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The Nuclear-Encoded Factor HCF173 Is Involved in the Initiation of Translation of the *psbA* mRNA in *Arabidopsis thaliana* [W]

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To gain insight into the biogenesis of photosystem II (PSII) and to identify auxiliary factors required for this process, we characterized the mutant *hcf173* of *Arabidopsis thaliana*. The mutant shows a high chlorophyll fluorescence phenotype (*hcf*) and is severely affected in the accumulation of PSII subunits. In vivo labeling experiments revealed a drastically decreased synthesis of the reaction center protein D1. Polysome association experiments suggest that this is primarily caused by reduced translation initiation of the corresponding *psbA* mRNA. Comparison of mRNA steady state levels indicated that the *psbA* mRNA is significantly reduced in *hcf173*. Furthermore, the determination of the *psbA* mRNA half-life revealed an impaired RNA stability. The *HCF173* gene was identified by map-based cloning, and its identity was confirmed by complementation of the *hcf* phenotype. *HCF173* encodes a protein with weak similarities to the superfamily of the short-chain dehydrogenases/reductases. The protein HCF173 is localized in the chloroplast, where it is mainly associated with the membrane system and is part of a higher molecular weight complex. Affinity chromatography of an HCF173 fusion protein uncovered the *psbA* mRNA as a component of this complex.

INTRODUCTION

Photosystem II (PSII) is one of the three multisubunit protein complexes of the photosynthetic electron transport chain. It is localized in the thylakoid membranes of photosynthetically active organisms and functions as a water-plastoquinone oxidoreductase. Like other complexes of the thylakoid membrane, PSII is a genetic mosaic consisting of plastid- and nuclear-encoded subunits (Wollman et al., 1999); therefore, a coordination of gene expression in the plastid and the cytosol is required (Goldschmidt-Clermont, 1998).

The core complex of the PSII reaction center consists of the proteins D1 and D2, which bind all important cofactors needed for the primary charge separation (i.e., the P680 chlorophyll *a*, pheophytin, and plastoquinone). The 32-kD D1 protein is encoded by the *psbA* gene of the chloroplast genome. Regulation of D1 protein synthesis is important for the correct biogenesis of PSII during chloroplast development and for the maintenance of a functional photosystem, since D1 exhibits a high turnover due to damage conferred by light. The accumulation of the D1 protein depends on environmental signals, such as light and the developmental stage of the plant (Klein et al., 1988; Gamble and Mullet, 1989; Klein and Mullet, 1990; Klaff and Gruissem, 1991; Kim et al., 1993; Staub and Maliga, 1994).

In general, the expression of plastid-encoded genes is mainly regulated by posttranscriptional mechanisms (Deng and Gruissem, 1987). Several nuclear-encoded factors have been identified to be involved in RNA-stabilizing processes, RNA degradation, and translation by in vitro UV cross-linking of RNA with proteins, in vitro translation experiments, and characterization of nuclear mutants of green algae and higher plants (reviewed in Goldschmidt-Clermont, 1998; Barkan and Goldschmidt-Clermont, 2000; Monde et al., 2000; Zerges, 2000; Leister and Schneider, 2003). One of the best-studied examples was described for the regulation of the *psbA* mRNA translation in *Chlamydomonas reinhardtii*. A complex of at least four distinct proteins (RB38, RB47, RB55, and RB60) seems to activate translation in a light- and redox state–dependent manner (Danon and Mayfield, 1991; Trebitsh et al., 2000; Kim and Mayfield, 2002; Somanchi et al., 2005). The RNA binding activity of the complex is thought to be regulated by the proteins RB60 and Tba1, which are able to modulate the redox state of RB47 (Kim and Mayfield, 2002; Somanchi et al., 2005). Binding of the complex to the 5′-untranslated region (5′-UTR) of the *psbA* mRNA is mediated by RB47 and RB38 (Yohn et al., 1998a, 1998b; Barnes et al., 2004). It has been proposed that RB47 takes part in D1 synthesis for de novo PSII biogenesis because it localizes to the low-density membrane system (Zerges and Rochaix, 1998), whereas a 63-kD protein, which also binds to the 5′-leader of the *psbA* RNA but is localized to the stroma thylakoids, might participate in D1 synthesis for PSII repair (Ossenbühl et al., 2002).

Less is known about protein binding to the 5′-UTR of the *psbA* transcript in higher plants. Cross-linking experiments identified

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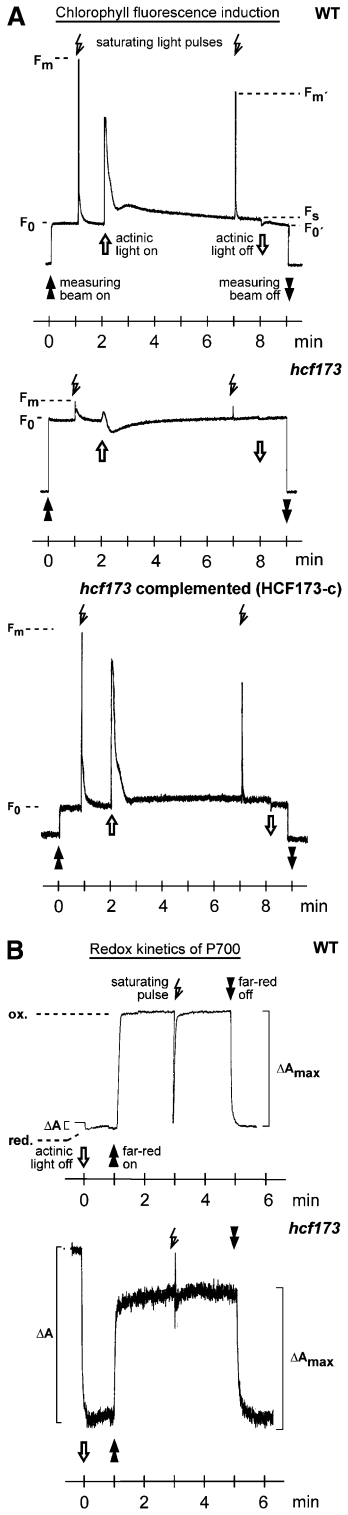

Figure 1. Spectroscopic Analyses of Wild-Type, *hcf173* Mutant, and Complemented *hcf173* Plants.

Chlorophyll fluorescence induction **(A)** and P700 absorbance kinetics **(B)** were measured with 3- or 4-week-old plants, respectively.

several distinct proteins, which bind to the 5′-leader of the *Spinacia oleracea psbA* mRNA. Among these, the protein CS1 is a homolog of the *Escherichia coli* ribosomal S1 protein (Alexander et al., 1998). In *Arabidopsis thaliana*, two proteins were identified, which bind to the 5′-UTR of the *psbA* mRNA in a redox-dependent manner. The binding occurs in a region comprising the ribosome binding sites, indicating a role in translation initiation of the D1 protein (Shen et al., 2001). However, the identity of these two proteins is still unclear.

We are interested in understanding the biogenesis of the multisubunit protein complex PSII. To identify genes that are involved in this process, we investigate nuclear mutants of *Arabidopsis* that exhibit the high chlorophyll fluorescence phenotype (*hcf*), which indicates defects in the photosynthetic light reactions and the electron transport (Miles, 1982). Here, we report on *hcf173*, a mutant that is especially affected in D1 protein synthesis. The altered polysome association of the *psbA* mRNA in *hcf173* suggests that translation initiation of this transcript is impaired. The

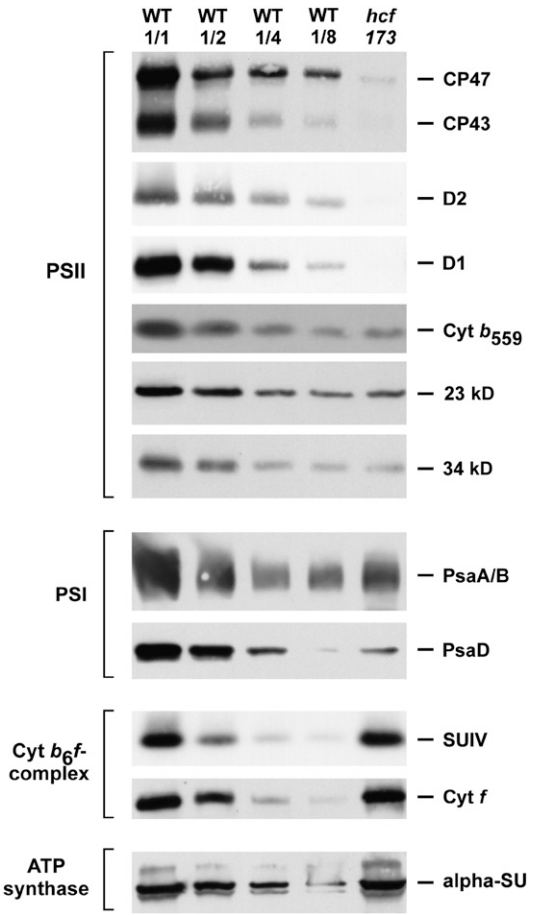

Figure 2. Immunoblot Analysis of Thylakoid Membrane Proteins from the Wild Type and the Mutant *hcf173*.

