

HCF164 Encodes a Thioredoxin-Like Protein Involved in the Biogenesis of the Cytochrome *b₆f* Complex in Arabidopsis

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To understand the biogenesis of the plastid cytochrome *b₆f* complex and to identify the underlying auxiliary factors, we have characterized the nuclear mutant *hcf164* of Arabidopsis and isolated the affected gene. The mutant shows a high chlorophyll fluorescence phenotype and is severely deficient in the accumulation of the cytochrome *b₆f* complex subunits. In vivo protein labeling experiments indicated that the mutation acts post-translationally by interfering with the assembly of the complex. Because of its T-DNA tag, the corresponding gene was cloned and its identity confirmed by complementation of homozygous mutant plants. *HCF164* encodes a thioredoxin-like protein that possesses disulfide reductase activity. The protein was found in the chloroplast, where it is anchored to the thylakoid membrane at its luminal side. *HCF164* is closely related to the thioredoxin-like protein TxIA of *Synechocystis* sp PCC6803, most probably reflecting its evolutionary origin. The protein also shows a limited similarity to the eubacterial CcsX and CcmG proteins, which are required for the maturation of periplasmic c-type cytochromes. The putative roles of *HCF164* for the assembly of the cytochrome *b₆f* complex are discussed.

INTRODUCTION

The cytochrome *b₆f* complex of the thylakoid membranes of higher plants takes a central position in photosynthetic electron transport. It functions as a plastoquinol-plastocyanin-oxidoreductase linking photosystems I and II (PSI and PSII). It also is involved in cyclic electron transport around PSI and acts as a proton translocase. The complex is composed of the four major subunits cytochrome *f* (*petA*), cytochrome *b₆* (*petB*), the Rieske-FeS protein (*petC*), and PetD (*petD*) and the four small subunits PetG, PetL, PetM, and PetN. A projection map at 8 Å resolution showed the presence of cytochrome *b₆f* complex dimers that are believed to be the functional form (Mosser et al., 1997).

The cytochrome *b₆f* complex consists of nucleus- and plastid-encoded subunits. Although the Rieske-FeS protein and PetM are encoded by the nucleus, all other subunits are encoded by the plastome. The biogenesis of the complex, therefore, requires coordinated gene expression in the nucleus and chloroplast. Several auxiliary and regulatory factors are necessary to ensure that the biogenesis of the

cytochrome *b₆f* complex proceeds in an ordered manner (reviewed by Rochaix, 1996; Wollman et al., 1999): factors that regulate processing and splicing of the plastome-encoded RNAs that are mostly transcribed in polycistronic transcription units (Barkan et al., 1994; Jenkins et al., 1997; Fisk et al., 1999); factors that control translation and the stoichiometric accumulation of the various subunits (Barkan, 1993; Kuras and Wollman, 1994; Choquet et al., 1998); and factors that target the proteins to their destinations (Voelker and Barkan, 1995a; Robinson and Mant, 1997). Finally, cofactors have to be attached to the cytochromes and the Rieske-FeS protein (Kuras et al., 1997; Xie et al., 1998), and the holosubunits have to be assembled into a functional complex.

There is convincing evidence that most of these factors are encoded in the nucleus. Nuclear mutants with defects in the cytochrome *b₆f* complex, therefore, can serve to identify such factors. These mutants have reduced or completely blocked electron transport and can be identified on the basis of their high chlorophyll fluorescence phenotype (*hcf*). This phenotype arises when the absorbed light energy cannot be used for photosynthesis and is emitted as red fluorescent light (Miles, 1994).

Here we report on the mutant *hcf164* of Arabidopsis, which was generated by T-DNA insertional mutagenesis (Bechtold et al., 1993) and which is deficient specifically in

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the biogenesis of the cytochrome *b₆f* complex. None of the major subunits of the complex accumulate, but they are synthesized. Therefore, the gene disrupted in this mutant encodes a factor that is essential for the assembly of the complex or its stability. The corresponding gene, *HCF164*, could be isolated because of its T-DNA tag. It encodes a thioredoxin-like protein that functions biochemically as a disulfide oxidoreductase. *HCF164* is located inside the chloroplast and is anchored to the thylakoid membrane at its luminal side. The protein is closely related to the thioredoxin-like protein TxlA of cyanobacteria. Additionally, there is limited homology with the eubacterial proteins CcsX of *Bordetella pertussis* and CcmG of *Escherichia coli* and other Gram-negative bacteria, both of which are involved in the maturation of periplasmic *c*-type cytochromes.

RESULTS

hcf164 Is Affected in the Intersystem Electron Transport Chain

The *hcf164* mutant of *Arabidopsis* ecotype Wassilewskija was generated by T-DNA insertional mutagenesis (Bechtold et al., 1993) and selected from a collection of the Institute National de la Recherche Agronomique (Versailles, France). *hcf164* segregated as a single recessive mutation. Cosegregation with the phosphinotricin resistance marker of the T-DNA indicated that the mutation was tagged by the T-DNA insertion. The mutant plants showed an *hcf* phenotype and were not able to grow photoautotrophically. They could be maintained, however, on sucrose-supplemented medium, but they did not develop any flowers.

Because the *hcf* phenotype indicated decreased photosynthetic electron transport rates, we measured chlorophyll fluorescence induction (Figure 1A). The ratio (F_v/F_m) of variable fluorescence ($F_v = F_m - F_o$; where F_v is variable fluorescence, F_m is maximum fluorescence, and F_o is minimal fluorescence) to maximum fluorescence in *hcf164* ($F_v/F_m = 0.76 \pm 0.02$) was comparable to that in wild type ($F_v/F_m = 0.79 \pm 0.01$). This revealed the presence of an active PSII in *hcf164*. In contrast, photochemical quenching ($qP = [F_m' - F_s]/[F_m' - F_o']$; where F_s is steady-state fluorescence) was drastically reduced in *hcf164* ($qP = 0.16 \pm 0.02$) compared with wild-type plants ($qP = 0.88 \pm 0.03$), suggesting that the electrons released by PSII accumulated in the plastoquinone pool and were not transported downstream of the photosynthetic electron transport chain. This could be attributable to a defect in intersystem electron transport or in PSI. To distinguish between these two possibilities, we studied PSI function by measuring the absorbance kinetics of P700 at 830 nm (Figure 1B). A clear absorbance change induced by far-red light similar to that in wild-type indicated that PSI was functional in *hcf164*. Application of a white light pulse in far-red background led to a short re-reduction of

