent to 10^5 cpm was separated by SDS–PAGE (lanes 1). For affinity purification of proteins binding to hcf136pA, thylakoids were solubilized and a radiolabeled protein amount equivalent to 5×10^5 cpm was subjected to IgG- (WT, 2), and IgG-hcf136pA Sepharose columns (PA, 2). Proteins released from the columns were concentrated and separated by SDS–PAGE (lanes 2, a and b).

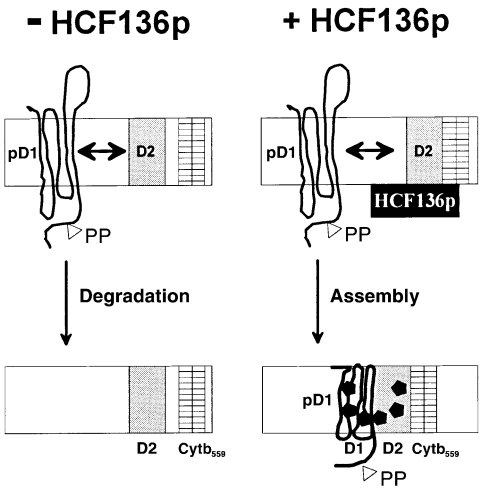

Fig. 6. Model for a HCF136p regulated step in assembly of PSII RCs. Biogenesis of PSII RC is proposed to require a Chl a independent HCF136p catalyzed interaction of D2 and cytb559. The RC precomplex forms the basis for assembly/disassembly cycles with pD1-protein [13]. In the presence of HCF136p, stable binding of Chl a (black pentagons) and assembly of RC complexes is possible. In the absence of HCF136p, pD1 is synthesized but assembly of pD1 with RC precomplexes into stable RC complexes is blocked and pD1 is degraded.

3.5. *The HCF136pA fusion protein concentrates two radiolabeled membrane proteins*

These results prompted us to investigate the selective interaction of HCP136p and PSII proteins by affinity chromatography. *Hcf136* mutants complemented by the HCF136–ProtA fusion gene and WT seedlings were radiolabeled in vivo in the presence of cycloheximide and membrane proteins equivalent to an amount of 10^5 cpm were separated by SDS–PAGE (Fig. 5 WT, PA; lane 1). For affinity chromatography with hcf136p radiolabeled and solubilized membrane proteins were subjected to IgG-Sepharose or to IgG-Sepharose coupled to the hcf136pA construct. Two radiolabeled protein bands were found to bind and to selectively elute upon an acid pH shift, or upon incubation with TEV protease (Fig. 5, WT, PA, lane 2). The results show that the hcf136pA construct increased the yield of both radiolabeled membrane proteins over the WT control (Fig. 5, lanes 2, a and b). The molecular mass and plastic origin of protein bands a and b denote that these proteins are D2 and Cytb559, respectively. The identification of D2 and Cytb559 in a putative complex with hcf136p by BN–PAGE makes it therefore highly likely that these thylakoid membrane proteins interact in *A. thaliana* chloroplasts.

4. Discussion

The nuclear encoded protein HCF136p was described earlier to be essential for the stability of PSII in higher plant chloroplasts. In vivo labeling and gel blot experiments of *hcf136* seedlings had shown synthesis, but not accumulation of PSII subunits D1, D2, CP43, CP47 and Cyt b_{559} [14]. Why a parallel defect in PSI accumulation occurred in *hcf136* mu-

tant thylakoids remained open. The light and PSII-/Cyt b_6f dependent levels of PSI shown here, indicate that the disturbed state of the photosynthetic electron transport in *hcf136* could serve as a signal to adjust, i.e. down-regulate, PSI levels. Alternatively, the reduced PSI levels could result from photooxidative processes which occur when the light-induced excitation of PSI and its antenna systems is not accompanied by a matching consumption of electrons at the donor or acceptor sides of the photosystem [23].

We demonstrated that HCF136p is essential for the PSII assembly process by BN–PAGE which was already used for separation of mitochondrial membrane protein complexes [19,22,24]. In WT plant thylakoids, the CCI complexes have been demonstrated to be the minimal functional assembly stage for enzymatic evolution of oxygen from water [25]. In contrast, an oxygen evolution with a very low yield was also detected in CCI complexes lacking CP43, but the level of a contaminating enzymatic activity of CCI complexes may have been too low for detection of the CCI subunit CP43 in gel blot or silver staining analysis after SDS–PAGE [26]. In this respect, neither *hcf136* nor *hcf107* assemble any functional PSII complexes. In the *hcf107* mutant, the RC complex accumulated despite the loss of psbH and psbB expression and showed that this mutant is a PSII assembly mutant. In *hcf136* thylakoids, the assembly intermediates RC, RC47, CCI and -CCII were not found, but pulse and pulse/chase labeling experiments demonstrate expression of the RC subunits D1, D2 and Cyt b_{559}. Since no accumulation of the D2-Cyt b_{559} RC precomplex occurs, it remains open whether hcf136p is required for assembly or stability of the RC precomplex. In dark-grown plants, accumulation of the RC precomplex has been detected by immunoprecipitation analysis in the absence of Chl a, indicating that hcf136p is required before Chl a binding [12].