Twenty micrograms (WT 1/1 and *hcf173*), 10 μg (WT 1/2), 5 μg (WT 1/4), and 2.5 μg (WT 1/8) of protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Specific proteins were immunodecorated with the indicated antisera.

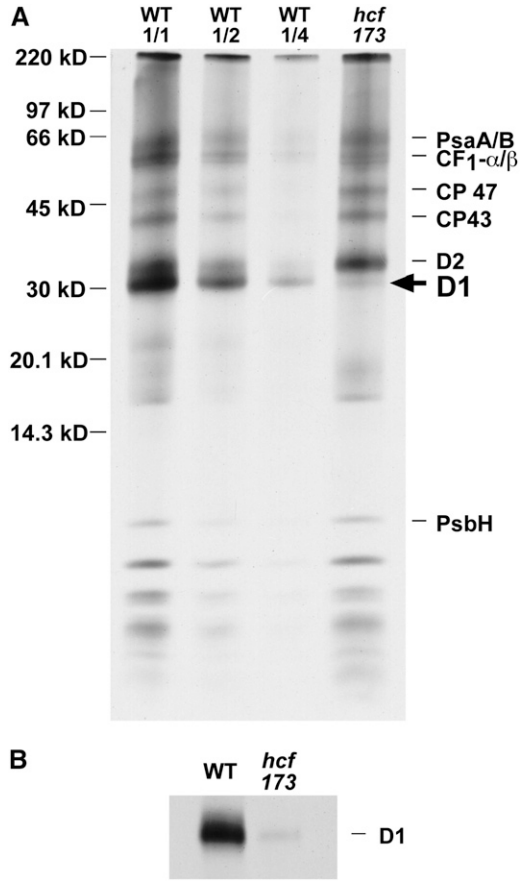

Figure 3. In Vivo Protein Synthesis in Wild-Type Plants and *hcf173* Mutants.

Newly synthesized proteins of 15-d-old plants were radiolabeled with ^{35}S-Met for 20 min.

(A) Wild-type and *hcf173* membrane proteins with equivalent amounts of radioactivity (100,000 cpm) or the indicated dilutions were separated by SDS-PAGE and analyzed by fluorography.

(B) SDS-solubilized membrane proteins with the equivalent of 200,000 incorporated cpm were used for immunoprecipitation with anti-D1. The *hcf173* sample was filled up with unlabeled wild-type membrane protein to guarantee equal amounts of D1 in both the wild-type and the mutant sample. Anti-D1 was used in excess for immunoprecipitation.

mutant phenotype was rescued by complementation experiments with the *At1g16720* cDNA. *At1g16720*/*HCF173* encodes a protein that shows weak similarities to the short-chain dehydrogenase/reductase (SDR) superfamily. The protein is mainly localized to the chloroplast membranes and part of a high molecular weight complex, which is associated with the *psbA* mRNA, emphasizing the possible role of HCF173 in *psbA* gene expression.

RESULTS

Hcf173 Is Affected in PSII Function

The mutant *hcf173* was isolated by its recessive high chlorophyll fluorescence phenotype from a collection of M2 families that were obtained by ethyl methanesulfonate (EMS) mutagenesis of *Arabidopsis* seeds as described (Meurer et al., 1996b). Mutant seedlings of *hcf173* were not able to grow photoautotrophically on soil but could be maintained on sucrose-supplemented medium. However, they did not develop any fertile flowers.

Chlorophyll fluorescence induction measurements (Figure 1A) revealed a drastically reduced ratio of variable fluorescence to maximal fluorescence (Fv/Fm) in *hcf173* (0.15 $\pm$ 0.02) when compared with the wild type (0.80 $\pm$ 0.01). This is indicative of a severe defect of PSII in *hcf173*, which was further supported by an extremely low quantum yield of PSII (ΦII), with 0.065 $\pm$ 0.02

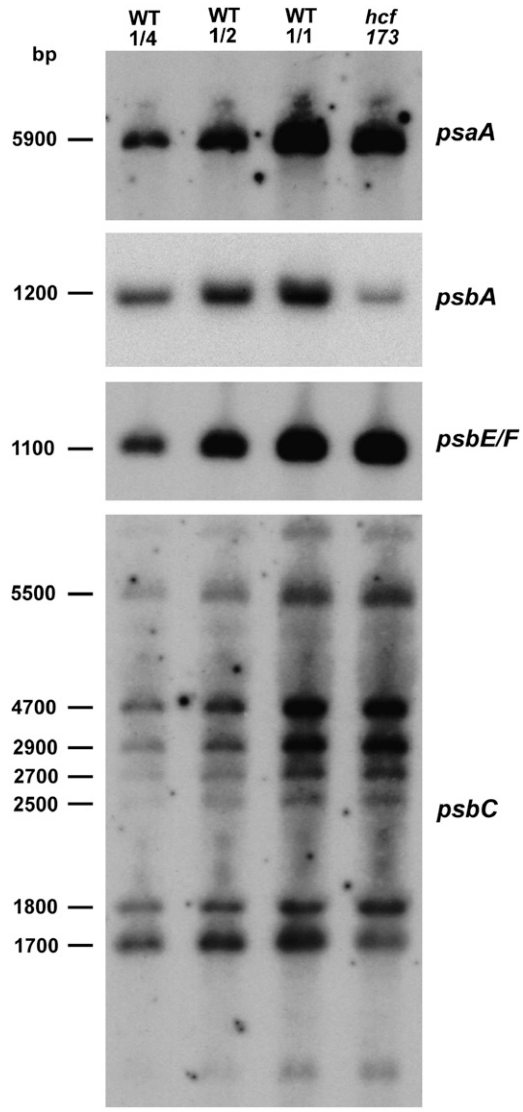

Figure 4. Transcript Analysis of *psaA*, *psbA*, *psbE/F*, and *psbC* in the *hcf173* Mutant.

Six micrograms or the indicated dilutions of total leaf RNA from 3-week-old wild-type and *hcf173* mutant plants were separated by denaturing agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with radiolabeled probes as described. The *psbC* mRNA is transcribed in a polycistronic transcription unit resulting in a complex transcript pattern (Meurer et al., 1996a).