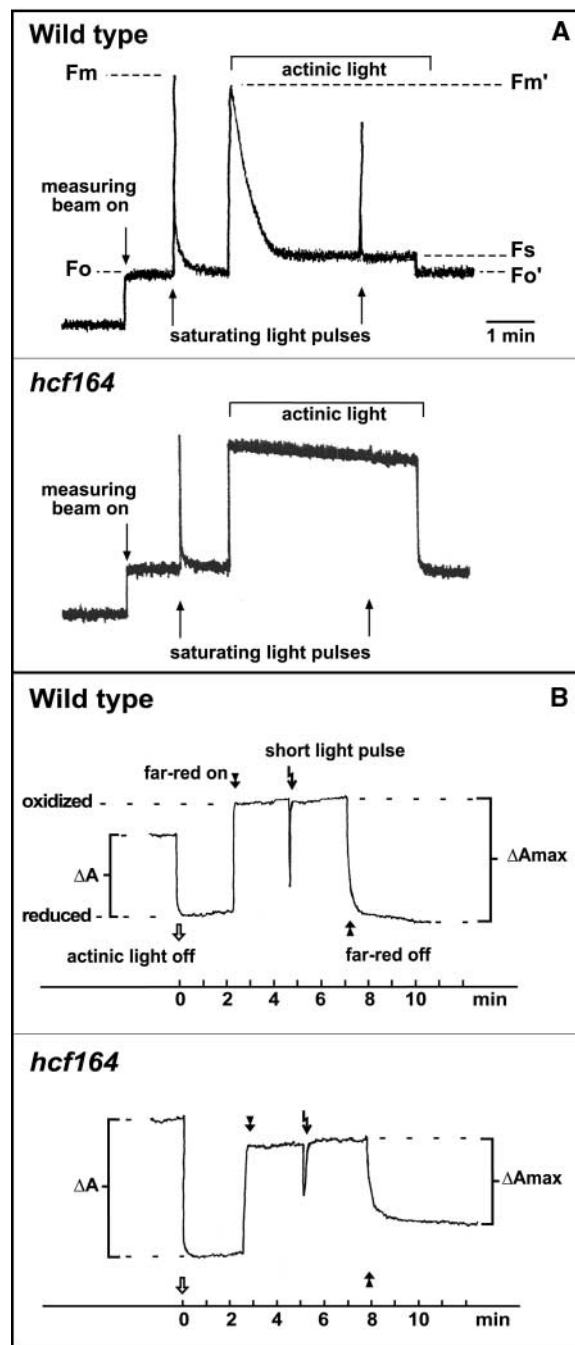


Figure 1. Spectroscopic Analyses of *hcf164* Mutants and Wild-Type Plants.

Measurements of chlorophyll fluorescence induction (A) and P700 absorbance kinetics (B) were performed with 2-week-old plants as described in Methods.

PSI by electrons released from PSII. This re-reduction was diminished in *hcf164*. Together, the spectroscopic data indicate that the defect is located in the intersystem electron transport chain and not in one of the two photosystems.

Subunits of the Cytochrome b_6f Complex Do Not Accumulate in *hcf164*

A block in the intersystem electron flow could be the result of a defect in the cytochrome b_6f complex. To address this possibility, we assayed thylakoid membranes of wild-type and mutants for the presence of cytochrome f and cytochrome b_6 holoproteins by virtue of their heme-associated peroxidase activities. Figure 2A shows that cytochrome f , with its covalently bound heme, can be detected clearly even when only 2.5 μg of total chloroplast proteins was analyzed. The peroxidase activity of cytochrome b_6 is drastically lower; therefore, at least 5 μg of chloroplast proteins have to be assayed. Figure 2A reveals that in mutant thylakoids, both proteins are below the level of detection—that is, their amounts are significantly less than 12.5% (cytochrome f) and 25% (cytochrome b_6) of the wild-type levels analyzed.

To measure the steady state levels of the subunits of the cytochrome b_6f complex directly, immunoblot analyses were performed. Under the experimental conditions used, the antisera to the subunits cytochrome f , the Rieske-FeS protein, and PetD detected less than 12.5% of the wild-type level in *hcf164* (Figure 2B). Cytochrome b_6 accumulated to amounts between 12.5 and 25%. These data indicate that the accumulation of the cytochrome b_6f complex is impaired considerably in the mutant.

In parallel, we analyzed the protein levels of the two photosystems. Representative PSI polypeptides (PsaA/B and PsaD) were reduced to 30 to 50% of wild-type amounts. For PSII representatives, we tested CP47, D2, and cytochrome b_{559} . These polypeptides were reduced to 50 to 70% in *hcf164* (Figure 2C). In contrast, when plants were grown under very low light intensities (PPFD = 5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$), neither PSI nor PSII subunits were reduced in *hcf164* mutants, whereas the levels of the subunits of the cytochrome b_6f complex still were diminished (data not shown). This suggests that the reduction in PSI and PSII subunits most likely are a secondary effect of the mutation.

We conclude from the spectroscopic analyses, the heme staining, and the immunoblot experiments that the *hcf164* mutation affects primarily the cytochrome b_6f complex and that this complex as a whole is reduced significantly in mutant thylakoids.

Plastome-Encoded Subunits of the Cytochrome b_6f Complex Are Synthesized in *hcf164*

To determine whether the reduced amounts of polypeptides of the cytochrome b_6f complex are caused by impaired RNA

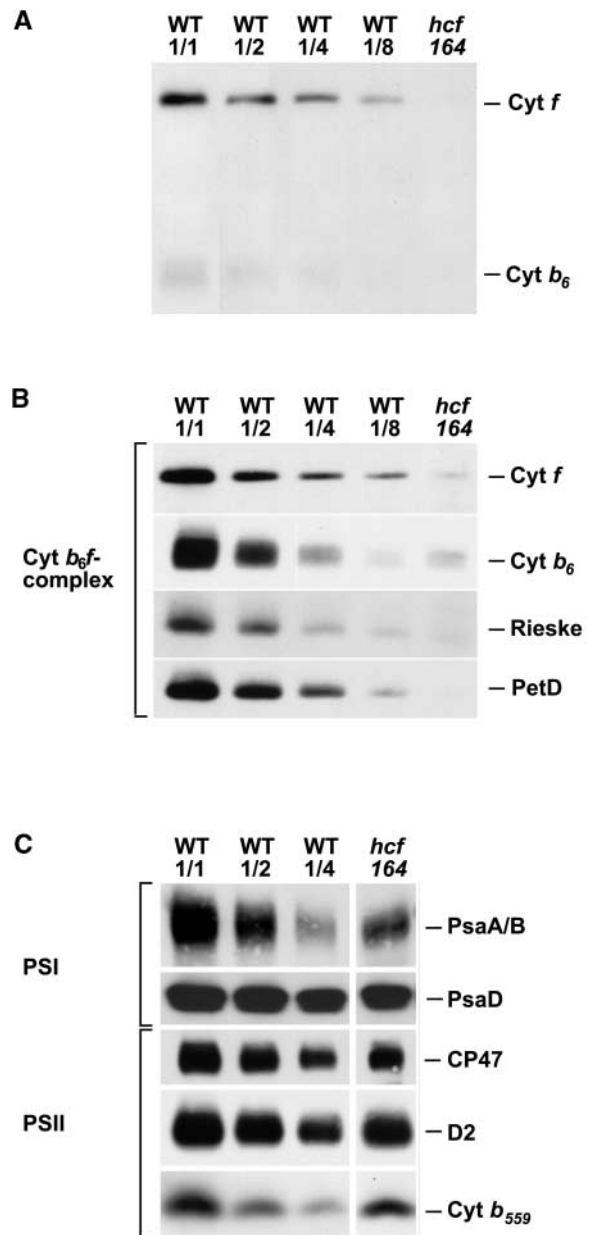


Figure 2. Analysis of Photosynthetic Membrane Proteins from the Mutant *hcf164* and the Wild-Type (WT).