Hcf136p has been located in stromal thylakoids [14], where translation of the plastic encoded subunits of PSII RC cores has been described and where some of the primary assembly steps of membrane inserted subunits occur [7,14,27]. Here, the binding of de novo synthesized D1 was described to occur cotranslationally, i.e. during membrane insertion of the nascent D1 protein [6]. HCF136p could operate from the lumenal side of the thylakoid as a membrane-attached folding or assembly catalyst, which may be inevitable to assist assembly during cotranslation insertion of D1 from the stroma side of the membrane. According to our data, we propose a model where HCF136p is required for assembly or stability of the RC precomplex (Fig. 6). A stable RC precomplex may be maintained in the absence of pD1, providing a mold for successive binding of Chl a to the RC heterodimer [12], a strategy comparable to binding of Chl a to natural and the development of artificial protein maquettes [10,28]. Hence, a specific function of HCF136p for Chl a binding is suggested since a chaperone binding to the lumen-exposed peptide loops may provide the flexibility for non-covalent attachment of Chl a to the membrane α-helix bundles (Fig. 6). The Protein A tag used in our approach may have altered the binding properties of native HCF136p; however, the fusion protein fully substitutes the native protein in mutant plants, complementing them to a WT phenotype, and tagged plus untagged proteins show formation of PSII precomplexes in BN–PAGE. It is therefore unlikely that the tag itself is responsible for binding of HCF136pA to D2 and Cyt b_{559}.

References

[1] Zouni, A., Witt, H.T., Kern, J., Fromme, P., Krauss, N., Saenger, W. and Orth, P. (2001) Nature 409, 739–743.
[2] Rhee, K.H., Morris, E.P., Barber, J. and Kuhlbrandt, W. (1998) Nature 396, 283–286.
[3] Hankamer, B., Nield, J., Zheleva, D., Boekema, E., Jansson, S. and Barber, J. (1997) Eur. J. Biochem. 243, 422–429.
[4] Barkan, A. and Goldschmidt-Clermont, M. (2000) Biochimie 82, 559–572.
[5] Pakrasi, H.B. (1995) Annu. Rev. Genet. 29, 755–776.
[6] Zhang, L., Paakkarinen, V., van Wijk, K.J. and Aro, E.M. (1999) J. Biol. Chem. 274, 16062–16067.
[7] van Wijk, K.J., Andersson, B. and Aro, E.M. (1996) J. Biol. Chem. 271, 9627–9636.
[8] Eichacker, L.A. and Henry, R. (2001) Biochim. Biophys. Acta 1541, 120–134.
[9] Robinson, C., Woolhead, C. and Edwards, W. (2000) J. Exp. Bot. 51 Spec. No., 369–374.
[10] Eggink, L.L. and Hoober, J.K. (2000) J. Biol. Chem. 275, 9087–9090.
[11] Ananyev, G.M. and Dismukes, G.C. (1996) Biochemistry 35, 14608–14617.
[12] Muller, B. and Eichacker, L.A. (1999) Plant Cell 11, 2365–2378.
[13] Wollman, F.A., Minai, L. and Nechushtai, R. (1999) Biochim. Biophys. Acta 1411, 21–85.
[14] Meurer, J., Plucken, H., Kowallik, K.V. and Westhoff, P. (1998) EMBO J. 17, 5286–5297.
[15] Rappsilber, J., Siniossoglou, S., Hurt, E.C. and Mann, M. (2000) Anal. Chem. 72, 267–275.
[16] Clough, S.J. and Bent, A.F. (1998) Plant J. 16, 735–743.
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Schagger, H., Cramer, W.A. and von Jagow, G. (1994) Anal. Biochem. 217, 220–230.
[20] Felder, S., Meierhoff, K., Sane, A.P., Meurer, J., Driemel, C., Plucken, H., Klaff, P., Stein, B., Bechtold, N. and Westhoff, P. (2001) Plant Cell 13, 2127–2141.
[21] Lennartz, K., Plucken, H., Seidler, A., Westhoff, P., Bechtold, N. and Meierhoff, K. (2001) Plant Cell 13, 2539–2551.
[22] Krimmer, T., Rapaport, D., Ryan, M.T., Meisinger, C., Kassenbrock, C.K., Blachly-Dyson, E., Forte, M., Douglas, M.G., Neupert, W., Nargang, F.E. and Pfanner, N. (2001) J. Cell. Biol. 152, 289–300.
[23] Voelker, R. and Barkan, A. (1995) Mol. Gen. Genet. 249, 507–514.
[24] Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R.A. and Schagger, H. (1999) J. Biol. Chem. 274, 36–40.
[25] Zheleva, D., Sharma, J., Panico, M., Morris, H.R. and Barber, J. (1998) J. Biol. Chem. 273, 16122–16127.
[26] Buchel, C., Barber, J., Ananyev, G., Eshaghi, S., Watt, R. and Dismukes, C. (1999) Proc. Natl. Acad. Sci. USA 96, 14288–14293.
[27] Aro, E.M., Virgin, I. and Andersson, B. (1993) Biochim. Biophys. Acta 1143, 113–134.
[28] Grosset, A.M., Gibney, B.R., Rabanal, F., Moser, C.C. and Dutton, P.L. (2001) Biochemistry 40, 5474–5487.