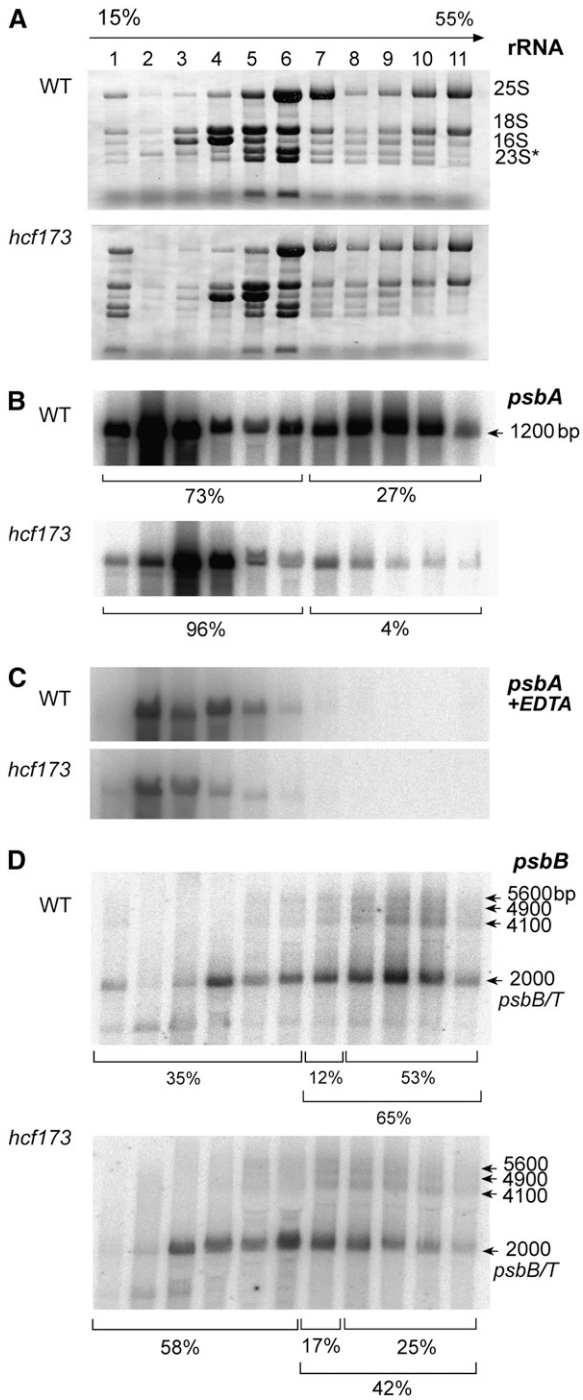

Figure 5. Association of the *psbA* Transcript with Polysomes in the *hcf173* Mutant.

Whole-cell extracts were fractionated in linear 15 to 55% sucrose gradients by ultracentrifugation. Gradients were divided into 11 fractions, and equal proportions of each fraction were analyzed.

(A) RNA gel blots of polysome gradient fractions were stained with methylene blue to visualize distribution of rRNAs.

(B) The blots shown in **(A)** hybridized with a probe that detects *psbA* mRNA. The RNA amounts of the different fractions were quantified by phosphor imaging.

(C) Cell extracts treated with EDTA to disrupt polysome association.

(D) RNA gel blots hybridized with a probe specific for *psbB*. The signals of the large primary transcripts are faint, but their positions are indicated.

in *hcf173* versus 0.74 ± 0.03 in the wild type. Photochemical quenching was less affected in the mutant plants [qp(*hcf173*) = 0.73 ± 0.07 and qp(WT) = 0.97 ± 0.02]. This shows that the few electrons released by PSII were transported through the electron transport chain, thus indicating an active cytochrome b_6f complex and photosystem I (PSI). This finding was supported by measurements of P700 redox kinetics in wild-type and *hcf173* mutant plants (Figure 1B). In *hcf173*, we detected a clear absorbance change induced by far-red light, suggesting that PSI is functional. Application of a saturating light pulse under far-red light induces PSII electron transport leading to a complete rereduction of P700 in the wild type. By contrast, only a weak rereduction of the PSI reaction center was detectable in the mutant, which is in line with its strong PSII defect.

Taken together, these spectroscopic analyses indicate that the mutant *hcf173* is affected mainly in PSII and not in the intersystem electron transport chain or PSI.

hcf173 Fails to Accumulate PSII Subunits

To corroborate the results obtained by spectroscopic measurements, we monitored the steady state levels of distinct subunits of the photosynthetic membrane complexes with specific antibodies (Meurer et al., 1996b). The plastid-encoded subunits D1, D2, CP43, and CP47 of PSII were hardly detectable in *hcf173* (Figure 2). The cytochrome b_{559} polypeptides were reduced to ~25% of wild-type amounts and the nuclear-encoded subunits of the oxygen-evolving complex (23- and 34-kD proteins) to ~25 or 12.5%, respectively. We further analyzed representative polypeptides of PSI (PsaD and PsaA/B), the cytochrome b_6f complex (SUIV and Cyt*f*), and the ATP synthase (α-SU). Reduced protein levels were found for PsaD (20%) and PsaA/B (50%). Subunits of the cytochrome b_6f complex and the ATP synthase accumulated to normal wild-type levels. Hence, the immunoblot analysis indicates a severe defect in PSII of *hcf173*. Obviously, subunits of the core complex and the inner antenna cannot accumulate, whereas subunits of the oxygen-evolving complex accumulate to low but detectable amounts. The reduction of PSI proteins was less severe than that found for PSII components. Furthermore, *hcf173* mutant plants grown under low-light conditions (2 to 5 $\mu mol \cdot m^{-2} \cdot s^{-1}$) accumulated much higher amounts of PSI proteins compared with plants cultivated under standard light conditions (50 to 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (Plücken et al., 2002). This suggests that the levels of PSI subunits in *hcf173* mutant plants are sensitive to light and therefore not directly influenced by the mutation.

Together, we conclude from these data that the nuclear mutation in *hcf173* leads primarily to a drastically impaired accumulation of PSII polypeptides.

The Synthesis of the PSII Reaction Center Protein D1 Is Drastically Reduced in *hcf173*

To define the mutational defect in the mutant *hcf173* more precisely, the synthesis of plastid-encoded subunits of the thylakoid membrane was examined by in vivo labeling of leaf proteins with

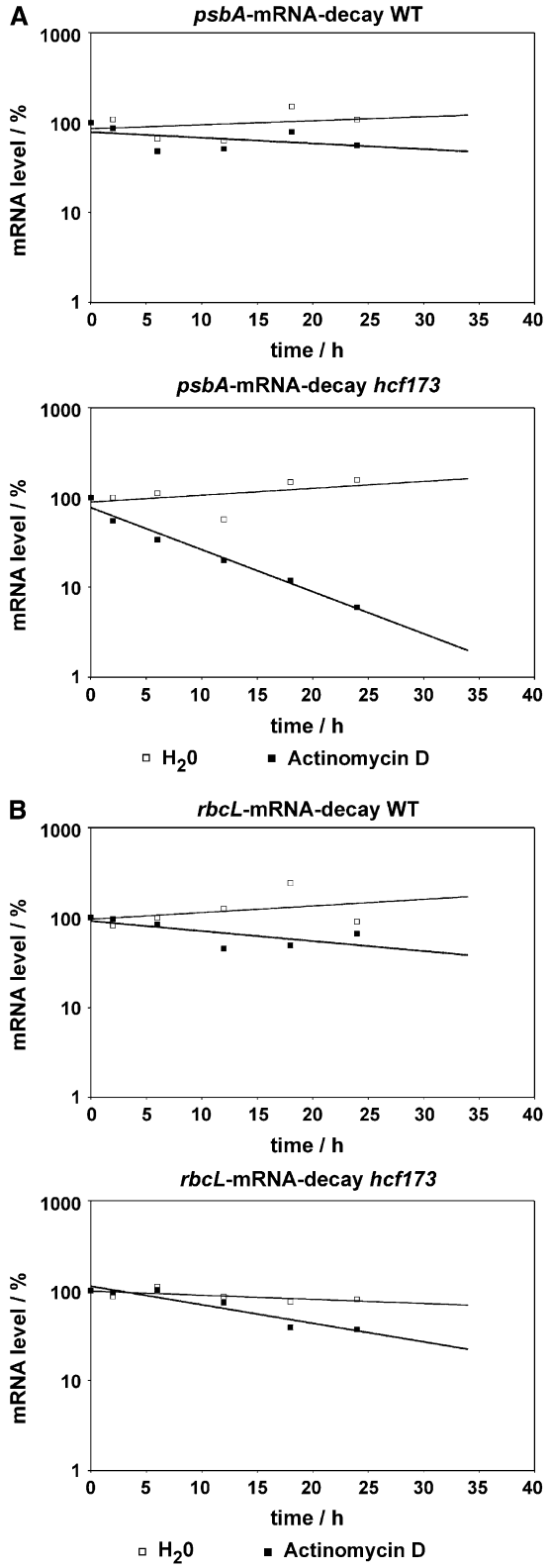

Figure 6. Stability of *psbA* and *rbcL* Transcripts in the *hcf173* Mutant.