Twenty micrograms (*hcf164*, WT1/1), 10 μg (WT1/2), 5 μg (WT1/4), and 2.5 μg (WT1/8) of chloroplast proteins were size separated by SDS-PAGE and electroblotted onto nitrocellulose membranes.

(A) Detection of the heme binding proteins cytochrome f and cytochrome b_6 by enhanced chemiluminescence.

(B) Immunoblot analysis of the cytochrome b_6f subunits cytochrome f , cytochrome b_6 , the Rieske-FeS protein (Rieske), and PetD.

(C) Immunodetection of the subunits PsaA/B and PsaD of PSI, the inner antenna protein CP47, and the reaction center proteins D2 and cytochrome b_{559} of PSII.

Cyt, cytochrome.

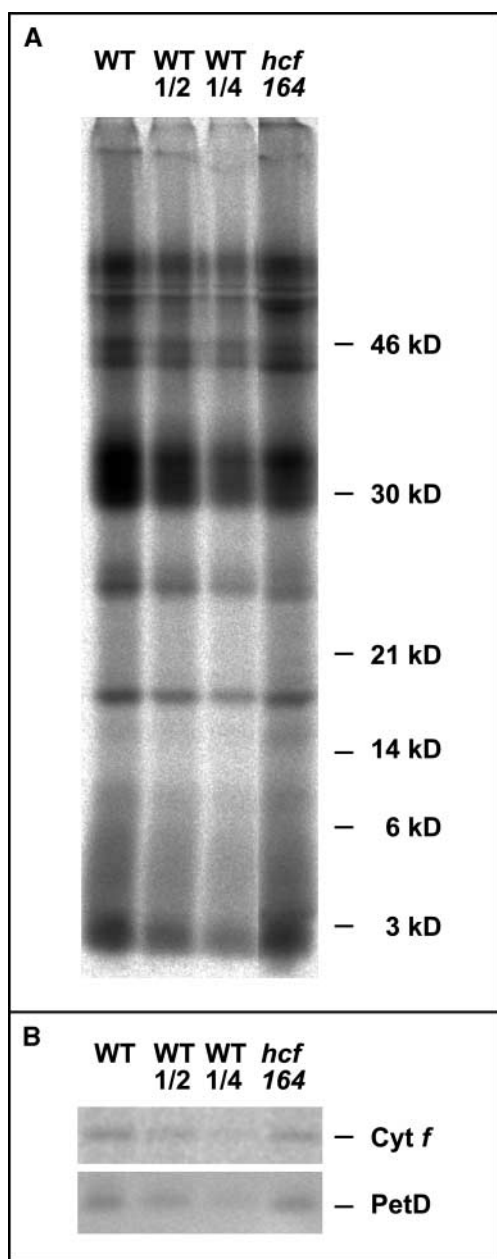


Figure 3. In Vivo Protein Synthesis in *hcf164* Mutants and Wild-Type (WT) Plants.

Primary leaves of 15-day-old plants were radiolabeled with ^{35}S -methionine for 30 min.

(A) Membrane proteins were isolated, separated by SDS-PAGE, and analyzed by autoradiography as described in Methods. Proteins equivalent to 100,000 incorporated cpm or the indicated dilutions of wild-type were loaded in each lane.

(B) Membrane proteins were solubilized, and the protein equivalent of 200,000 incorporated cpm was used for immunoprecipitation with antisera against cytochrome *f* (Cyt *f*) and the PetD subunit. Lanes WT and *hcf164* were loaded with 50% of the appropriate immunoprecipitation reaction, and lanes WT1/2 and WT1/4 were loaded with

accumulation, RNA gel blot hybridizations with probes for the four major subunits of the cytochrome *b₆f* complex were performed. The plastid-encoded genes *petA* (cytochrome *f*), *petB* (cytochrome *b₆*), and *petD* (PetD) are transcribed in polycistronic transcription units resulting in complex RNA patterns (Barkan, 1988; Westhoff and Herrmann, 1988; Gray, 1992). In contrast, the nucleus-encoded *petC* (Rieske-FeS protein) is transcribed into a monocistronic transcript. No differences in transcript patterns and accumulation were observed between mutant and wild-type, indicating that the processes of transcription, RNA processing, and RNA stability are not affected by the mutational lesion (data not shown).

To determine whether the impaired accumulation of the subunits of the cytochrome *b₆f* complex is caused by decreased translation or protein stability, the rates of synthesis of chloroplast-encoded thylakoid membrane proteins were studied by pulse labeling of mutant leaves with ^{35}S -methionine in the presence of cycloheximide as an inhibitor of cytoplasmic translation.

Figure 3A shows that the protein labeling patterns in mutant and wild-type plants were similar. Because the subunits of the cytochrome *b₆f* complex could not be identified easily in the protein patterns, immunoprecipitation experiments were performed. After 30 min of labeling, membrane proteins were extracted from the leaves and individual subunits of the cytochrome *b₆f* complex were immunoprecipitated using specific antisera raised against cytochrome *f*, cytochrome *b₆*, and PetD. To ensure a quantitative immunoprecipitation, a surplus of the corresponding antibodies was used in the assay and, as a control, the supernatants from the first immunoprecipitation were subjected to a second round of immunoprecipitation. These experiments revealed that all proteins could be precipitated quantitatively from wild-type and mutant extracts in the first round of immunoprecipitation (data not shown).

Gel electrophoretic analysis of the immunoprecipitates showed that both cytochrome *f* and PetD are synthesized in the mutant plants (Figure 3B). Only one band of cytochrome *f* could be precipitated from the mutant protein extracts. Its apparent molecular weight was indistinguishable from that obtained with wild-type extracts, suggesting that in the mutant the precursor of cytochrome *f* is processed correctly at the N-terminus (Gray, 1992). The antiserum against cytochrome *b₆* was not able to immunoprecipitate radiolabeled protein; thus, the synthesis of this subunit could not be analyzed. The quantitative analysis of the immunoprecipitates by phosphorimaging revealed that the accumulation of radioactive label in cytochrome *f* and PetD is comparable in wild-type and *hcf164* mutant plants (Figure 3B). We conclude, therefore, that the *hcf164* mutation does not affect primarily

25 and 12.5% of the WT precipitation, respectively. For experimental details, see Methods.

the synthesis of the subunits of the cytochrome *b₆f* complex but rather leads to an increased rate of their degradation.

