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A nuclear-encoded protein of prokaryotic origin is essential for the stability of photosystem II in *Arabidopsis thaliana*

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To understand the regulatory mechanisms underlying the biogenesis of photosystem II (PSII) we have characterized the nuclear mutant *hcf136* of *Arabidopsis thaliana* and isolated the affected gene. The mutant is devoid of any photosystem II activity, and none of the nuclear- and plastome-encoded subunits of this photosystem accumulate to significant levels. Protein labelling studies in the presence of cycloheximide showed that the plastome-encoded PSII subunits are synthesized but are not stable. The *HCF136* gene was isolated by virtue of its T-DNA tag, and its identity was confirmed by complementation of homozygous *hcf136* seedlings. Immunoblot analysis of fractionated chloroplasts showed that the *HCF136* protein is a lumenal protein, found only in stromal thylakoid lamellae. The *HCF136* protein is produced already in dark-grown seedlings and its levels do not increase dramatically during light-induced greening. This accumulation profile confirms the mutational data by showing that the *HCF136* protein must be present when PSII complexes are made. *HCF136* homologues are found in the cyanobacterium *Synechocystis* species PCC6803 (slr2034) and the cyanelle genome of *Cyanophora paradoxa* (ORF333), but are lacking in the plastomes of chlorophytes and metaphytes as well as from those of rhodo- and chromophytes. We conclude that *HCF136* encodes a stability and/or assembly factor of PSII which dates back to the cyanobacterial-like endosymbiont that led to the plastids of the present photosynthetic eukaryotes.

Keywords: *Arabidopsis*/high-chlorophyll fluorescence mutants/nuclear regulator gene/photosystem II assembly

Introduction

Photosystem II (PSII) is one of the four major multisubunit protein complexes of the thylakoid membrane of oxygenic photosynthetic organisms. The reaction centre core of this photosystem consists of the P680-binding subunits D1 (*PsbA*) and D2 (*PsbD*), cytochrome b_{559} (*PsbE–PsbF*) and the *PsbI* protein, and performs the primary charge separation. The minimal oxygen-evolving PSII complex in addition contains CP47 (*PsbB*) and CP43 (*PsbC*), two proteins of the inner chlorophyll *a* antenna, as well as the extrinsic, lumenal 34 kDa protein (*PsbO*). There are ~15–20 additional proteins which have been identified as constituent subunits of PSII, but their functions have only been partly elucidated. PSII, like the other thylakoid membrane complexes, is a genetic mosaic and consists of nuclear- and plastome-encoded subunits (reviewed in Hankamer *et al.*, 1997).

The functioning of PSII as a light-triggered water-plastoquinone oxidoreductase is reasonably well understood due to numerous biochemical and biophysical studies (reviewed in Hankamer *et al.*, 1997). A two-dimensional structure at 8 Å resolution is also available (Rhee *et al.*, 1997) that may provide a starting point towards the elucidation of its three-dimensional structure. Compared with these detailed functional and structural informations, relatively little is known about the biogenesis of this membrane complex. Greening studies with etiolated seedlings of angiosperms demonstrated that the biosynthesis of a functional PSII depends on light (Westhoff *et al.*, 1990). However, truncated PSII complexes which lack the chlorophyll *a*-binding subunits D1, D2, CP47 and CP43 can accumulate in dark-grown seedlings (Westhoff *et al.*, 1990). This suggests that the assembly of PSII proceeds stepwise and involves the formation of intermediate subcomplexes (Van Wijk *et al.*, 1996).

The stoichiometry of PSII with respect to the other thylakoid membrane complexes is not constant but varies depending upon light conditions and species (reviewed in Melis, 1991). The fact that PSII biogenesis can be regulated separately from the other thylakoid membrane complexes is evident from its differential accumulation in mesophyll and bundle-sheath cells of NADP-malic enzyme type C4 plants (Schuster *et al.*, 1985; Oswald *et al.*, 1990). A selective regulation of the PSII activity is also observed when cells of nitrogen-fixing filamentous cyanobacteria differentiate into heterocysts (Wolk *et al.*, 1997). The molecular and genetic basis of this capacity for differential PSII biogenesis in both eukaryotic and prokaryotic photosynthetic organisms is unclear.

In general, posttranscriptional controlling mechanisms are thought to be very important for chloroplast biogenesis (reviewed in Rochaix, 1992; Mayfield *et al.*, 1995) and can therefore be expected to play a dominant role in PSII biogenesis as well. Several *cis*- and *trans*-regulatory components have been identified which are involved in the processing and stability of plastid mRNAs (Rochaix, 1992; Mayfield *et al.*, 1995). The translation of plastid mRNAs requires *trans*-regulatory factors which at least in part interact with the 5′ untranslated regions of the target mRNAs (Danon and Mayfield, 1991; Zerges *et al.*, 1997). The activity of these *trans*-regulatory factors may be controlled by the redox state of the photosynthetic apparatus as has been shown for the *psbA* mRNA (Danon and Mayfield, 1994).

There is a particular interest in understanding the final step in the biogenesis of thylakoid membrane complexes; that is, how the plastid- and nuclear-encoded subunits assemble to functional complexes. The correct folding of both types of plastidial proteins is mediated by molecular chaperones of the *GroEL–GroES* (Viitanen *et al.*, 1995) and *DnaK–DnaJ–GrpE* families (Marshall *et al.*, 1990; Schlicher and Soll, 1997). All available evidence indicates that the plastidial homologues of these chaperones act similarly to their eubacterial and mitochondrial counterparts. However, it is not yet clear whether the actual assembly step requires additional complex-specific factors. A complex-specific factor has recently been described for the cyanobacterial photosystem I, but its mechanism of action has yet to be investigated in detail (Bartsevich and Pakrasi, 1997).

We are interested in understanding the biogenesis of the thylakoid membrane and particularly that of PSII at the molecular level. To identify genes that are involved in these processes we are pursuing a genetic approach using *Arabidopsis thaliana* as the experimental system (Meurer *et al.*, 1996b). *Arabidopsis*, besides *Chlamydomonas* (Rochaix, 1995) and maize (Miles, 1982), offers a number of attractive features which makes it the system of choice for studying the biogenesis of the photosynthetic apparatus in photosynthetic eukaryotes. With the high chlorophyll fluorescence (*hcf*) phenotype (Miles, 1982) as a selection criterion, a systematic search for mutants defective in photosynthetic light reactions and electron transport was initiated. The mutations were generated by ethyl methanesulfonate treatment (Meurer *et al.*, 1996b) or by T-DNA insertion mutagenesis (Feldmann, 1991). To date, ~60 different recessive nuclear mutants have been isolated (Meurer *et al.*, 1996b; J.Meurer and K.Meierhoff, personal communication). The mutants were characterized by chlorophyll fluorescence induction, P700 absorption kinetics, immunoblotting, and RNA gel blot analysis. This allowed us to classify the mutants with respect to the thylakoid membrane complex being affected by the mutation.

Here we report on mutant *hcf136* which is specifically deficient in PSII activity but not primarily affected in the functions of the other thylakoid membrane complexes. RNA gel-blot and immunoblotting analyses revealed that all known plastid- and nuclear-encoded mRNAs for PSII subunits are present in the mutant, but none of the corresponding subunits. Protein labelling studies revealed that the plastome-encoded PSII subunits are synthesized but do not accumulate. This indicates that the *HCF136* gene encodes a factor that is essential for the stability or assembly of PSII. The *HCF136* gene could be cloned because of its T-DNA tag and was found to encode a protein that has homologues in the cyanobacterial genome and the cyanelle genome of *Cyanophora paradoxa*. This is the first report on the molecular identification of a nuclear-encoded gene that is essential for the biogenesis of PSII in higher plants and that originated from the prokaryotic ancestor of extant plastids.

Results

Selection and phenotype of the hcf136 mutant

The *hcf136* mutant of *A.thaliana* ecotype Wassilewskya was generated by T-DNA insertion mutagenesis and isolated from the mutant collection of K.Feldmann (Feldmann, 1991). Genetic analysis showed that the mutation is recessive. The kanamycin resistance marker carried by the T-DNA and the mutant phenotype co-segregated, thus indicating that the mutation was due to the insertion of the T-DNA (data not shown).