Wild-type and mutant plants were harvested and incubated with a solution of the transcriptional inhibitor actinomycin D and water as control. RNAs were isolated 0, 2, 6, 12, 18, and 24 h after the start of actinomycin D or water treatment. Amounts of *psbA* mRNA **(A)** and *rbcL* mRNA **(B)** were quantified by RNA gel blot analysis and subsequent phosphor imaging.

^{35}S-Met. Cytoplasmic translation of nuclear-encoded subunits was blocked by application of cycloheximide. Figure 3A shows that after a labeling period of 20 min, the rates of synthesis of thylakoid membrane polypeptides were similar in wild-type and mutant leaves with the exception of the PSII reaction center protein D1. The incorporation of radioactivity into this protein was eight times lower in *hcf173* compared with the wild type. We repeated the in vivo labeling experiments with a shorter pulse of only 5 min. Even under these conditions a reduced D1 labeling was observed, indicating that indeed D1 synthesis is affected and not D1 protein stability (see Supplemental Figure 1 online).

To confirm the identity of the supposed D1 protein band, immunoprecipitation experiments were performed. After a labeling period of 20 min, membrane proteins were solubilized and incubated with a specific antiserum raised against the D1 protein. Gel electrophoresis of immunoprecipitated proteins and subsequent autoradiography (Figure 3B) confirmed the reduced labeling of the D1 protein in the mutant *hcf173*. Together, these results provide strong evidence that a reduced synthesis rate of the PSII reaction center protein D1 is the primary mutational defect in *hcf173*.

psbA mRNA Accumulation and Ribosomal Loading Are Impaired in *hcf173* Mutants

To confirm that the reduced D1 synthesis rate in the *hcf173* mutant is caused by a defect in translation, we first determined the amount of the *psbA* mRNA. Figure 4 shows that the level of the monocistronic *psbA* transcript was reduced to ~12% of the wild-type amount in the mutant *hcf173*. We further examined the transcript levels of other PSII, PSI, cytochrome b_6f complex, and ATP synthase polypeptides (i.e., *psbC*, *psbD*, *psbE/F*, *psbO*, *psbP*, *psaA*, *psaF*, *petA*, *petD*, and *atpA*), but no significant differences between wild-type and mutant transcript levels were detected (Figure 4; data not shown).

To address the question whether the mutation in *hcf173* affects the ability of the *psbA* mRNA to associate with ribosomes, we examined ribosomal loading of this transcript. To this end, leaf extracts were fractionated in sucrose gradients under conditions that maintain intact polysomes (Barkan, 1993). Efficiently translated ribonucleic acids are highly associated with ribosomes and therefore migrate deep into the gradient. Specific mRNAs were localized in the gradients by performing RNA gel blot hybridizations with RNA purified from gradient fractions. The distribution of plastidic and cytosolic rRNAs in wild-type and *hcf173* polysome gradients was determined, and both gradients showed an equal pattern of rRNA distribution, revealing that there is no general difference in the distribution of ribosomes between the mutant and the wild type (Figure 5A). By contrast, different sedimentation behavior was observed for the *psbA* mRNA in the wild type and *hcf173* (Figure 5B). Quantification of the signals by phosphor imaging revealed that 27% of wild-type *psbA* mRNA sedimented in the polysomal region of the gradient (fractions 7 to 11). In *hcf173* mutants, the bulk of the *psbA* mRNA was localized in the fractions containing monosomes or free

RNA, and only 4% were detected in the polysomal fraction, indicating a low translational activity of this transcript. To confirm the distribution of small RNA particles (monosomes and free RNA) and polyribosomes in the sucrose gradients, crude polysomal RNA from the wild type and *hcf173* was treated with EDTA, which disrupts ribosome association. This treatment resulted in a shift of the wild-type *psbA* mRNA into the first six fractions (Figure 5C), revealing that the RNA migrating deeper into the gradient represents polysomes. We further examined polysomal association of the *psbB* mRNA, which is encoded in the *psbB-psbT-psbH-petB-petD* polycistronic transcription unit (Westhoff and Herrmann, 1988). In the wild type, 65% of the *psbB/T* transcript was found in the polysomal fractions of the gradient (fraction 7-11; Figure 5D). In the mutant, the corresponding fractions contained lower amounts (42%) of the *psbB/T* mRNA, and a major amount of the transcript was associated with small polysomes (fraction 7). Because there is no indication for RNA degradation in the polysomal fractions of the mutant, this difference could be an indication of impaired translation of *psbB*-containing transcripts in *hcf173* mutant plants.

Together, these results imply that the reduced labeling of the D1 protein in *hcf173* is mainly the result of drastically impaired translation. The lack of polysomal association suggests that the *hcf173* mutation affects the initiation of *psbA* mRNA translation or very early steps of translation elongation. Moreover, a direct or indirect effect of the mutation on the translational efficiency of the *psbB/T* transcript is possible, although the in vivo labeling experiments did not corroborate this assumption. The 5-min pulse indeed showed weaker labeling of CP47 but also for all other synthesized proteins in the mutant (see Supplemental Figure 1 online). Moreover, the 20-min pulse resulted in similar signals of CP47 in the wild type and mutants (Figure 3A).

The altered ribosomal loading possibly causes the diminished accumulation of the *psbA* mRNA in the mutant. To test whether the reduced *psbA* mRNA level is caused by decreased mRNA stability, *psbA* mRNA decay in wild-type plants and *hcf173* mutants was investigated in the presence of the transcriptional inhibitor actinomycin D. Total RNA was isolated from single plants after incubation in the inhibitor solution for 0, 2, 6, 12, 18, or 24 h. Isolated RNAs were analyzed by gel blot hybridization with probes for *rbcL* and *psbA* mRNAs and subsequent autoradiography. Figure 6A shows that the stability of the *psbA* mRNA in the mutant was drastically reduced ($t1/2_{psbA}$ *hcf173* $\approx$ 6 h) compared with wild-type plants ($t1/2_{psbA}$ WT $\approx$ 47 h). As a control, the stability of the *rbcL* mRNA was measured. The half-life of this RNA was similar in the wild type ($t1/2_{rbcL}$ WT $\approx$ 22 h) and mutants ($t1/2_{rbcL}$ *hcf173* $\approx$ 15 h) (Figure 6B). Thus, the observed RNA degradation is not the result of a general RNA instability in the mutant plants. The reduction of *psbA* mRNA stability in *hcf173* by a factor of eight is sufficient to explain the low steady state level of the *psbA* transcript. Therefore, our result suggests that the reduced amount of *psbA* mRNA in the mutant results from increased RNA turnover and not from a reduced transcription rate.

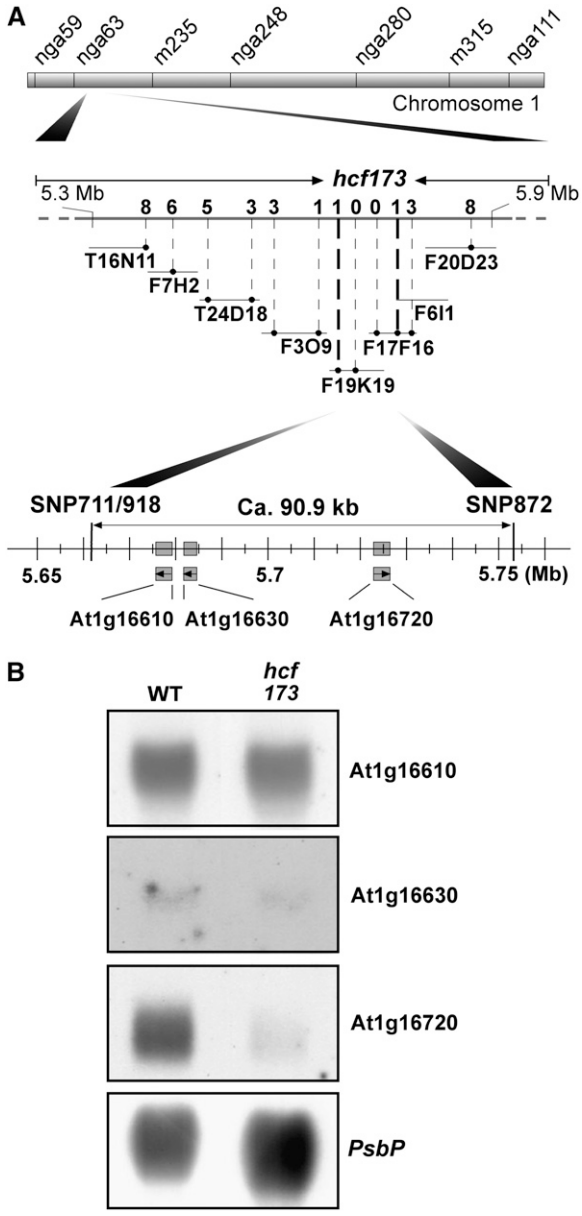

Figure 7. Mapping of the *HCF173* Locus.