Molecular Cloning of *HCF164*

Cosegregation of the *hcf164* mutation with the phosphino-tricin resistance marker of the T-DNA indicated the presence of a single T-DNA tagging the mutated gene. This was confirmed by DNA gel blot analysis (data not shown). To isolate the genomic sequences flanking the left border of the T-DNA, inverse polymerase chain reaction (PCR) was used (Ochman et al., 1993). The amplified sequences were cloned, sequenced, and subjected to database searches. One hundred percent identity was found between the isolated PCR fragment and a putative gene (GenBank accession number CAB16778) on chromosome IV of *Arabidopsis*. This sequence information was used to design primers, and an *HCF164* cDNA was amplified by reverse transcriptase-mediated PCR. The cDNA clone contained an open reading frame of 783 nucleotides. The ATG start codon is preceded by a stop codon, indicating that the isolated cDNA harbors the complete *HCF164* coding sequence. In the mutant line, the T-DNA insertion maps 34 bp upstream of the ATG start codon.

To study the effect of the T-DNA insertion on *HCF164* expression, the *HCF164* cDNA was used as a probe in RNA gel blot hybridization. Figure 4 shows that in wild-type RNA, a single transcript of 1.2 kb was present. This *HCF164* transcript still could be detected in heterozygous plants, al-

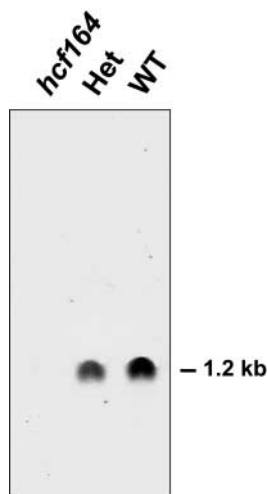


Figure 4. *HCF164* Transcript Analysis of Wild-Type, Heterozygous, and Homozygous *hcf164* Mutant Plants.

Ten micrograms of poly(A⁺) RNA of 3-week-old wild-type (WT), heterozygous (Het), and homozygous *hcf164* mutant plants was separated on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled *HCF164* cDNA probe.

though at lower levels, but it was completely absent in RNA from homozygous mutant plants.

To prove that the *HCF164* gene disruption was responsible for the mutant phenotype, the isolated cDNA was used for complementation analysis. The *HCF164* cDNA was fused to the 35S promoter of the *Cauliflower mosaic virus* in the plant transformation vector pPCV91 (Strizhov et al., 1996), and roots of homozygous *hcf164* mutants were transformed by means of *Agrobacterium tumefaciens* (Koncz et al., 1994). Forty-five independent transgenic plants were regenerated. They were able to grow photoautotrophically, and their chlorophyll fluorescence induction kinetics were indistinguishable from those of wild-type plants (data not shown). We conclude from this complementation experiment that the T-DNA insertion in the *HCF164* gene is responsible for the mutant phenotype and that the isolated cDNA encodes a functional HCF164 protein.

HCF164 Encodes a Thioredoxin-Like Protein

The *HCF164* cDNA encodes a protein of 261 amino acids with a predicted molecular mass of 29 kD. Hydropathy analysis revealed that the protein is composed of three domains: an N-terminal hydrophilic domain up to amino acid 97; a hydrophobic part from amino acids 98 to 113; and a large C-terminal hydrophilic part from amino acids 114 to 261 (data not shown). The first two domains exhibit typical features of plastid transit peptides of luminal proteins, suggesting that the HCF164 protein is localized in the chloroplast. The C-terminal hydrophilic part contains a WCxxC motif (boxed in Figure 5), which is a typical signature of the active site of thioredoxins. Database searches and protein sequence alignments revealed that this segment of the HCF164 protein exhibits significant similarities to thioredoxins, thioredoxin-like proteins, and protein disulfide isomerases from various organisms. The strongest similarity was observed with the thioredoxin-like protein TxIA of *Synechocystis* sp PCC6803 (44% identity, 59% similarity) and its homologs of *Synechococcus* sp PCC7249 (38% identity, 57% similarity) and *Anabaena* sp PCC7120 (33% identity, 53% similarity) (Figure 5). In addition, we noted a limited sequence similarity between HCF164 and the thioredoxin-like proteins CcsX of *B. pertussis* (Beckett et al., 2000) (15% identity, 28% similarity) and CcmG of *E. coli* (15% identity, 32% similarity) and its homologs in other Gram-negative bacteria (Beckman and Kranz, 1993; Vargas et al., 1994; Fabianek et al., 1997, 1998). CcsX and CcmG are periplasmic proteins that are involved in the maturation of c-type cytochromes (Fabianek et al., 1998; Beckett et al., 2000).

Recombinant HCF164 Protein Exhibits Disulfide Reductase Activity in Vitro

To determine whether the HCF164 protein shows disulfide reductase activity, insulin reduction assays were performed.



Figure 5. Amino Acid Sequence Alignment of HCF164 and Thioredoxin-Like Proteins.

Multiple alignment of the amino acid sequences of HCF164 and the Tx1A homologs of *Synechocystis* sp PCC6803 (accession number P73920), *Synechococcus* sp PCC7942 (accession number P35088), and *Anabaena* sp (Cyanobase, Kazusa Research Institute, Kisarazu, Japan). Identical amino acids are marked with asterisks, and conserved exchanges are marked with dots. The thioredoxin motif is boxed, and the hydrophobic region is shaded.

Such assays are used commonly to test proteins for thioredoxin activities (Holmgren, 1979). In the presence of DTT, thioredoxin reduces the intermolecular disulfide bonds between the insulin A and B chains, and as a consequence, the insoluble B chain precipitates. The assay was performed with recombinant HCF164, which lacked the N-terminal segment up to amino acid 115. Figure 6 shows that recombinant HCF164 was able to accelerate the reduction of insulin in the presence of DTT. Under the same assay conditions, purified *Spirulina* sp thioredoxin also catalyzed the insulin reduction. This demonstrates that HCF164 possesses disulfide reductase activity in vitro. However, compared with the purified thioredoxin, the disulfide reductase activity of HCF164 is 1 to 2 orders of magnitude lower.

HCF164 Is a Thylakoid Membrane Protein with Its Hydrophilic C-Terminal Part Exposed to the Lumen

The sequence analysis suggested that the HCF164 protein is a plastid protein to be targeted into the thylakoid lumen (Robinson and Mant, 1997): the protein starts with the motif MA and possesses a serine-rich region from amino acids 62

to 84, which is typical for plastid transit peptides. The luminal targeting is suggested by the presence of a twin arginine motif at amino acids 96/97, which is diagnostic for the ΔpH-dependent translocation pathway (Hynds et al., 2000), and furthermore by the hydrophobic domain of 16 amino acids directly after the twin arginines.