The *hcf136* mutant was selected by its high chlorophyll fluorescence which could be detected by eye under UV light in the dark (Miles, 1982). When germinated on soil, homozygous mutant seedlings were found to be lethal. They developed pale green cotyledons but no primary leaves. Cultivation on a sucrose-supplemented Gelrite medium rescued the mutant seedlings leading to a wild-type-like habitus. However, the mutant seedlings became somewhat paler during culture in the light, indicating that even a relatively low photon flux density of ~20–50 μmol/m^2/s causes photooxidative damage and results in the progressive loss of chlorophyll. Mutant plants did not produce any flowers.

Hcf136 is deficient in PSII activity

The functional state of PSII can easily be monitored by non-invasive chlorophyll fluorescence measurements (Krause and Weis, 1991). Analysis of *hcf136* mutant seedlings revealed no variable fluorescence with this technique (Figure 1A), indicating that PSII was completely inactive and that the linear electron flow was interrupted. Measurements of the absorbance kinetics of the P700 reaction pigment at 820 nm (Figure 1B) supported this inference. A strong light-pulse, given after illumination with far-red light, induced a further increase in the absorbance in mutant plants, whereas a decrease was detected in the wild type. Such an increase is also observed in plants treated with the herbicide DCMU [3-(3′,4′-dichlorophenyl)-1,1-dimethylurea] which completely blocks the electron transport between PSII and photosystem I (PSI) (compare with Meurer *et al.*, 1996b). The altered redox kinetics of P700 in mutant plants are therefore due to a lacking electron flow from PSII, and are not caused by deficiencies in PSI.

Chlorophyll fluorescence measurements at 77K verified the above findings (Figure 1C). The emission band at 733–735 nm, which is characteristic for a functional PSI, was clearly detectable in the mutant, corroborating evidence that PSI is functional or at least present in mutant plants. In contrast, the two emission peaks at 688 and 695 nm, which are indicative of the PSII reaction centre, were undetectable. Instead, the two bands were replaced by a new emission peak at 685 nm which is not found in the wild type. This additional band probably originates from the increased emission of the peripheral light-harvesting complex of PSII (LHCII) which in turn is caused by the inhibition of exiton transfer from the peripheral to the inner light-harvesting complex in the mutant (Krugh and Miles, 1995). Taken together, all spectroscopic data indicate that PSII is completely inactive in *hcf136* and that this defect represents the primary lesion.

Chloroplast ultrastructure in hcf136

PSII and its associated light-harvesting complex are preferentially located in the stacked grana regions, while the cytochrome b_6f complex and PSI are found in the margins of the grana as well as in stroma thylakoids (Anderson,

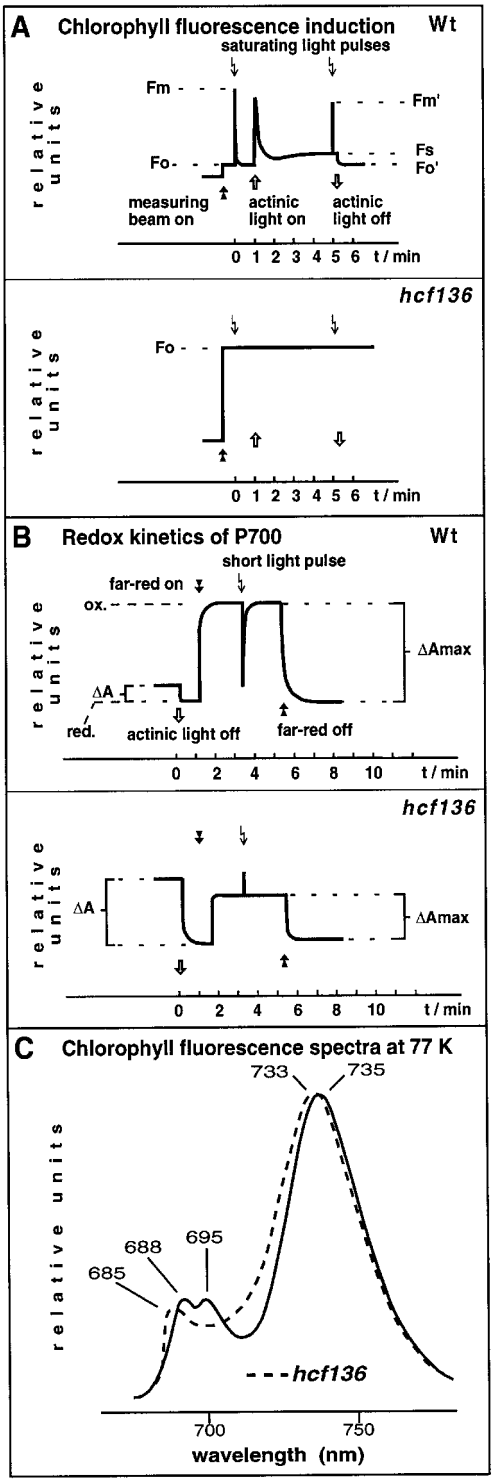

Fig. 1. Spectroscopic analysis of *hcf136* mutant and wild-type plants by measurements of chlorophyll fluorescence induction (**A**), P700 redox kinetics (**B**) and chlorophyll fluorescence emission at 77 K (**C**). For experimental details see Materials and methods.

1986). Wild-type chloroplasts display the typical differentiation into stroma and grana lamellae, whereas *hcf136* thylakoids reveal a strikingly different ultrastructure (Figure 2). The grana appear enlarged by 6- to 8-fold and extend almost throughout the chloroplast. Mutant grana lamellae are closely appressed to each other, indicating

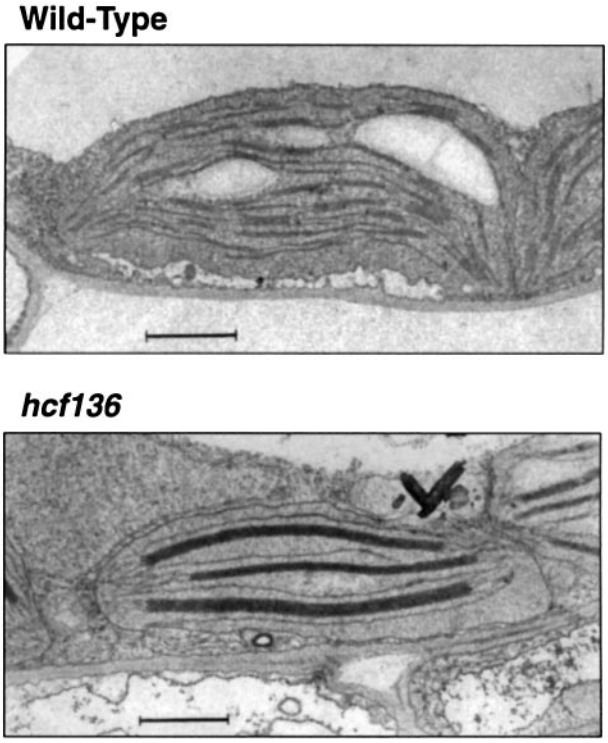

Fig. 2. Electron micrographs of chloroplasts from wild-type and *hcf136* leaves. Both wild-type and mutant plants were grown on a sucrose-supplemented agar medium, and plants of comparable growth stages were selected for the electron microscopic analysis. Bar = 1 μm.

that the spacing of the thylakoid membranes within one granum is reduced.

PSII polypeptides do not accumulate in mutant thylakoids

The spectroscopical analyses indicated that the mutational defect of *hcf136* resides in PSII. To support this contention and to investigate the PSII subunits affected by the mutation, thylakoid membranes of *hcf136* were analysed by immunoblotting (Figure 3) using a collection of antisera raised against individual PSII polypeptides and representative polypeptides of other photosynthetic membrane complexes (Meurer *et al.*, 1996b). The plastome-encoded subunits of PSII analysed are almost undetectable (CP47, CP43, D2 and D1) or drastically depleted (cytochrome b_{559}) in mutant thylakoids. Even the nuclear-encoded subunits of the oxygen-evolving complex, the 34 and 23 kDa proteins, fail to accumulate to significant levels in *hcf136*. In contrast, the amount of the 24 kDa LHCII polypeptide is not reduced as estimated by silver-stained SDS gels (data not shown).