(A) The *HCF173* locus was localized on the upper arm of chromosome 1 between the two CAPS markers SNP711/918 (BAC clone F19K19) and SNP872 (BAC clone F17F16). Within the mapped region, three genes (*At1g16610*, *At1g16630*, and *At1g16720*) were identified, which encode proteins with a putative chloroplast transit peptide.

(B) The transcripts of the genes *At1g16610*, *At1g16630*, and *At1g16720* were analyzed by RNA gel blot hybridization with gene-specific probes. To this end, 5 μg of poly(A)$^+$ RNA of 3-week-old wild-type plants and *hcf173* mutants were separated on a denaturing agarose gel.

Molecular Cloning of *HCF173*

The mutant *hcf173* was generated by EMS mutagenesis of *Arabidopsis* seeds. A set of microsatellite and cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994) was used to map the gene *HCF173* on the upper arm of chromosome 1 (Figure 7A). To determine the *HCF173* locus more precisely, several simple sequence length polymorphism and CAPS markers were developed and used for

genotyping 1279 plants of the mapping population. The markers SNP711/918 on BAC clone F19K19 (one recombination) and SNP872 on BAC clone F17F16 (one recombination) were found to flank the *HCF173* locus. The DNA region between these markers (91 kb) comprised three genes that encode proteins with a predicted chloroplast transit peptide (*At1g16610*, *At1g16630*, and *At1g16720*). The transcript levels of these genes were investigated in wild-type and mutant plants. Indeed, one of these genes, *At1g16720*, showed a drastically reduced RNA level in the *hcf173* mutant (Figure 7B). To test whether *At1g16720* was affected by a mutation, we amplified the whole sequence by PCR using genomic DNA from *hcf173* mutants as a template. Sequence analysis revealed a point mutation in exon 10 at base pair position 2524 (Figure 8). The exchange from cytosine to thymine at this site caused a premature termination codon and would very likely lead to synthesis of an incomplete HCF173 protein. The incorrect RNA is probably submitted to the pathway of nonsense-mediated mRNA decay (Baker and Parker, 2004), which would explain the reduced transcript level. This mutant allele was referred to as *hcf173-1*. The discovery of a second allele (*hcf173-2*) in the GABI-Kat collection (Rosso et al., 2003; line 246C02) supports the assumption that *At1g16720* encodes the nuclear factor HCF173. Line 246C02 contains a T-DNA insertion in exon 3 of *At1g16720* (Figure 8). The mutant exhibited the *hcf* phenotype under UV light and at the molecular level showed the same defects as *hcf173-1* (data not shown).

We further confirmed the identity of the gene *At1g16720* by complementation analysis. The cDNA of *At1g16720* under the control of the 35S promoter was transferred into heterozygous *hcf173-1* plants by *Agrobacterium tumefaciens*–mediated transformation. In the progeny of these transformants, we identified two transgenic plants that were homozygous for the mutation *hcf173* and were able to grow photoautotrophically on soil. Chlorophyll fluorescence induction measurements showed that the transgenic plants exhibit the same kinetics as wild-type plants (Figure 1A), indicating that PSII is fully active. Thus, the introduced cDNA of *At1g16720* is able to complement the *hcf173* mutant phenotype. Together, the data of the identified T-DNA mutant and the complementation analysis revealed that the gene *At1g16720* encodes the factor HCF173.

HCF173 Encodes a Protein with Similarity to the SDR Superfamily

The *HCF173* gene encodes a protein of 598 amino acids with a predicted molecular mass of 65.7 kD. The WolfPSORT program (Horton et al., 2006; http://wolfpsort.seq.cbrc.jp) predicted a chloroplast transit peptide of 83 amino acids (Figure 9).

BLAST analyses (Altschul et al., 1994) indicated two possible HCF173 homologs in rice (*Oryza sativa*; Figure 9): Os-HCF173-1 consists of 648 amino acids and is encoded on chromosome 6; Os-HCF173-2 has a length of 598 amino acids, and the corresponding gene is localized on chromosome 2. According to prediction programs, both proteins possess an N-terminal chloroplast transit peptide of 89 and 83 amino acid residues, respectively. In pairwise comparisons between the protein sequences of *Arabidopsis* HCF173 and the rice proteins, 74% identical amino acid residues were observed. Additional BLAST searches in the database of *Chlamydomonas* revealed an HCF173 homolog in the green alga. The sequence alignment showed an amino acid identity of 45% and total sequence similarity of 65% between the *Arabidopsis* and the predicted *Chlamydomonas* protein. However, it has to be mentioned that the deduced protein sequence of *Chlamydomonas* HCF173 starts with a Leu instead of a Met residue, suggesting that the 5′-end of the proposed gene model is incomplete. Moreover, BLAST analysis revealed similarities of HCF173 with the UOS1 protein and its homologs (see Supplemental Figure 2 online). UOS1 stands for UV-B and ozone similarly regulated protein 1, as the UOS1 transcript decreases due to UV-B irradiation or ozone application (Sävenstrand et al., 2002). HCF173 and several UOS1 from cyanobacteria (*Nostoc* sp PPC7120 and *Anabaena variabilis* ATCC 29413) and higher plants (*Pisum sativum* and *Arabidopsis*) shared total sequence similarities between 35 and 38% (20 to 23% sequence identity). The distinct role of the UOS1 protein and its site of function are still unknown and therefore allowed no functional prediction for HCF173.

Furthermore, database searches revealed two conserved domains in the protein sequence of HCF173 (Figures 9 and 10). These domains are located between amino acids 161 to 260 and 461 to 560. Many proteins were identified showing sequence similarities with these two regions. The conserved regions of

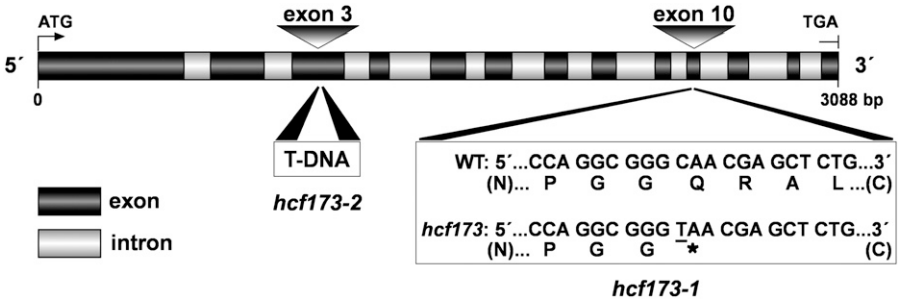

Figure 8. Gene Structure of *HCF173* and Mutant Alleles.

HCF173 represents an open reading frame of 3088 bp consisting of 13 exons separated by 12 introns. Sequence analysis of the mutant allele *hcf173-1* discovered a point mutation at base pair 2524 in exon 10. This base pair exchange causes a premature stop codon. In *hcf173-2* (GABI-Kat line 246C02), the T-DNA insertion was detected in exon 3.