To determine the intracellular localization of HCF164, a polyclonal antiserum was raised against recombinant HCF164 protein (amino acids 85 to 261). The affinity-purified antiserum detected a 26-kD protein in wild-type leaf protein extracts (Figure 7A). No protein of that size could be detected in leaf protein extracts of the *hcf164* mutant, which was to be expected from the RNA gel blot analysis described above.

To prove the assumption that HCF164 is a chloroplast protein, intact chloroplasts were prepared from spinach and fractionated into envelopes and thylakoid membranes, and the protein fractions obtained were subjected to immunoblot analyses. Figure 7B demonstrates that HCF164 is detectable only in the thylakoid membrane and not in the envelope fraction. Washing of sonicated thylakoids with 0.25 M NaCl, 0.2 M Na₂CO₃, or 1 M CaCl₂ did not release HCF164 from the membrane, in contrast to the 23-kD ex-

trinsic PSII subunit that was used as a control (Figure 7C). This finding supports the conclusion that the HCF164 protein is firmly associated with the thylakoid membrane. The only hydrophobic part of HCF164 that could bind to the membrane is part of the transit peptide. We conclude, therefore, that this segment is still present in the mature HCF164 protein and serves as a membrane anchor of the otherwise hydrophilic protein.

To investigate whether the large hydrophilic part of HCF164 is located toward the stroma or the thylakoid lumen, thermolysin protection assays were performed (Figure 8). When intact thylakoids were incubated with thermolysin, the HCF164 protein remained unaffected for at least 5 min of incubation. The luminal 23-kD subunit of PSII showed the same protection, whereas the stromal ferredoxin NADP oxidoreductase was degraded within 1 min of incubation. In contrast, when sonicated thylakoids that contained ~50% right-side out and 50% inside-out vesicles were treated with thermolysin, the HCF164 protein and the 23-kD PSII subunit were no longer protected and thus were degraded rapidly within 1 min of incubation.

We conclude from these data that the HCF164 protein is inserted into the thylakoid membrane via its N-terminal hydrophobic segment and that the C-terminal hydrophilic part that harbors the thioredoxin motif extends into the thylakoid lumen.

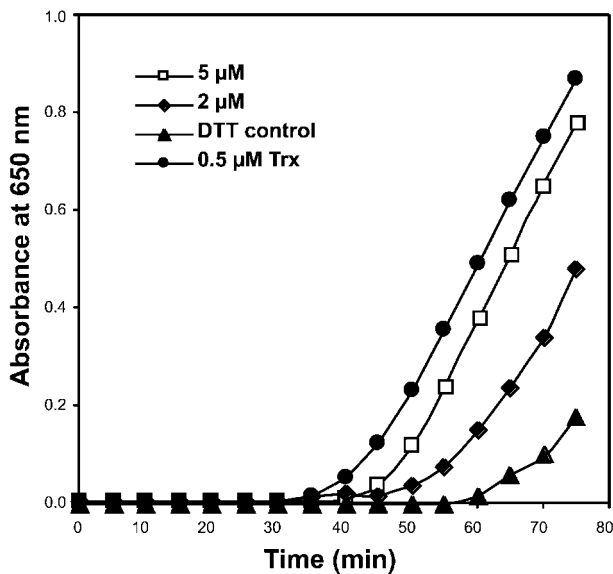


Figure 6. Measurement of Disulfide Reductase Activity of Recombinant HCF164 Using the Turbidimetric Assay of Insulin Reduction.

The incubation mixture contained 100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mg/mL insulin, 0.35 mM DTT, and 2 or 5 μ M HCF164 protein. As a positive control, insulin reduction by 0.5 μ M thioredoxin from *Spirulina* sp was assayed. The nonenzymatic insulin reduction by DTT served as a negative control.

DISCUSSION

In recent years, a number of nuclear mutations affecting the biogenesis of the cytochrome *b₆f* complex have been characterized in the higher plants *Arabidopsis* (Dinkins et al., 1994; Meurer et al., 1996), maize (Barkan et al., 1994; Voelker and Barkan, 1995b; Fisk et al., 1999), and *Lemna perpusilla* (Bruce and Malkin, 1991) as well as in the green alga *Chlamydomonas reinhardtii* (Howe and Merchant, 1992; Kuras and Wollman, 1994; Gumpel et al., 1995; Howe et al., 1995) (reviewed by Goldschmidt-Clermont, 1998; Wollman et al., 1999). In most cases, the corresponding gene has not been identified; as a consequence, the corresponding factor could not be characterized. We report here on the nuclear mutant *hcf164* of *Arabidopsis*, which allowed us to isolate a gene that is involved in the biogenesis of the cytochrome *b₆f* complex in chloroplasts of higher plants.

The mutant *hcf164* displayed a severe deficiency in the cytochrome *b₆f* complex. The accumulation of its component subunits was reduced to less than 10 to 20% of wild-type amounts. This is in line with spectroscopic data revealing a defect in intersystem electron transport rates. There also was a reduction in the amounts of PSI and PSII complexes; the remaining complexes, however, were fully functional, as inferred from spectroscopic analyses. Wild-type levels of PSI and PSII subunits were observed when *hcf164* mutants were grown under very low light intensities. We conclude, therefore, that the reduction of these complexes most likely is a secondary effect of the mutation and that *hcf164* has to be classified as a mutant affected specifically in the cytochrome *b₆f* complex.

Cytochrome *b₅₅₉* of PSII accumulated to normal amounts in *hcf164* mutants grown under very-low-light conditions. This indicates that the loss of the cytochrome *b₆f* complex is not a result of a defect in heme biosynthesis. In vivo labeling of chloroplast proteins showed no principal difference between wild-type and mutant, and the overall rate of plastid protein synthesis in *hcf164* was comparable to that in the wild-type. Immunoprecipitation experiments demonstrated that at least the synthesis of cytochrome *f* and PetD was similar in the wild-type and mutant, suggesting that the translation of these subunits is not affected primarily by the mutation. Therefore, we conclude that HCF164 operates at the post-translational level and is involved in the maturation or the assembly of the cytochrome *b₆f* complex.