Reduced levels (~70%) are also observed for subunits A/B and D of PSI which were investigated as representative polypeptides of this photosystem (Figure 3). These reductions are already detectable in 1-week-old seedlings (data not shown), suggesting that the *hcf136* mutation affects also PSI. However, it is often observed with PSII mutants that their PSI content is also affected, indicating that the reduced PSI levels in PSII mutants are due to secondary effects of the mutation. In the case of *hcf136* this view is supported by the spectroscopic analyses (see above) which revealed that the PSI reaction centres accumulating are

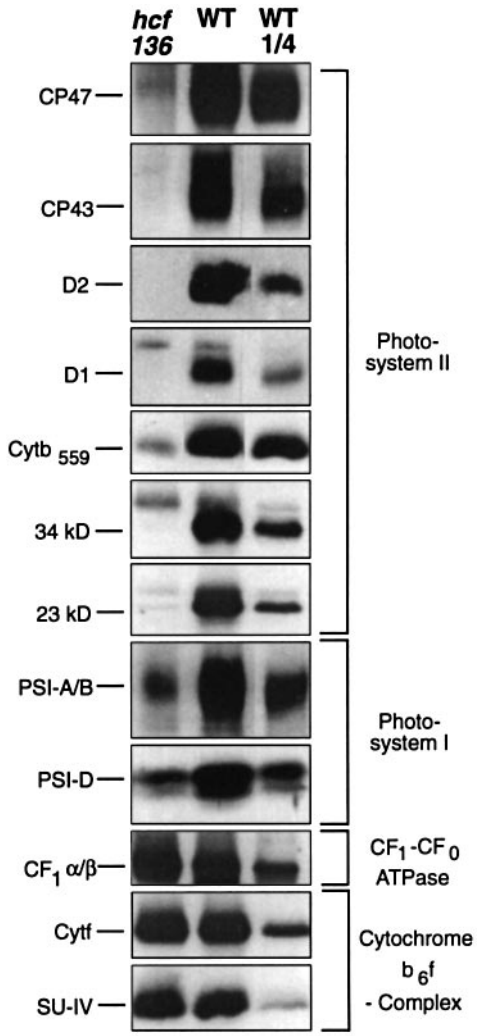

Fig. 3. Immunoblot analysis of thylakoid membrane proteins from *hcf136* and wild-type. Three-week-old plants were used for the analysis. Size fractionated membrane proteins were transferred to nitrocellulose, and specific polypeptides were immunodetected with the indicated antisera. The lanes were loaded with 8 μg (WT and *hcf136*) or 2 μg (WT 1/4) protein, respectively. The nature of the high molecular weight band reacting with the antiserum to the 34 kDa protein in both the mutant and wild-type is not known.

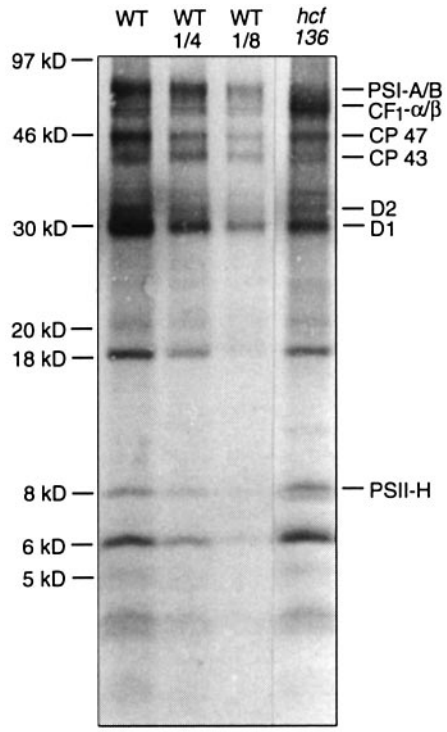

Fig. 4. *In vivo* protein synthesis with primary leaves of 12-day-old mutant and wild-type seedlings. Incorporation of [^{35}S]methionine in the presence of cycloheximide was performed as described in Materials and methods. Wild-type and mutant proteins with equivalent amounts of radioactivity [100 000 c.p.m. (WT and *hcf136*) or 25 000 c.p.m. (WT 1/4) and 12 500 c.p.m. (WT 1/8)] were electrophoresed on polyacrylamide–SDS gels, blotted onto a nitrocellulose membrane and analysed by fluorography. The identity of the indicated bands was determined as described (Meurer *et al.*, 1996a). The molecular masses (in kDa) were estimated by co-electrophoresis with commercially available size standards.

functional. Therefore, a direct effect of the *hcf136* mutation on PSI appears to be unlikely but cannot be excluded at the current stage of investigation.

No differences in steady-state levels were observed for subunits of the cytochrome b_6f complex (cytochrome *f* and subunit IV) and of the chloroplast ATP synthase (α and β subunits), demonstrating that the *HCF136* gene product is not needed for the assembly of these thylakoid membrane complexes. Taken together, the immunoblot analyses indicate that *hcf136* is primarily affected in PSII and that the reduction in PSI reaction centres is likely due to secondary effects of the mutation.

The plastome-encoded PSII polypeptides are synthesized but not stable in hcf136

To understand why PSII polypeptides do not accumulate in *hcf136* plants, the levels and patterns of the plastome- and nuclear-encoded PSII transcripts were investigated by RNA gel blot hybridization. Representative plastome- and nuclear-encoded genes for components of other thylakoid membrane complexes were included in this analysis (Meurer *et al.*, 1996b). The experiments revealed that both the amount and the sizes of all analysed transcripts were not altered in the mutant (data not shown). The inability of *hcf136* to accumulate PSII subunits is therefore not caused by a missing transcript encoding one of these structural PSII proteins, but must be due to a translational defect or, alternatively, to the instability of the PSII subunits once they have been synthesized.

To distinguish between these two possibilities, the rate of synthesis of thylakoid membrane proteins was investigated in intact mutant seedlings by pulse-labelling experiments with [^{35}S]methionine (Figure 4). To obtain an easily interpretable labelling pattern, the synthesis of the nuclear-encoded chloroplast proteins was blocked with cycloheximide, and only the plastome-encoded PSII proteins were investigated. Figure 4 shows that the protein labelling patterns of mutant and wild type are similar with respect to the numbers of polypeptides detectable and their intensity of labelling. The PSII subunits CP47, CP43, D1 and D2 can be identified due to their known electrophoretic mobilities (Kim *et al.*, 1994). They are synthesized in the mutant, but the incorporation of [^{35}S]methionine into these proteins is reduced by ~50% as compared with the wild type. Therefore, the lack of the plastome-encoded PSII subunits in *hcf136* cannot be

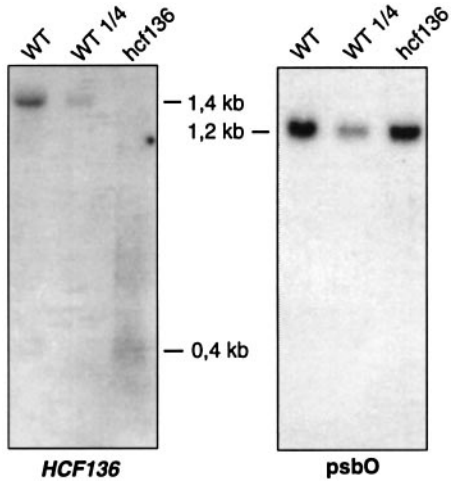

Fig. 5. Expression analysis of the *HCF136* gene. Eight micrograms of leaf RNA from 3-week-old mutant and wild-type plants grown under the same conditions was analysed by Northern hybridization as described in Materials and methods. As a probe, a 530 bp *Dra*I restriction fragment of the inverse PCR amplified products was used which contained exclusively genomic sequences directly adjacent to the T-DNA. To check for equal loading the filter was stripped and rehybridized with a *psbO* probe (Meurer *et al.*, 1996a).

explained by a translational deficiency, but must be predominantly caused by an increased instability of these PSII subunits in the mutant background.