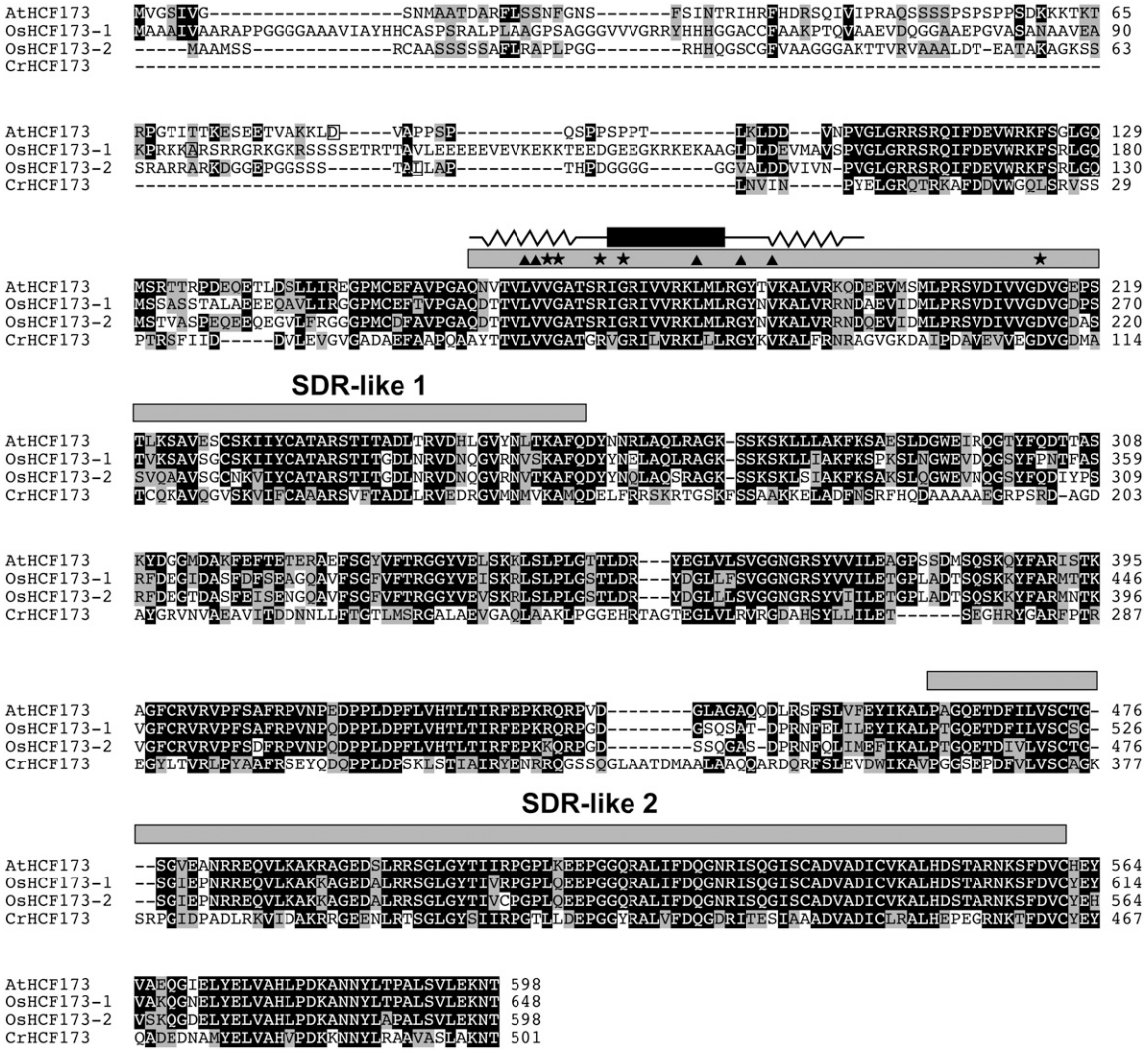

Figure 9. Sequence Alignment of *Arabidopsis* HCF173 (At-HCF173) and Putative HCF173 Homologs from Rice (Os-HCF173-1 and Os-HCF173-2) and *C. reinhardtii* (Cr-HCF173).

Predicted ends of chloroplast transit peptides are boxed: white on black, identical to At-HCF173; black on gray, similar to At-HCF173. Gray bars indicate SDR-like regions 1 and 2. The predicted secondary structure of the putative dinucleotide binding site of HCF173 is schematically indicated above the sequences: black bar, α-helix; zigzag, β-strand. Positions of the Gly-rich motif and the conserved Asp are indicated by asterisks; other conserved amino acid residues in the βαβ-turn are marked by triangles.

HCF173 shared highest sequence similarity with a 219–amino acid YCF39-like protein from *Synechocystis* sp PCC6803, showing 34% identical and 54% similar amino acids (Figure 10). Analysis of the *Synechocystis* protein sequence by the Conserved Domain Database (Marchler-Bauer et al., 2005) revealed highest similarity to the clusters of the orthologous groups COG0451 and COG0702. These clusters comprise nucleoside-diphosphate-sugar epimerases or predicted nucleoside-diphosphate-sugar epimerases, respectively. The epimerases of COG0451 are members of the SDR superfamily. Predictions of the secondary structure of HCF173 with the web tool Jpred (Cuff and Barton, 2000) suggested a pattern of alternating α-helices and β-strands that is typically observed for SDR proteins (Jörnvall et al., 1995). Therefore, we decided to designate HCF173 as SDR-like protein and the two conserved sequence elements as SDR-like domains.

HCF173 Is Associated with Chloroplast Membranes

The N-terminal part of the HCF173 polypeptide was predicted to function as a chloroplast transit peptide. To test this assumption, we transformed heterozygous *hcf173* mutants with two fusion genes encoding C-terminally tagged proteins, which allow the localization of the HCF173 protein: The fusion gene At-HCF173-tHA consisted of *HCF173* and the DNA sequence of a triple hemagglutinin tag. In the second construct, At-HCF173-TAP *HCF173* was fused to the DNA sequence of the so-called twin affinity purification (TAP) tag (Puig et al., 2001). We identified

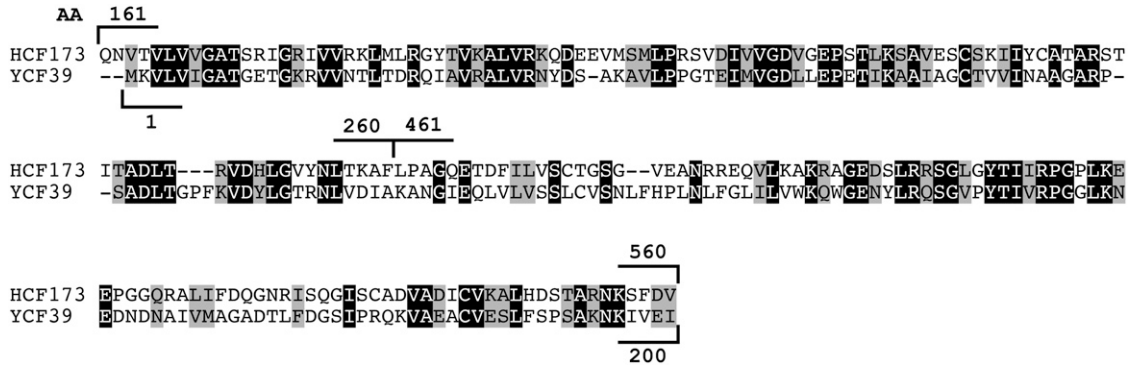

Figure 10. Partial Alignment of *Arabidopsis* HCF173 and a YCF39-Like Protein from *Synechocystis* sp.

Amino acid residues 161 to 260 and 460 to 560 of HCF173 were aligned to the first 200 of a total of 219 amino acid residues of the YCF39-like protein. White letters on black indicate identical residues; black letters on gray mean similar residues.

five transgenic HCF173-ctHA plants and seven transgenic HCF173-cTAP plants that were homozygous for the mutation *hcf173* and were able to grow photoautotrophically on soil. Spectroscopic measurements and analyses of the accumulation of thylakoid membrane proteins confirmed a fully functional PSII and therefore the complementation of the mutant phenotype (data not shown).