HCF164 encodes a thioredoxin-like protein. In accordance with this fact, the protein behaves biochemically as a thiol disulfide oxidoreductase. The cyanobacterial thioredoxin-like TxIA proteins were found to be the closest relatives of HCF164. This finding shows that HCF164 is a thioredoxin-like protein with an evolutionary origin in the cyanobacterial ancestor of chloroplasts. The function of the TxIA proteins is not yet clear. Partial gene knockouts yielded a severe defect in photoautotrophic growth (Collier and Grossman, 1995). However, the results remained inconclusive

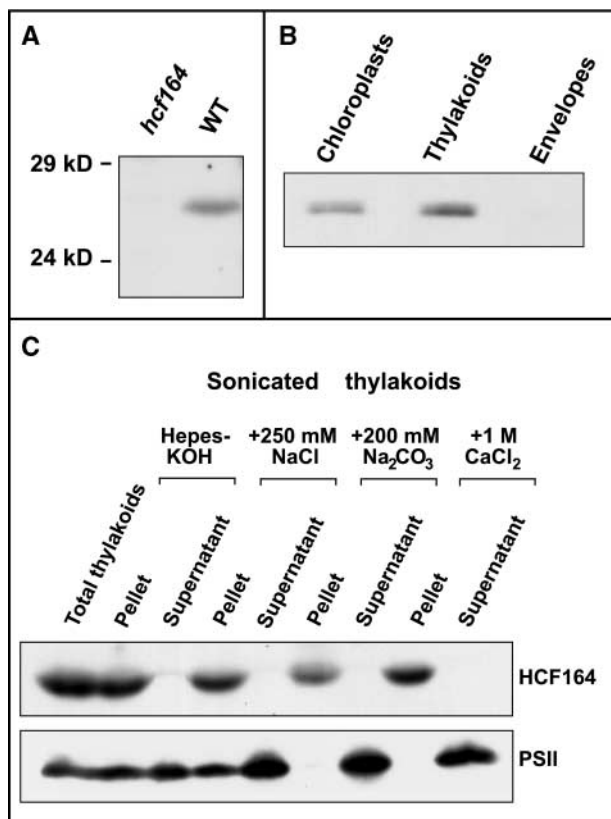


Figure 7. Immunolocalization of the HCF164 Protein.

(A) Immunoblot analysis of the HCF164 protein from wild-type (WT) and mutant. Total membrane proteins were isolated from Arabidopsis leaves, separated by SDS-PAGE, and analyzed by immunoblotting. Each lane was loaded with a protein equivalent of 10 μ g of chlorophyll.

(B) Chloroplast localization of HCF164. Chloroplast proteins isolated from spinach were fractionated by a sucrose step gradient as described in Methods. In each lane, 30 μ g of protein was separated by SDS-PAGE in the presence of 6 M urea and analyzed by immunoblotting.

(C) Salt washing of thylakoid membranes. Spinach thylakoids were sonicated in the salt conditions indicated and separated into membrane and soluble proteins by centrifugation. The protein equivalent of 10 μ g of chlorophyll was analyzed. The 23-kD luminal protein of PSII served as a marker for a membrane-associated but releasable hydrophilic thylakoid protein.

because the cells grew poorly, and therefore detailed biochemical analyses could not be performed.

Interestingly, HCF164 also showed a limited similarity to the thioredoxin-like proteins CcsX of the β proteobacterium *B. pertussis* (15% identity, 28% similarity) and CcmG of *E. coli* (15% identity, 32% similarity) and its homologs in Gram-negative bacteria. Both proteins are necessary for periplas-

mic thiol reduction and are believed to reduce the cysteine thiols of the heme binding motif (CxxCH) in apocytochrome *c* before the covalent binding of the heme group (Beckman and Kranz, 1993; Vargas et al., 1994; Fabianek et al., 1997, 1998; Page and Ferguson, 1997; Page et al., 1998; Beckett et al., 2000). Three distinct pathways, designated systems I, II, and III, with different assembly proteins have evolved for the covalent heme attachment to apocytochrome *c* (Kranz et al., 1998). CcmG participates in system I cytochrome *c* biogenesis, which is active in α and γ proteobacteria, plant and protozoal mitochondria, and some archaea. CcsX is associated with the system II pathway, which operates in Gram-positive bacteria, some β , ϵ , and δ proteobacteria, and cyanobacteria and chloroplasts.

Is HCF164 a component of the system II-type pathway of cytochrome *c* biogenesis in plastids? The protein localization experiments support this notion. HCF164 is a thylakoid protein that is anchored to the membrane, presumably via its uncleaved signal peptide, and whose major part extends into the lumen. Thus, it shows the same topology as CcsX/CcmG proteins that extend into the periplasm, the equivalent of the thylakoid lumen, and that are anchored to their membranes via hydrophobic patches of their uncleaved signal sequences (Monika et al., 1997; Fabianek et al., 1997, 1998; Beckett et al., 2000). If this functional assignment is correct, HCF164 is the functional equivalent of CcsX in a system II-type pathway of cytochrome *c* biogenesis in higher plants and is involved in the maturation of a *c*-type cytochrome (most likely cytochrome *f*).

Although these suggestions are persuasive, the results of the *in vivo* protein synthesis experiments do not corroborate the hypothesis that HCF164 represents a cytochrome *f* maturation factor. These experiments revealed that cytochrome *f* is synthesized and accumulates normally in the mutant during the labeling experiment (30 min). Provided that covalent binding of the heme group does not occur, the detected protein should be apocytochrome *f*. In *Chlamydomonas*, however, it has been shown that the inability to convert apocytochrome *f* to holocytochrome *f* results in increased turnover of the apoprotein, with a half-life of \sim 10 to 25 min (Howe et al., 1995; Kuras et al., 1997). Because of this discrepancy, we cannot exclude that HCF164 is necessary for processes other than cytochrome *f* maturation. For instance, it could be involved in heme attachment to cytochrome *b₆*. For this cytochrome, it has been suggested that one of the two heme groups is bound covalently to the protein (Kuras et al., 1997). Mutational analysis with *Chlamydomonas* showed that nuclear factors are necessary to catalyze this cytochrome *b₆* maturation process (Nakamoto et al., 2000); unfortunately, none of these factors has been cloned yet. HCF164 might be one of these unidentified proteins. Alternately, HCF164 could be a chaperone-like factor that is specific for the assembly of the cytochrome *b₆f* complex. An increasing number of such protein complex-specific assembly factors are being discovered in eubacteria and their evolutionary descendants, the mitochondria and

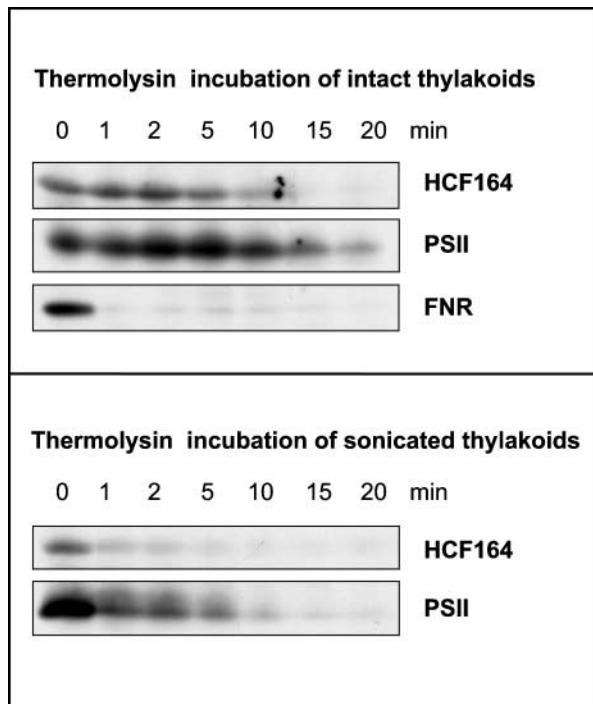


Figure 8. Luminal Localization of the HCF164 Protein.