Figure 4 also reveals that the synthesis of PSA-A/B is significantly reduced in the *hcf136* mutant. This can be due to enhanced proteolysis within the short incubation time of 15 min or to an effect on synthesis of these proteins in the mutant background.

Cloning of HCF136 sequences

Since segregation and Southern blot data indicated that the mutated *hcf136* gene was tagged by T-DNA, the genomic regions flanking the right border of the T-DNA were isolated by inverse PCR (Ochman *et al.*, 1993) as described in Materials and methods. Sequence analysis of the cloned PCR-amplified fragment and Southern hybridization with genomic DNA from *hcf136* and wild-type plants (data not shown) confirmed that the isolated PCR fragments indeed flank the right T-DNA border.

To examine the effect of the T-DNA insertion on the expression of the *HCF136* gene the isolated PCR genomic fragment was used for Northern hybridization (Figure 5). With RNA from wild-type plants the probe hybridized to a single transcript of ~1.4 kb in length. No hybridizing RNA of that size could be detected in RNA from mutant plants but some degradation products appeared at ~0.4 kb. Reprobing the filters with *psbO* sequences which encode the 34 kDa polypeptide of the oxygen-evolving complex of PSII revealed no differences in the hybridization intensity of the *psbO* mRNA, thus demonstrating that equal RNA amounts have been analysed in both cases (Figure 5). It follows from this that the T-DNA insertion leads to the inactivation of the presumptive *HCF136* gene by inhibiting the stable accumulation of its transcript.

To determine the sequence of the affected gene, a λZAP-cDNA library prepared from *Arabidopsis* leaves was screened with the genomic sequence that had been used for Northern hybridizations. Twenty-two of the

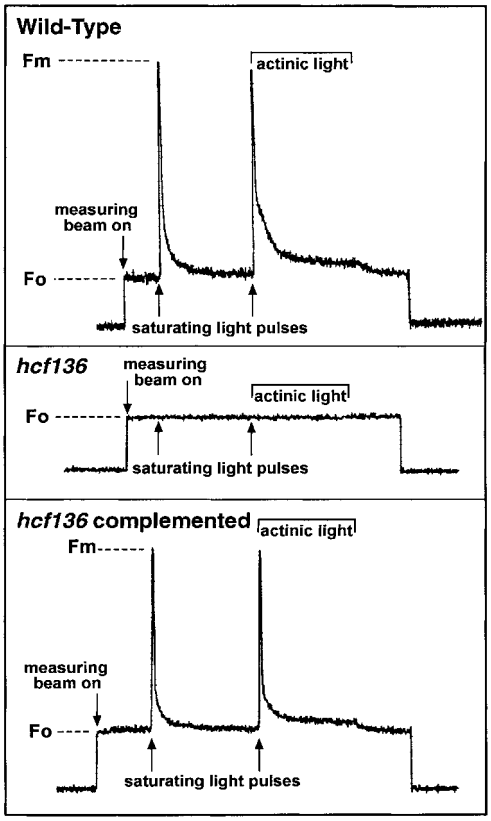

Fig. 6. Chlorophyll fluorescence induction kinetics of *hcf136* seedlings transformed with the *HCF136* cDNA under control of the 35S promoter. For details see Materials and methods.

positive cDNAs obtained were partially sequenced from both ends and found to contain identical sequences. The largest clone of these cDNAs, pcAt136–28, was 1400 bp in size and was sequenced on both strands. Sequence analysis revealed an open reading frame of 403 amino acids encoding a protein of 44.1 kDa.

To confirm that the *HCF136* gene disruption was indeed responsible for the high chlorophyll fluorescence phenotype of *hcf136*, the pcAt136–28 cDNA was fused to the 35S promoter of the Cauliflower Mosaic Virus (see Materials and methods), and the chimeric gene was introduced into homozygous mutant plants via *Agrobacterium tumefaciens* using a root transformation protocol (Valvekens *et al.*, 1988). In each of the 14 independently generated transformants the *HCF136* cDNA was able to complement the mutation and to restore wild-type characteristics. The transformed mutants turned normal green and showed chlorophyll fluorescence induction kinetics indistinguishable from wild type (Figure 6). In particular, the ratio of the variable to the maximal fluorescence (Fv/Fm) which reflects the efficiency of the primary photochemical events in PSII was fully restored in the complemented lines (Figure 6). The complementation experiment thus ultimately demonstrates that the mutant gene disrupted by the T-DNA is the causative factor of the mutation, and that the cDNA sequences isolated encode a functional *HCF136* protein.

Characteristics and evolution of the HCF136 protein

The *HCF136* reading frame present in the cDNA pcAt136–28 encodes a protein of 403 amino acids (Figure 7). The

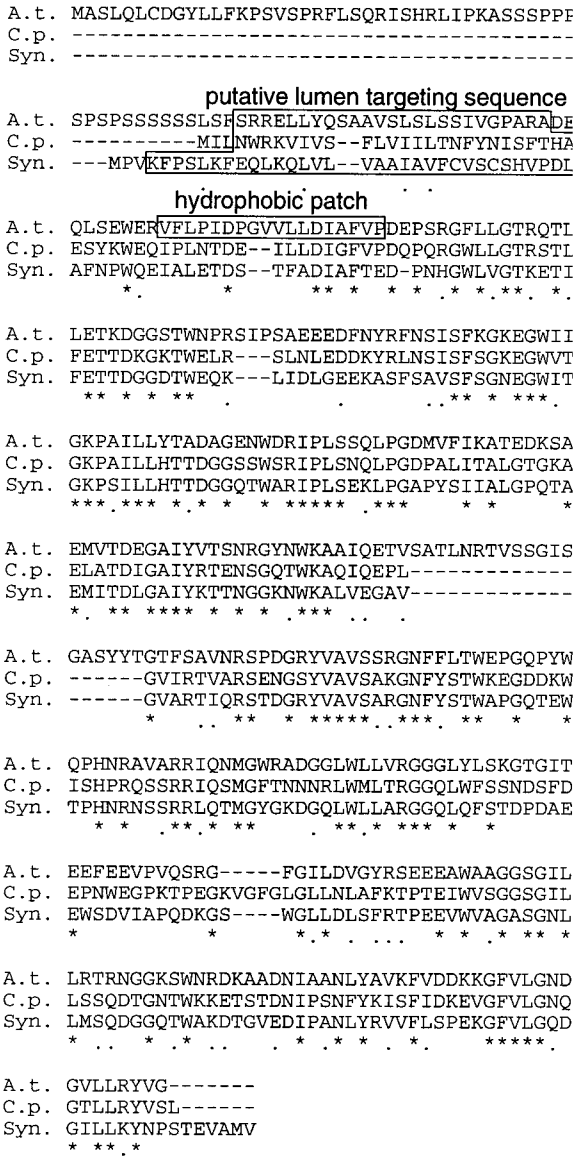

Fig. 7. Amino acid sequence of *HCF136* and comparison with ORF333 of *Cyanophora paradoxa* (C.p.) and ORF342 (slr2034) of *Synechocystis* sp. PCC6803 (Syn). Identical amino acids are star-marked, conserved exchanges are dotted. The nucleotide sequence of the *HCF136* cDNA has been submitted to DDBJ/EMBL/GenBank databases under the accession No. Y15628.

flanking region of its ATG start codon matches the consensus motif CA ATG GC which has been found around the translational initiation sites of a large number of plant genes (Lütcke *et al.*, 1987) suggesting that this ATG is the translational initiation codon. However, the genomic sequence of *HCF136* (http://www.kazusa.or.jp) revealed that there is an additional in-frame ATG codon. This putative translational initiation codon is located 23 amino acids in front of the one present in pcAt136–28, but lacks the sequence context typical for plant translational initiation sites. Because of this, and since the complementation test demonstrated that the *HCF136* reading frame present in pcAt136–28 encodes a functional protein we conclude that this upstream AUG codon is not used as the translational initiation codon.