For localization of HCF173, we prepared total leaf, total chloroplast, chloroplast membrane, and stroma proteins from HCF173-ctHA and wild-type plants. The proteins were separated by gel electrophoresis, electrotransferred to nitrocellulose membranes, and incubated with a high-affinity antihemagglutinin peroxidase conjugate. In extracts obtained from complemented plants, a protein of ~70 kD could be detected (Figure 11A), which was expected from the HCF173-tHA protein sequence. As predicted, the HCF173-tHA protein was indeed detectable in isolated chloroplasts. The purity of this fraction was confirmed by the cytosolic marker protein α-tubulin. Compared with the total protein fraction, no enrichment of HCF173-tHA was observed in the total chloroplast protein fraction. This stands in contrast with the chloroplast proteins PsaD and RbcL and indicates that the overexpressed protein is not completely imported into the organelle (Figure 11A). Separation of extracts from isolated chloroplasts into membrane and soluble fractions revealed a heterogeneous distribution of HCF173-tHA within the plastid. Approximately 20% of the protein was localized in the stroma, but the bulk of HCF173-tHA was associated with chloroplast membranes. PsaD and RbcL, two proteins that are membrane or predominantly stroma localized, respectively, served as controls for this experiment.

To confirm if the observed membrane association of HCF173-tHA is a genuine feature or rather due to overexpression of the fusion protein, we performed a membrane flotation experiment, which was originally used to separate different membrane systems on a sucrose gradient by their buoyant densities (Cline et al., 1981). To this end, lysed chloroplasts were placed at the bottom of the gradient. During ultracentrifugation, the chloroplast membranes floated through the gradient to a zone of equal density. Proteins that are associated with the membrane system should migrate in the same manner. Figure 11B clearly shows that HCF173-tHA comigrated with PsaD and therefore with chloroplast membranes into fraction 5, whereas RbcL was evenly distributed in the first five fractions. This result revealed that HCF173-tHA is a bona fide membrane-bound protein and not artificially attached to the thylakoids. Washing of chloroplast membranes with sodium chloride did not release the protein from the membrane; however, with sodium carbonate, ~50% of HCF173-tHA was removed (Figure 11C). This finding indicates a firm association of the fusion protein with the chloroplast membranes. Although it is possible that membrane association is mediated by the tHA tag itself, it is unlikely because immunolocalization experiments with fusion constructs of HCF173 containing the TAP tag demonstrated membrane binding as well (data not shown). In addition, the protein Mbb1 was identified as a stroma component of the chloroplast via a triple hemagglutinin tag (Vaistij et al., 2000). Thus, it appears that HCF173 is indeed mainly attached to chloroplast membranes in vivo.

HCF173 Is Part of a Higher Molecular Weight Complex

To investigate whether HCF173 is associated with other proteins, solubilized chloroplast membrane proteins from HCF173-cTAP, HCF173-ctHA, and wild-type plants were analyzed by nondenaturing blue native PAGE (Schägger et al., 1994) in the first dimension followed by separation on denaturing SDS gels in the second dimension. For detection of the fusion proteins, the gels were subjected to immunoblot analyses. As shown in Figure 12A, in the first dimension, the majority of HCF173-TAP was detected in the range between 130 and 150 kD. In addition, minor amounts could be detected in several complexes ranging from ~210 to >670 kD. This finding suggested that HCF173-TAP is part of at least one higher molecular weight complex in vivo. In the case of HCF173-tHA, most of the protein was detected in the range of 100 to 110 kD (Figure 12B). In addition, the protein was located to complexes of ~140, 200, 230, and 300 kD, emphasizing the finding that HCF173 interacts with other factors. It is not yet clear if the strong signals at 130 to 150 kD and 100 to 110 kD, respectively, are caused by HCF173 fusion protein monomers or if the protein is already bound to an interaction partner. Although the monomers are smaller (HCF173-TAP ≈ 90 kD; HCF173-tHA

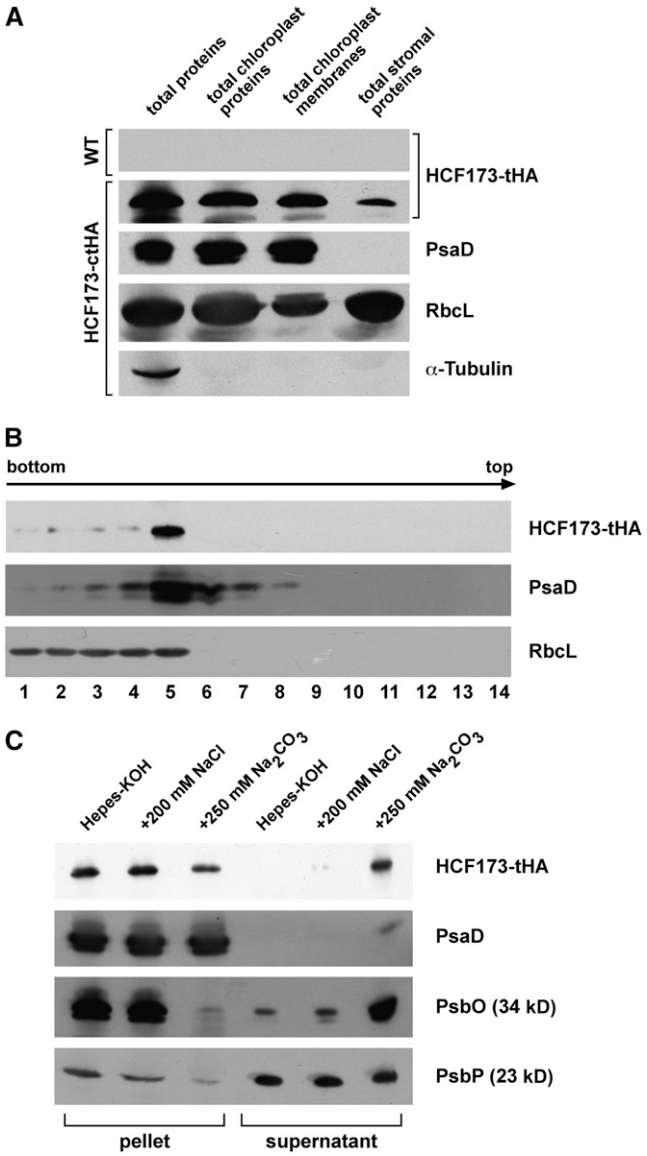

Figure 11. Immunolocalization of HCF173-tHA.

(A) Chloroplasts were isolated from 3- to 4-week-old wild-type and HCF173-ctHA plants by Percoll gradient centrifugation, lysed, and separated into chloroplast membranes and stroma proteins. A protein equivalent to 15 μg of chlorophyll was used for SDS-PAGE and gel blot analysis. In addition, a total leaf protein extract containing the same protein amount as the total chloroplast extract was analyzed.

(B) Intact chloroplasts were isolated from 3-week-old HCF173-ctHA plants by Percoll gradient centrifugation and ruptured in a glass homogenizer. Chloroplast membranes were floated by discontinuous sucrose gradient centrifugation. Fractions (340 μL) were collected from the bottom to the top of the gradient. Aliquots of the fractions were used for SDS-PAGE and gel blot analysis.

(C) Chloroplast membranes were isolated from HCF173-ctHA plants and sonicated in 200 mM NaCl or 250 mM Na_2CO_3, respectively. Proteins of repelleted membranes and the supernatants were separated by SDS-PAGE. The protein equivalent of 20 μg chlorophyll was analyzed. As controls, a membrane-associated protein that is not releasable (PsaD) ≈ 70 kD), the strong intensity of the detected signals indicate that they represent the overexpressed fusion protein monomers.