Intact and sonicated spinach thylakoids were protease treated with thermolysin (final concentration, 0.1 mg/mL). The reaction was stopped at the times indicated by the addition of EDTA to a final concentration of 50 mM. Membranes were recovered by centrifugation, and the equivalent of 10 μ g of chlorophyll was loaded in each lane for immunoblot analysis. Ferredoxin NADP oxidoreductase (FNR) was used as a control for a peripheral thylakoid protein that is exposed to the stromal side, and the 23-kD protein of PSII was used as control for a luminal protein.

chloroplasts (Glerum et al., 1997; Suzuki et al., 1997; Meurer et al., 1998). HCF164 is unlikely to be a stoichiometric component of the cytochrome *b₆f* complex. Centrifugation of detergent-treated thylakoid membranes on sucrose density gradients revealed that HCF164 and the cytochrome *b₆f* complex do not comigrate (data not shown). Moreover, a stoichiometric component of the cytochrome *b₆f* complex of ~26 kD would not have escaped detection in earlier investigations aimed at the protein chemical characterization of that complex (Hauska et al., 1996).

Together, the evidence discussed here and the reports of other groups lead us to favor the model that HCF164 is the functional equivalent of CcsX that is involved in the maturation of cytochrome *f*. Whether the protein has other functions during the biogenesis of the cytochrome *b₆f* complex, for instance, the attachment of the heme group to cytochrome *b₆*, must be left for future experimentation.

METHODS

Mutant Isolation and Growth Conditions

The *hcf164* mutant was recovered from a collection of T-DNA insertion lines of *Arabidopsis thaliana* ecotype Wassilewskija produced by Nicole Bechtold and co-workers (Institut National de la Recherche Agronomique, Versailles, France). Selection of the mutant and its propagation was essentially as described by Meurer et al. (1998) except that phosphinotricin (10 mg/L final concentration) was used for mutant selection.

Spectroscopic Measurements

Chlorophyll fluorescence and P700 absorbance measurements were performed as described by Meurer et al. (1996) except that for the P700 absorbance kinetics, the pulse amplitude modulated fluorometer was modified with a P700 dual-wavelength emitter/detector unit (Waltz, Effeltrich, Germany).

PAGE, Heme Staining, and Immunoblot Analysis of Proteins

Isolation of protein extracts from wild-type and mutant leaves, electrophoresis of proteins on SDS-polyacrylamide gels (Laemmli, 1970; Schägger and von Jagow, 1987), and immunodecoration of electroblotted proteins were performed according to the methods described by Meurer et al. (1996, 1998) with the following variations. Plant material was ground for 20 sec in a Waring blender (capacity, 70 mL) with 25 mL of homogenization buffer (350 mM sucrose, 50 mM Tris-HCl, pH 8.0, 7 mM EDTA, and 10 mM DTT). After filtration through two layers of Miracloth, the homogenate was centrifuged for 10 min at 4000g (HB4 rotor; Sorvall, Norwalk, CT). The pellet containing the chloroplasts was resuspended in homogenization buffer without saccharose and adjusted to a final concentration of 2 to 5 mg protein/mL. For the detection of heme-containing proteins, samples were treated as described for the immunoblotting procedures. Proteins were electroblotted onto nitrocellulose filters, and these were incubated immediately for 30 min with the reagent of the chemiluminescence assay (ECL Plus; Amersham Pharmacia Biotech, Uppsala, Sweden). Exposure times of 1 hr produced a visible signal for the lowest concentration of the dilution series.

In Vivo Labeling of Proteins and Immunoprecipitation

Primary leaves of 20 15-day-old plants were preincubated in 200 μ g/mL cycloheximide for 30 min and radiolabeled with 5 μ Ci/ μ L ³⁵S-methionine (specific activity >1000 Ci/mmol; Amersham Pharmacia Biotech) in the presence of 100 μ g/mL cycloheximide for 30 min at 25°C. After washing five times with homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM EDTA), leaves were ground with a conical stainless steel rod in an Eppendorf tube with 300 μ L of the same buffer. Membranes were pelleted by centrifugation at 15,000g for 10 min and resuspended in 100 μ L of homogenization buffer. SDS was added to a final concentration of 2% (w/v), and proteins were solubilized for 10 min at 25°C followed by a 30-sec incubation at 70°C. For immunoprecipitation, aliquots

equivalent to 200,000 cpm of incorporated label were diluted 10-fold with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% [v/v] Nonidet P-40) and incubated with 5 μ L of specific antiserum at 4°C overnight. Antibody-antigen complexes were adsorbed by adding 30 μ L (bed volume) of protein A-Sepharose (Amersham Pharmacia Biotech) and by incubation of the suspension for 1 hr at 20°C under slow rotation. The protein A-Sepharose then was collected by centrifugation at 900g for 5 min and washed four times with immunoprecipitation buffer. Antibody-antigen-protein A complexes were dissociated by incubation in gel loading buffer (Laemmli, 1970) for 30 sec at 70°C. To ensure a quantitative precipitation of the proteins, the supernatants from the first immunoprecipitation were treated a second time with the same amount of antiserum. Proteins were electrophoresed by PAGE according to Laemmli (1970). Radiolabeled proteins were detected on the dried gels by autoradiography and quantified using a BioImager (BioImaging Analyser BAS-1800; Fuji Photo Film Co., Düsseldorf, Germany) and the software packages Image Reader BAS 1.4 and Image Gauge 3.0 (Fuji Photo Film Co., Düsseldorf, Germany).

RNA Gel Blot Analysis

Isolation of RNA, electrophoresis of glyoxylated RNA on agarose gels, and RNA gel blot hybridization analysis were performed as described elsewhere (Westhoff et al., 1991; Meurer et al., 1996). For isolation of poly(A⁺) RNA, the total RNA was treated with Oligotex (Amersham Pharmacia Biotech) according to the protocol supplied by the manufacturer.

Inverse Polymerase Chain Reaction Amplification of T-DNA Flanking Genomic Sequences, Cloning of the *HCF164* cDNA, and Mutant Complementation

Genomic DNA was isolated from 3-week-old mutants according to Dellaporta et al. (1983), restricted with EcoRI, and self-ligated. Standard polymerase chain reaction (PCR) reactions with the primers LB-TAG5 (5'-CTACAAATTGCCCTTTCTTATCGA-3') and LB-TAG17 (5'-GTCCGCTCTACCGAAAGTTACG-3') resulted in a 2040-bp product. This was reamplified with the nested primers LB-TAG14 (5'-GGT-AATAGGACACTGGGATTCGTC-3') and LB-TAG18 (5'-CGACAA-CATGTGCGAGGCTCAGC-3'), yielding a 1820-bp DNA fragment. The PCR product was cloned into the EcoRV site of pBluescript II KS+ (Stratagene, La Jolla, CA) as described (Marchuk et al., 1991), and the resulting clone, pAt-HCF164, was sequenced. Based on genomic sequences that matched pAt-HCF164 (EMBL accession numbers Z99707 and CAB16778.1), the primers 164FOR (5'-GCG-GATCCCGACGATGGCTCGCTTAG-3') and 164REV (5'-CGGATC-CGCATGATAGTGATGCCACTC-3') were designed and used for reverse transcriptase-mediated PCR amplification of the complete *HCF164* coding sequence from ecotype Wassilewskija. Because the primers 164FOR and 164REV contained a BamHI restriction site, the PCR product of 814 bp was cloned into the BamHI site of pBluescript II KS+, and several of the resulting cDNA clones were sequenced.