Database searches failed to detect any sequence motives or domains that could shed some light on the function of the *HCF136* protein. These searches did, however, identify significant similarities with two ORFs of yet unknown function (Figure 7). ORF333 is encoded in the plastome of the glaucocystophycean alga *Cyanophora paradoxa* and ORF342 (slr2034) by the genome of the cyanobacterium *Synechocystis* sp. PCC6803. The three proteins revealed 34% identical and 14% similar amino acid positions which are more or less evenly distributed. In addition to three minor insertions, the central part of the *HCF136* protein contains 19 amino acids which are not found in the glaucocystophycean and cyanobacterial homologues. We conclude that the *HCF136* gene was present in the genome of the cyanobacterial-like endosymbiont that gave rise to the extant plastids of all photosynthetic eukaryotes, but was transferred to the nucleus before the rhodophyte/chromophyte and chlorophyte lineages separated.

In both the cyanobacterium and the glaucocystophyte, the *HCF136* homologues are located directly upstream of the *psbE-F-L-J* operon, whose gene order and composition has been conserved in both the cyanobacteria and the plastomes of all photosynthetic eukaryotes sequenced so far (http://www.ebi.ac.uk/). The *psbE-F-L-J* operon exclusively encodes PSII proteins, and the clustering of the *HCF136* homologues with this operon may be taken as further support that *HCF136* is functionally associated with PSII.

HCF136 encodes a lumenal protein that is located in stromal thylakoids

The N-terminal region of the *HCF136* reading frame is similar to transit sequences that direct nuclear-encoded proteins into the chloroplast (von Heijne *et al.*, 1989), suggesting that the *HCF136* protein is located in this organelle. To prove this assumption and to determine precisely the intracellular location of the *HCF136* protein, immunoblot analyses were carried out with fractionated intact chloroplasts. As a prerequisite, an antiserum was raised to *HCF136* protein which had been produced in *Escherichia coli*, and the antiserum was furthermore affinity-purified.

The antiserum labelled a 37 kDa protein in crude extracts prepared from *Arabidopsis* and spinach plants but did not detect any protein in *hcf136* extracts (data not shown). This finding demonstrated the specificity of the antiserum produced and corroborated that the T-DNA insertion prevented the accumulation of *HCF136* protein.

The apparent molecular mass of the detected protein was ~7 kDa less than the predicted molecular mass of the entire *HCF136* reading frame (Figure 8A). Such a reduced size was to be expected if the N-terminal part functions as a plastidial transit peptide and is removed after import into the organelle. In line with this expectation, the *HCF136* protein was found to be located in intact chloroplasts (Figure 8A). Subfractionation of the intact chloroplasts by sucrose density gradient centrifugation showed that the *HCF136* protein is associated with the thylakoid membrane and not with the envelope (Figure 8B). Washing with 0.2 M Na_2CO_3 (Figure 8A) released the *HCF136* protein from the chloroplast membrane fraction suggesting that it is attached to this membrane. The hydropathy analysis (data not shown) supported this inference and showed that the first 18 N-terminal amino acids of the

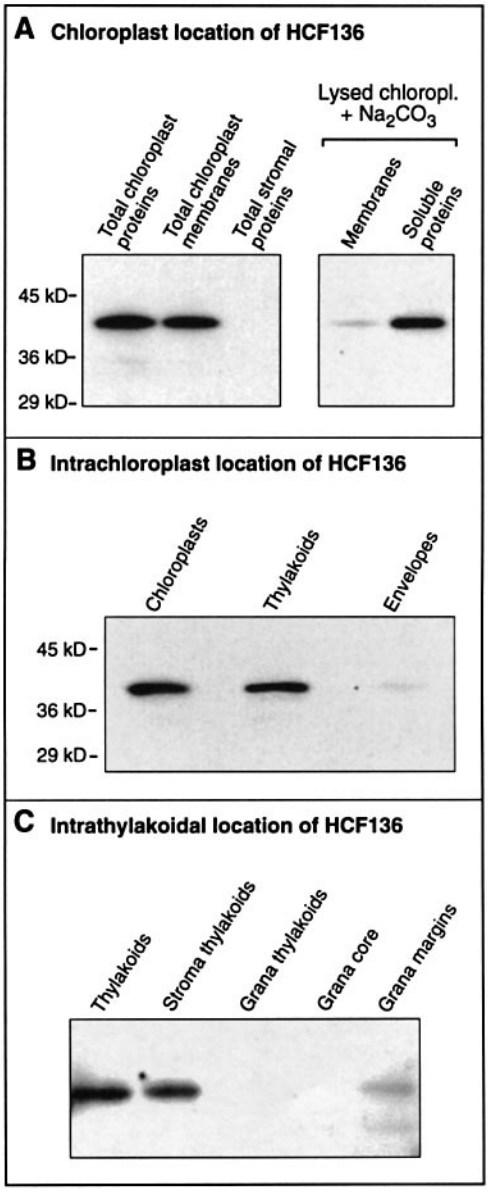

Fig. 8. Subcellular localization of the *HCF136* protein examined by protein blot analysis. (**A**) Chloroplast location of *HCF136* protein. Intact chloroplasts were isolated from *Arabidopsis* leaves, lysed and separated into total membranes and stromal proteins by centrifugation. In a separate experiment the lysed chloroplasts were first incubated with 0.2 M Na_2CO_3 and then separated by centrifugation into membranes and soluble proteins. In each lane the protein equivalent of 10 μg chlorophyll has been analysed. (**B**) Intrachloroplast location of *HCF136* protein. Thylakoid and envelope membranes were prepared according to Cline *et al.* (1981). Gradient fractions containing envelope and thylakoid membrane proteins, respectively, were identified by their pigment content and analysed by immunoblotting. Each lane was loaded with 30 μg of protein. Because of the low yield of envelopes which could be isolated from *Arabidopsis*, spinach chloroplasts were used for this experiment. (**C**) Intrathylakoidal location of *HCF136* protein. Spinach thylakoids were fractionated by the method of Wollenberger *et al.* (1994) and the various fractions obtained were analysed by immunoblotting. Proteins equivalent to 3 μg of chlorophyll were analysed in each lane.

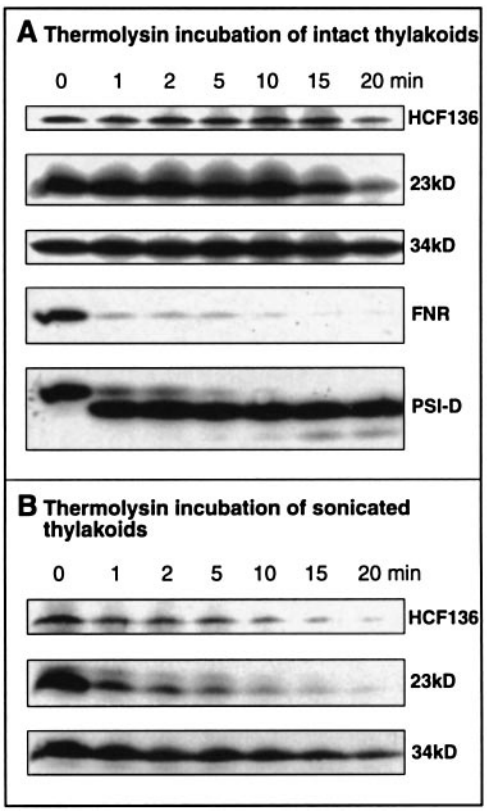

Fig. 9. Lumenal localization of *HCF136* protein by protease protection experiments. Intact thylakoid membranes from spinach (**A**) and a mixture of right-side and inside-out thylakoid vesicles (**B**) was incubated with thermolysin as described in Materials and methods. At the times indicated aliquots were removed and examined by immunoblot analysis. The 23 and 34 kDa proteins of the oxygen-evolving apparatus of PSII were used as lumenal marker proteins, while ferredoxin NADP oxidoreductase (FNR) and subunit D of PSI (PsaD) served as examples of thylakoid membrane proteins which are attached to the stromal site of that membrane.

mature *HCF136* protein form a hydrophobic patch, while the remainder of the protein is entirely hydrophilic. Fractionation of the thylakoid membranes into subdomains by aqueous two-phase partitioning (Wollenberger *et al.*, 1994) revealed further that the *HCF136* protein is located in stromal, and not in granal, thylakoids (Figure 8C).

The above experiments did not allow us to deduce at which site of the thylakoids the *HCF136* protein is located. To obtain this information protease digestion experiments were carried out. Thylakoid membranes were isolated as gently as possible and incubated with thermolysin, a protease that is commonly used in *in vitro* import experiments to probe the translocation of proteins through membranes (Cline *et al.*, 1984). Figure 9A shows that the *HCF136* protein, like the 23 and 34 kDa lumenal subunits of PSII, is protected from protease treatment. As expected, ferredoxin NADP oxidoreductase and the PsaD subunit of PSI, both of which are attached to the stromal site of the thylakoids, are rapidly degraded by thermolysin. When inside-out thylakoid vesicles were produced by sonication and the mixture of outside-out and inside-out vesicles was incubated with thermolysin, both the *HCF136* and the two lumenal PSII subunits were no longer resistant to protease digestion (Figure 9B). It follows that the *HCF136* protein has a similar accessibility to thermolysin as the 23 and 34 kDa extrinsic PSII subunits. Hence, it should be located in the same compartment, i.e. the thylakoid lumen. A lumenal location of the *HCF136* protein can also be inferred from the bipartite structure of the transit peptide

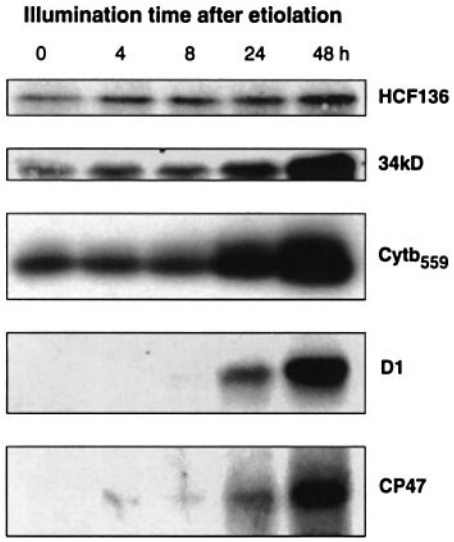

Fig. 10. Comparison of *HCF136* protein accumulation and PSII biogenesis during light-induced greening of etiolated *Arabidopsis* seedlings. *Arabidopsis* seeds were germinated for 1 day under normal light conditions and then grown for 5 days in darkness. After etiolation seedlings were illuminated for 48 h with continuous white light. Seedlings were harvested at the times indicated and processed for immunoblot analysis. Protein loadings were based on equal amounts of seedling fresh weight.

(Figure 7) which is similar to that of other lumenal proteins (Robinson and Mant, 1997).

The HCF136 protein accumulates in dark-grown Arabidopsis seedlings

The mutational analysis had indicated that the *HCF136* protein is absolutely required for the stable accumulation of all PSII subunits that were investigated in this study. This suggested that the *HCF136* protein should be available in plastids when the PSII complex is built up. To test this inference, the biogenesis of PSII was investigated during light-induced greening of etiolated *Arabidopsis* seedlings and compared with the accumulation of the *HCF136* protein.

The pigment-binding PSII subunits D1 and CP47 cannot be detected in etiolated seedlings, they appear after ~24 h of illumination (Figure 10). In contrast, cytochrome b_{559} and the 34 kDa PSII subunit accumulate in the dark, but there is a further light-induced increase in steady-state levels in parallel to that of D1 and CP47. The biogenesis of PSII in *Arabidopsis* is thus comparable with that of spinach and shows the same stepwise accumulation of its subunits (Westhoff *et al.*, 1990). The accumulation pattern of the *HCF136* protein is similar to that of cytochrome b_{559} and the 34 kDa protein, i.e. the protein accumulates already in dark-grown seedlings. *HCF136* protein levels increase after prolonged periods of illumination, but this light-induced increase is much less pronounced than that of cytochrome b_{559} and the 34 kDa PSII subunit. Taken together these data suggest that the *HCF136* protein has to be present prior to, or at least concomitantly with, the accumulation of the core subunits of the PSII complex. Thus, both the mutational and the biogenetic analyses point to the *HCF136* protein as being a central component for the assembly and/or stability of PSII.

Discussion

Considerable progress has been made in recent years in identifying and defining nuclear genes which regulate the biogenesis of the photosynthetic machinery in plastids of algae and higher plants. These regulatory genes may act at the transcriptional and post-transcriptional levels of the gene expression pathways in the organelle and the nucleo-cytosolic compartment. Alternatively, they may be required for the translocation of the photosynthetic proteins into and within the plastids or for their final assembly into the multisubunit complexes that are characteristic for photosynthetic electron transfer and ATP production. In this report we describe the identification and molecular characterization of a novel nuclear-encoded gene that is essential for the biogenesis of PSII in *A.thaliana* and traces back its origin to the prokaryotic endosymbiont that gave rise to the plastids of photosynthetic eukaryotes.

Homozygous *hcf136* plants are devoid of any functional PSII, which in turn is due to the complete lack of all core subunits of PSII as well as of those of the oxygen-evolving complex. The loss of the PSII complex has profound effects on the organization of the thylakoid membrane into grana and stroma thylakoids as has also been found for other PSII-deficient mutants (Simpson *et al.*, 1989). The mutation does not affect the accumulation of the light-harvesting complex of PSII, the cytochrome b_6f complex and the chloroplast ATP synthase, but results in reduced amounts of PSI. The spectroscopic analyses indicate that the PSI complexes accumulating in *hcf136* are functional. Since it is often observed that mutations in PSII and the cytochrome b_6f complex (Meurer *et al.*, 1996b) secondarily lead to deficiencies in PSI, it is tempting to conclude that the *hcf136* mutation does not affect PSI primarily, and hence *hcf136* should be classified as a PSII-specific mutation.

The *hcf136* mutation is accompanied by a complete loss, or at least a drastic reduction, of all core subunits of PSII as well as of the 34 and 23 kDa proteins of the oxygen-evolution complex. Two PSII mutants of *Chlamydomonas reinhardtii*, *nac2* (Kuchka *et al.*, 1989) and FuD44 (Mayfield *et al.*, 1987) show similar strong deficiencies in PSII subunit levels. However, it is not known whether these mutants, like *hcf136*, are also depleted in cytochrome b_{559}. The absence of all PSII core subunits in *hcf136*, as well as of the 34 and 23 kDa proteins of the oxygen evolving complex, suggests that the *HCF136* protein functions at a very central step in the biogenesis of the entire PSII complex.

The presence of a plastidial transit peptide as well as the evolutionary origin of *HCF136* indicates that the *HCF136* protein is a chloroplast protein. This inference was confirmed by cell fractionation and immunoblot experiments. These experiments revealed furthermore that the *HCF136* protein is a lumenal protein. The finding is consistent with the presence of a stretch of amino acids in the putative transit sequence that is reminiscent of a lumen targeting domain (Robinson and Mant, 1997). The *HCF136* protein could almost be completely removed from the membrane by alkaline washing, suggesting that the protein is attached to the membrane. Hydropathy analyses (data not shown) support this view. The mature protein contains a short hydrophobic patch of 18 amino

acids just at the N-terminus, but the remainder is hydrophilic with no evidence of membrane-spanning segments (Figure 7). Fractionation of the thylakoid membranes demonstrated that the *HCF136* protein is restricted to the stromal lamellae, is not found in grana cores, and is present only in small amounts in grana margins.