One of the clones with a correct *HCF164* sequence, pAt-HCF164, was used for the complementation of homozygous *hcf164* plants by *Agrobacterium tumefaciens*-mediated root transformation essentially as described by Meurer et al. (1998). Forty-five independent

transgenic plants were recovered and analyzed by chlorophyll fluorescence induction for a complemented mutant phenotype.

Enzymatic Analysis of Recombinant HCF164

The nucleotide sequences encoding the soluble part of HCF164 (amino acids 116 to 261, corresponding to nucleotide positions 380 to 788 of pcAt-HCF164) were amplified by PCR using the primers 5'-AGGATTGCTAGCGATTTTGGGATTTCTTTGAAGG-3' and 5'-TAC-TCAAGCTTATCCATGGCTTAAGGGATC-3'. The resulting DNA fragment was cleaved with NheI and HindIII and fused in frame with the signal peptide sequence present in the expression plasmid pAH33 (Seidler, 1994). The resulting plasmid, pEX-HCF164, when introduced into *Escherichia coli*, directed the soluble recombinant HCF164 protein into the periplasmic space. An N-terminal His tag allowed purification by metal chelate affinity chromatography.

Expression of the protein was performed in *E. coli* strain SF110 (Meerman and Georgiou, 1994) in tryptone phosphate (TP) medium (2% [w/v] tryptone, 1.5% [w/v] yeast extract, 0.8% [w/v] NaCl, 0.2% [w/v] Na₂HPO₄, and 0.1% [w/v] KH₂PO₄) (Moore et al., 1993). Cells were grown at 25°C to OD₆₀₀ = 0.6, and the expression of recombinant HCF164 was induced by adding isopropylthio- β -D-galactoside to a final concentration of 0.5 mM. Growth was continued for another 12 to 14 hr, and periplasmic proteins were isolated as described by Seidler (1994), except that lysozyme was omitted. The periplasmic protein fraction was dialyzed against buffer A (20 mM Hepes, pH 7.5, and 500 mM NaCl) and loaded onto a metal-affinity chromatography column (Chelating Sepharose FF; 1.5 \times 12 cm; Amersham Pharmacia Biotech) precharged with Zn²⁺. The column was washed with 30 mL of buffer A containing 20 mM imidazole. Elution of the His-tagged HCF164 was performed with a linear gradient of 20 to 350 mM imidazole in 150 mL of buffer A.

The recombinant HCF164 protein was assayed for disulfide reductase activity according to Holmgren (1979). The reaction mixture with a final volume of 1 mL contained 100 mM sodium phosphate, pH 7.0, 1 mM EDTA, 1 mg/mL insulin (bovine insulin; Sigma, München, Germany), and 2 or 5 μ M recombinant HCF164 protein. The reaction was started by the addition of 0.35 mM DTT and performed at room temperature. The insulin disulfide reduction was measured by the absorbance change at 650 nm. The nonenzymatic reduction of insulin by DTT served as a negative control, and as a positive control, 0.5 μ M thioredoxin from *Spirulina* sp (Sigma, Deisenhofen, Germany) was used.

Antiserum Production

The nucleotide sequence encoding amino acids 85 to 261 of the HCF164 protein (nucleotide positions 258 to 788 of pcAt-HCF164) was fused in frame to the glutathione S-transferase sequence of the expression vector pGEX-4T3 (Amersham Pharmacia Biotech), and the construct was transformed into *E. coli* strain BL21(DE3) pLysE. Expression of the 46-kD glutathione S-transferase HCF164 fusion protein was induced with 1 mM isopropylthio- β -D-galactoside. The *E. coli* cells were recovered by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 0.1 mM EDTA, and 0.01% (w/v) Triton X-100. After incubation for 30 min at room temperature in the presence of lysozyme at a final concentration of 1 mg/mL, the cell suspension was sonicated four times for 30 sec at an output intensity of 7 (Branson Sonifier B12; Branson, Danbury, CT) while kept on ice.

Because the fusion protein was found exclusively in inclusion bodies, the sonicated cell suspension was centrifuged and the pellet was solubilized in 2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and 10% (w/v) glycerol. This crude fraction was subjected to preparative PAGE (Hager and Burgess, 1980). The fusion protein was eluted in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.75% (w/v) *N*-lauryl-sarcosine, and 0.5% (v/v) 2-mercaptoethanol. After adjusting the eluate to a final concentration of 1.5% (v/v) Triton X-100, the fusion protein was cleaved with thrombin (Amersham Pharmacia Biotech). The released HCF164 protein was purified again by preparative PAGE and finally dissolved in 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3, 2.7 mM KCl, 140 mM NaCl, and 0.2% (w/v) SDS. A polyclonal antiserum was raised in rabbits and affinity purified on an HCF164 protein column by BioGenes (Berlin, Germany).

Immunolocalization Studies with Protein Gel Blots

The intracellular localization of the HCF164 protein was determined by immunoblot analysis. Thylakoid membranes and envelope fractions were prepared by fractionation of lysed spinach chloroplasts on a discontinuous sucrose density gradient according to Cline et al. (1981).

The membrane association of HCF164 was analyzed by salt washing experiments. Spinach thylakoids were resuspended to a final concentration of 50 µg/mL chlorophyll in 10 mM Hepes/KOH, pH 8.0, 10 mM MgCl₂, 330 mM sorbitol, 2 mM phenylmethylsulfonyl fluoride, and 2 mM benzamidine supplemented with 250 mM NaCl, 200 mM Na₂CO₃, or 1 M CaCl₂, respectively, without further supplement as a control. These suspensions were sonicated three times for 15 sec at an output intensity of 5 (Branson Sonifier B12) while kept on ice. The membrane vesicles were separated from the soluble components by ultracentrifugation at 100,000g for 2 hr at 4°C. Proteins of the supernatant were recovered by trichloroacetic acid precipitation (15% final concentration). Protein equivalents of 10 µg of chlorophyll of the soluble (supernatant) and membranous proteins were subjected to immunoblot analyses using the HCF164 antiserum.

The intraorganellar location of HCF164 was studied further by protease protection studies using thermolysin as a probe as described by Meurer et al. (1998).

Accession Number

The nucleotide sequence of the HCF164 cDNA has been submitted to the DDBJ/EMBL/GenBank databases under accession number AJ293262.

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