Both *HCF136* mRNA and protein accumulate to low levels, suggesting that the *HCF136* protein is not a stoichiometric component of the mature PSII complex. The localization of the *HCF136* protein to the stroma lamellae but not to the grana where the majority of PSII resides (Guenther and Melis, 1990) supports this conclusion. Although the *HCF136* protein is apparently not a constituent component of mature PSII complexes, it cannot be ruled out that the protein is attached to PSII complexes in the stroma lamellae that are in *statu nascendi* or being repaired after photoinhibitory damage (Guenther and Melis, 1990). Co-immunoprecipitation experiments of cross-linked proteins or protein–protein interaction analyses with the yeast two-hybrid system (Brent and Finley, 1997) may be suitable experimental approaches to tackle this question.

In vivo labelling experiments demonstrated that at least the plastome-encoded PSII subunits CP47 (PsbB), CP43 (PsbC), D1 (PsbA), D2 (PsbD), and PsbH are synthesized in the mutant background at approximately wild-type levels, although they are not stable. This indicates that the *HCF136* protein does not affect the translation of the above proteins but is required for their stable accumulation. Since the 34 and 23 kDa proteins of the oxygen-evolving complex and cytochrome b_{559} also fail to accumulate in the mutant background, one may conclude that the *HCF136* protein is essential for the stability and hence accumulation of all PSII subunits.

As far as the mechanism of function is concerned, several possibilities are conceivable. Although the *HCF136* protein is not necessary for the translation of CP47, CP43, D1, D2 and PsbH, it could be required for the translation of other plastome-encoded PSII subunits, i.e. PsbI, PsbK, PsbM, PsbN or PsbT. The loss of these PSII subunits in turn could result in the instability of the other PSII subunits and hence explain the observed phenotype of *hcf136*. However, the available mutant data do not support this possibility (Monod *et al.*, 1994; Takahashi *et al.*, 1994; Künstner *et al.*, 1995).

The clustering of the *HCF136* homologue with *psbE-F-L-J* in *Synechocystis* and *Cyanophora* suggests another explanation, that the *HCF136* protein is functionally connected to the biogenesis of these proteins, particularly to that of cytochrome b_{559}, a complex of the PsbE and PsbF proteins (Cramer *et al.*, 1993). The greening studies with etiolated *Arabidopsis* seedlings support this view. They show that the *HCF136* protein and cytochrome b_{559} accumulate with similar kinetics. The two proteins are present in etiolated seedlings, but their steady-state levels increase after ~24 h illumination. Thus, both the mutational data and the biogenesis studies suggest that the *HCF136* protein has to be present before cytochrome b_{559} can accumulate. Since gene inactivation experiments in *Synechocystis* PCC6803 (Pakrasi *et al.*, 1989, 1991) have shown that cytochrome b_{559} is essential for the biogenesis of the PSII complex, a lack of the *HCF136* protein and hence the absence of cytochrome b_{559} would explain the PSII-less mutant phenotype.

The greening studies show also that the *HCF136* protein accumulates in parallel with the 34 kDa protein of the oxygen-evolving apparatus. Mutational inactivation of this latter protein leads to a PSII deficiency (Mayfield *et al.*, 1987). Therefore, it is conceivable that the *HCF136* protein is needed for the assembly of the 34 kDa protein. The lumenal location of the *HCF136* protein is in line with this suggestion.

Regardless of which PSII subunit is the primary target of the *HCF136* protein, all available evidence indicates that this protein behaves as a PSII-specific stability and/or assembly factor. Protein complex-specific stability or assembly factors are becoming increasingly uncovered in eubacteria and their evolutionary descendents, the mitochondria and chloroplasts. The respiratory nitrate reductase complex of *E.coli*, a membrane-bound electron transfer complex that is composed of three subunits, needs a private chaperone in order to become assembled (Liu and DeMoss, 1997). In mitochondria, several factors have been identified that are required for the assembly of the respiratory electron transport complexes and the ATP synthase (Tzagoloff *et al.*, 1994; Glerum *et al.*, 1995, 1997; Altamura *et al.*, 1996). Some of these mitochondrial factors belong to a family of membrane-associated energy-dependent proteases that also act as chaperones during protein assembly (Suzuki *et al.*, 1997). Finally, in cyanobacteria and *Chlamydomonas* PSI-specific factors have been detected that are necessary for the stability or assembly of this photosystem (Wilde *et al.*, 1995; Bartsevich and Pakrasi, 1997; Boudreau *et al.*, 1997). To our knowledge, *HCF136* is the first PSII-specific accumulation factor that has been identified and molecularly characterized in photosynthetic eukaryotes. Its homologues in cyanobacteria and in the plastome of *C.paradoxa* reflect the prokaryotic origin of this gene. *HCF136* and its prokaryotic homologues do not reveal any similarities to proteins of known function. Hence, the mechanism of action of these evolutionarily conserved proteins remains unclear at present.

Materials and methods

Growth conditions and mutant selection

Surface-sterilized seeds of *A.thaliana* were sown on Petri dishes containing 0.3% (w/v) solidified Gelrite (Roth, Karlsruhe, Germany) supplemented with 0.5× MS nutrients (Murashige and Skoog, 1962) and 2% (w/v) sucrose. In mutant selection experiments kanamycin was added to a final concentration of 70 mg/l. Seed germination was synchronized by placing the plates after sowing for 48 h at 4°C. Seedlings were grown with a 16 h photoperiod at a photon flux density (PFD) of ~20–50 μmol/m^2/s and at a constant temperature of 23°C.

The *hcf136* mutation was identified in a collection of T-DNA insertion mutants (Feldmann, 1991). Mutants exhibiting the high chlorophyll fluorescence phenotype were initially selected in the dark under a strong- and long-wavelength UV light (Bachofer, Reutlingen, Germany) as described elsewhere (Miles, 1982; Meurer *et al.*, 1996b). Since *hcf136* seedlings were found to be lethal, the mutation was maintained in the heterozygous state. For production of seeds, heterozygous seedlings were selected with kanamycin and then grown to maturity on soil in continuous light at 80 μmol/m^2/s using the Arasystem (Beta Tech, Gent, Belgium).

Spectroscopic analyses

Chlorophyll *a* fluorescence measurements were performed with 3-week-old plants using the pulse amplitude modulated fluorometer PAM 101/

103 (Walz, Effeltrich, Germany; Meurer *et al.*, 1996b). For recording P700 absorbance changes, the PAM fluorometer was equipped with an ED 800 T emitter detector unit (Walz, Effeltrich, Germany) and the measurements were carried out as described (Meurer *et al.*, 1996b). Chlorophyll fluorescence changes at 77 K were measured with a fluorometer (F-3010; Hitashi, Tokyo, Japan) interphased with a personal computer for data collection (Weis, 1985) as described in Meurer *et al.* (1996a).

Transmission electron microscopy

Leaves from 3-week-old mutant and wild-type plants which had been cultivated on sterile media were cut into 1 mm pieces and simultaneously fixed in 2.5% glutaraldehyde and 1% OsO_4 in 0.05 M cacodylate buffer pH 7.0, for 2 h at 4°C. Dehydration in graded ethanol series and embedding in Epon 812 followed standard procedures. Ultrathin sections were cut with a diamond knife and mounted on single-hole grids covered with Pioloform/Formvar. They were double-stained with 1% aqueous uranyl acetate (30 min) followed by lead citrate (5–10 min) and viewed under a Philips electron microscope EM 301.

Polyacrylamide gel electrophoresis and immunoblotting

Electrophoresis of crude leaf proteins (Meurer *et al.*, 1996b) in sodium dodecyl sulfate polyacrylamide gels was carried out according to Schägger and von Jagow (1987). The separated proteins were visualized by silver-staining (Blum *et al.*, 1987). Radiolabelled proteins were detected by fluorography (Laskey, 1980). Proteins to be analysed by immunodecoration were transferred to nitrocellulose membranes (PH 79, 0.1 μm; Schleicher and Schüll, Dassel, Germany), incubated with specific antibodies (Meurer *et al.*, 1996b), and visualized by the enhanced chemiluminescence technique as described by the manufacturer (Amersham Buchler, Braunschweig, Germany).

In vivo labelling of chloroplast proteins

Primary leaves of 12-day-old mutant and wild-type seedlings were vacuum-infiltrated in 50 μl reaction medium containing 1 mM KH_2PO_4 pH 6.3, 0.1 % (w/v) Tween 20, 50 μCi [^{35}S]methionine (specific activity >1000 Ci/mmol; Amersham Buchler, Braunschweig, Germany) and 20 μg/ml cycloheximide. After 15 min incubation at 25°C in ambient light the leaves were washed twice with 500 μl [20 mM KH_2PO_4, 10 mM dithiothreitol (DTT)]. To isolate thylakoid membranes, the leaves were ground with a conical stainless steel rod fitting into the bottom of an Eppendorf tube, and the homogenate was then centrifuged for 10 min at 15 000 r.p.m. The pelleted membranes were washed once and finally resuspended in 100 mM Na_2CO_3, 10% (w/v) sucrose and 50 mM DTT. Trichloroacetic acid-insoluble radioactivity was measured according to Mans and Novelli (1961).

RNA gel blot analysis

Northern analysis of total leaf RNA by using DNA or RNA probes was carried out as described (Westhoff *et al.*, 1991; Meurer *et al.*, 1996b). Strand-specific RNA probes for the hybridization of plastid transcripts were obtained by run-off transcription using the appropriate phage RNA polymerases (Westhoff *et al.*, 1991), while the probes for nuclear RNAs were labelled by random priming of the corresponding DNA fragments (Meurer *et al.*, 1996b). The hybridization probes used are listed in Meurer *et al*. (1996b) and Kubicki *et al*. (1996).

Inverse PCR

Genomic sequences flanking the T-DNA right border were amplified by using inverse PCR (Ochman *et al.*, 1993). Genomic DNA of *hcf136* was isolated from frozen tissue (Dellaporta *et al.*, 1983) and digested with the enzymes *Hha*I or *Ssp*I. After self-ligation and digestion with *Sac*II, genomic sequences were amplified by PCR following standard protocols including 20 μM of each primer: R1, 5′-ATG AGC TAA GCA CAT ACG TCA G-3′; R2, 5′-GCG CGT TCA AAA GTG GCC TA-3′; F1, 5′-GTG GCT CCT TCA ATC GTT GCG G-3′; F2, 5′-CTT GTC GTT TCC CGC CTT CAG-3′. PCR amplification of the *Ssp*I-digested DNA with primers F1/R1 resulted in a 1280 bp product, while amplification of the *Hha*I-digested DNA with the same primers yielded a 2150 bp DNA fragment. The amplified products were cloned into the *Eco*RV site of Bluescript II KS+ (Stratagene, La Jolla, USA) resulting in clones pnAt136-1 (containing the 1280 bp fragment) and pnAt136–2 (containing the 2150 bp fragment). Both clones were sequenced.

Cloning and sequence analysis of HCF136 cDNA

A cDNA library prepared from RNA of the aerial parts of *Arabidopsis* plants, ecotype Columbia (supplied by the E.U. Stock Center, Max-Planck-Institut für Züchtungsforschung, Köln, Germany) was screened with a 530 bp *Dra*I genomic restriction fragment, which was excised from the cloned inverse PCR product and located adjacent to the right border of the inserted T-DNA. Approximately 1.5×10^6 plaques were analysed under stringent hybridization conditions, resulting in 94 positive signals. Twenty-two phages were purified by plaque hybridization. Eight of them were confirmed as positive and partially sequenced. The largest clone identified, pcAt136-28, was completely sequenced on both strands (Chen and Seeburg, 1985) using a nested set of unidirectional deletions (Henikoff, 1984) or *HCF136*-specific synthetic oligonucleotides. Nucleic acid and protein sequences were analysed with the programmes MacMolly Tetra (Softgene GmbH, Bocholt, Germany) and CLUSTAL W (Thompson *et al.*, 1994). The BLAST server (Altschul *et al.*, 1994) of the National Center for Biotechnology Information (Bethesda, MD) was used for database searches.

Complementation of the hcf136 mutant

The *HCF136* cDNA insert of pcAt136-28 was ligated into the plant expression vector pPCV91 (Strizhov *et al.*, 1996) under the control of the Cauliflower Mosaic Virus 35S promoter, and the construct was transformed into homozygous mutant plants using a root transformation protocol (Koncz *et al.*, 1994) with minor alterations: the roots were obtained from 2-week-old mutant seedlings grown in tissue culture, and the hygromycin concentration was reduced to 10 mg/l to allow the growth of transgenic tissue. Fourteen independent transgenic plants were recovered. They were easily distinguished from regenerated non-transformed *hcf136* mutants due to their fully green appearance. The successful complementation was confirmed by chlorophyll fluorescence measurements. In addition, the presence of the transgene within the complemented mutant seedlings was verified by PCR using two sets of primers that specifically allowed the amplification of *HCF136* cDNA fragments.

Antiserum production

The nucleotide sequence encoding the entire mature *HCF136* protein without the transit peptide part (nucleotide positions 264–1252 of pcAt136-28) was amplified by PCR. The 5′ and 3′ primers contained a *Bam*HI or a *Xho*I restriction site, respectively, for in-frame fusion with the glutathione *S*-transferase sequence of the pGEX-4T3 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The amplification product was restricted with *Bam*HI and *Xho*I, purified by agarose gel electrophoresis, and inserted into pGEX-4T3. The construct was introduced into the expression host *E.coli* strain BL21(DE3)pLysE by transformation, and expression was induced with 1 mM isopropylthio-β-D-galactoside. Recombinant clones expressing a protein of the expected size of 62 kDa were selected and a crude protein extract was prepared by sonicating the bacteria suspended in 50 mM Tris–HCl pH 7.9, 0.1 M NaCl, 0.1 mM EDTA, 0.01% (w/v) Triton X-100. The protein extract was clarified by centrifugation, and the supernatant was incubated with glutathione–Sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) at 4°C overnight. After extensive washing, the recombinant glutathione *S*-transferase *HCF136* fusion protein was cleaved with thrombin as described by the manufacturer (Amersham Pharmacia Biotech), and the released *HCF136* protein was purified by preparative polyacrylamide gel electrophoresis. A polyclonal antiserum was raised in rabbits by BioGenes (Berlin, Germany) and further purified by affinity chromatography on a *HCF136* protein column. The specificity of the antiserum was tested by immunoblotting with purified *HCF136* fusion protein and crude protein extracts were prepared from wild-type and mutant leaves.

Immunolocalization studies with protein gel blots

Intact chloroplasts were isolated from 2-week-old *Arabidopsis* wild-type plants or greenhouse-grown spinach by Percoll gradient centrifugation essentially as described (Meurer *et al.*, 1996a). Chloroplasts were lysed in ice-cold 10 mM HEPES–KOH pH 8.0, 10 mM $MgCl_2$, and stromal and membrane proteins were separated by 10 min centrifugation at 17 000 *g*. To test for the association of the *HCF136* protein with membranes Na_2CO_3 (200 mM final concentration) was added to the suspension of lysed chloroplasts, and stromal and membrane proteins were recovered by centrifugation as above.

Thylakoid and envelope membranes were separated from each other by rupturing intact chloroplasts by freezing and thawing in hypertonic medium, followed by sucrose density gradient centrifugation (Cline *et al.*, 1981). The two chloroplast membrane fractions were identified by their pigment content. Thylakoid membranes were fractionated

into stroma lamellae, grana cores and grana margins as described (Wollenberger *et al.*, 1994).

The intraorganellar location of the *HCF136* protein was further studied by protease protection experiments using thermolysin as a probe (Cline *et al.*, 1984). Thylakoid membrane proteins from spinach prepared as above were adjusted to a chlorophyll concentration of 50 μg/ml. A mixture of inside-out and outside-out thylakoid vesicles was prepared by sonicating thylakoid membranes for 45 s at 0°C. The sonifier used (Branson Sonifier B12, Danbury, USA) was equipped with a microtip and the output was set to 3. Thermolysin (0.1 mg/ml final concentration) was added to both membrane preparations and incubation was continued at room temperature. Aliquots were removed from the assays after varying times of incubation and the thermolysin activity was stopped by adding EDTA to a final concentration of 50 mM. Gel electrophoretic and immunoblot analysis of the protein samples was carried out as above.

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