The HCF173-tHA Complex Is Associated with *psbA* RNA

The involvement of HCF173 in RNA translation and stabilization implies that the HCF173-tHA complex could be associated with RNA. To test this assumption, affinity chromatography was performed with protein extracts from wild-type and HCF173-ctHA plants. Chloroplast membranes were solubilized as described in Methods, and the protein extracts were incubated with an anti-HA affinity matrix. Purified proteins were competitively eluted by HA peptide, and nucleic acids were purified by phenol/chloroform extraction followed by alcohol precipitation. RT-PCR and quantification of RNA amounts were performed as described in Methods. Within the exponential phase of the polymerase chain reactions, the ratios of the transcript amounts obtained by HCF173-tHA affinity chromatography to the transcript amounts purified by wild-type affinity chromatography were determined (Figure 13A). The examined transcripts could be detected in both the wild-type and HCF173-ctHA samples. Therefore, we conclude that the anti-HA affinity chromatography resin unspecifically binds nucleic acids. Nevertheless, the data indicate that in all three independent experiments, the *psbA* mRNA was enriched in the HCF173-ctHA sample as the ratio of *psbA* $mRNA_{HCF173\text{-}ctHA}$ to *psbA* $mRNA_{WT}$ was 2.4- to 6.2-fold higher compared with all other transcript ratios. Moreover, RNA samples of two additional experiments were submitted to slot blot hybridization and analyzed with a *psbA* and a *psbB* probe, respectively (Figure 13B). Both experiments showed a clear enrichment of the *psbA* mRNA in the HCF173-ctHA sample. The factor of *psbA* mRNA enrichment correlates with the different amounts of purified HCF173-tHA (Figure 13C). Therefore, we assume that the HCF173-tHA complex is indeed specifically associated with *psbA* mRNA.

DISCUSSION

PSII is a genetic mosaic consisting of nuclear- and plastid-encoded subunits. Therefore, many predominantly nuclear-encoded auxiliary factors are needed for correct and coordinated biogenesis of PSII. Here, we describe the nuclear mutant *hcf173* of *Arabidopsis*, which is specifically affected in the accumulation of the PSII reaction center protein D1 due to defects in initiation of *psbA* mRNA translation. In addition, impaired stability of the *psbA* mRNA was observed. We report on the identification of the defective nuclear gene by map-based cloning and further discuss possible functions of the HCF173 protein based on biochemical studies with HCF173 fusion proteins.

The Mutant *hcf173* Is Primarily Affected in D1 Expression

Spectroscopic measurements revealed a severe defect in PSII of the *hcf173* mutant, while the cytochrome b_6f complex and PSI

and the 34-kD (PsbO) and 23-kD (PsbP) proteins of the oxygen-evolving complex, which are releasable by sodium carbonate treatment or sonification, respectively, were used.

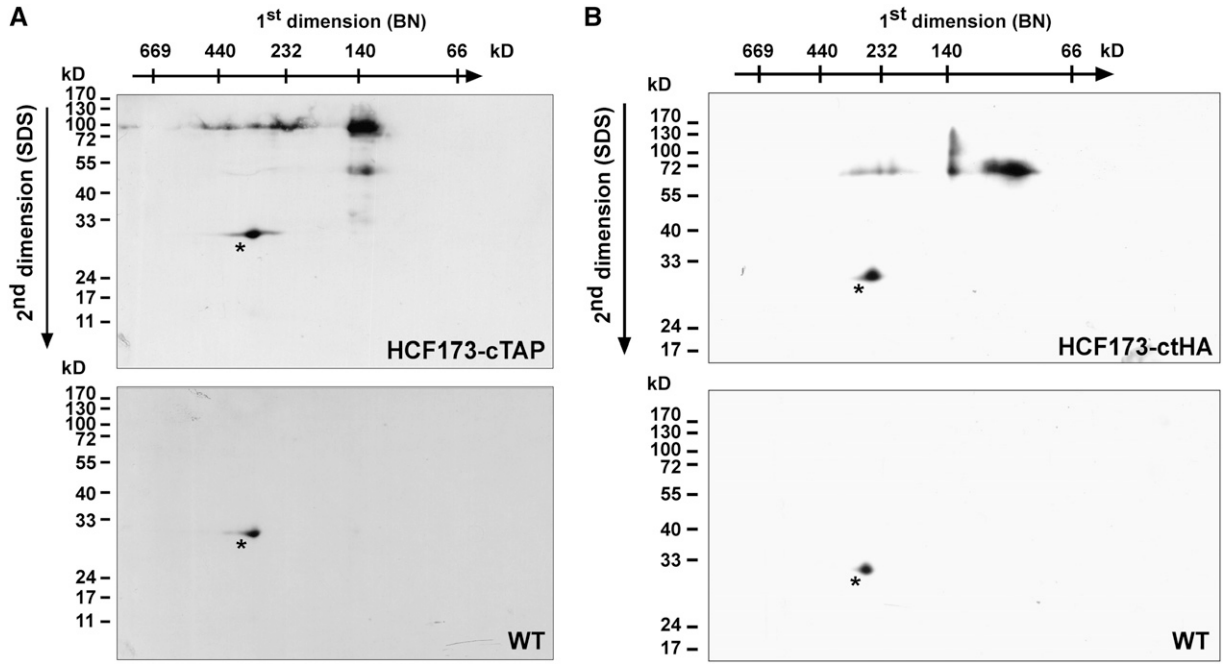

Figure 12. Identification of HCF173-TAP/HCF173-tHA–Containing Complexes by Two-Dimensional Blue Native/SDS-PAGE.

Solubilized chloroplast membrane protein complexes of wild-type, HCF173-cTAP, and HCF173-ctHA plants were separated according to their molecular masses by blue native (BN) PAGE (first dimension). Subsequent denaturing SDS-PAGE separated the complexes into their subunits (second dimension). Complexes containing HCF173-TAP **(A)** or HCF173-tHA **(B)** were identified by gel blot analysis. The cytochrome *f* subunit of the cytochrome b_6f complex dimer was detected due to its heme-associated peroxidase activity and is indicated by an asterisk.

are functional. The levels of thylakoid membrane proteins in the mutant corroborate these results. Whereas polypeptides of the cytochrome b_6f complex, PSI (under low light), and the ATP synthase accumulated to nearly wild-type amounts, subunits of PSII and the oxygen-evolving complex were drastically reduced, though to a different degree. Together, these data indicate that the mutation in *hcf173* primarily affects the accumulation of PSII, whereas the reduction of PSI protein amounts is caused by secondary effects of the mutational lesion. Radioactive labeling of de novo synthesized plastidic proteins indicates that the synthesis rate of the D1 protein was drastically impaired. This result was confirmed by the observation that only a minority of the *psbA* mRNA in *hcf173* mutants was associated with polyribosomes. The reduced D1 synthesis rate was accompanied by a decreased stability of the *psbA* transcript, which resulted in a low steady state level of this RNA. Together, these data provide strong evidence that the nuclear-encoded factor HCF173 is primarily involved in translation of the *psbA* mRNA. The pattern of polysomal association in the mutant indicates that the factor possibly acts during initiation of translation. Moreover, HCF173 may directly or indirectly confer stability to the *psbA* mRNA. Alternatively, high RNA turnover could also be a secondary effect induced by altered ribosomal loading of this transcript in the mutant.

Recently, it was observed that decreased synthesis of D1 in *Chlamydomonas* results in impaired translation of the *psbB* transcript (Minai et al., 2006). This relationship, found also for other photosynthesis proteins, was termed control by epistasy of synthesis (Wollman et al., 1999; Choquet and Wollman, 2002). The polysome association analysis point to a comparable situation in the *Arabidopsis* mutant *hcf173* because the polysome profile of the *psbB* transcript is significantly shifted to smaller polysomes and monosomes, indicating impaired translational efficiency of this transcript. However, pulse labeling experiments do not show a reduced synthesis rate of CP47 and further studies will be necessary to obtain a conclusive result.

The lack of accumulation of other PSII proteins tested indicated an increased degradation rate of these polypeptides in the mutant. This clearly shows that D1 is a prerequisite for stable PSII assembly, which is in accordance with the fact that D1 and D2 together with cytochrome b_{559} and PsbI represent the primary intermediate of PSII assembly (Rokka et al., 2005).

The Identified Nuclear Gene Encodes an SDR-Like Protein That Is Associated with Chloroplast Membranes

HCF173 was predicted to possess a functional chloroplast transit peptide in its N terminus. Immunoblot analyses of isolated chloroplasts confirmed the plastidic localization of HCF173. The separation of chloroplasts into membrane and soluble fractions revealed that minor amounts of the protein are localized in the stroma, whereas most of the protein is associated with the chloroplast membranes, indicating the existence of two different HCF173 pools. This finding was further supported by membrane flotation experiments in which the bulk of the HCF173 fusion protein comigrated with the chloroplast membranes. These

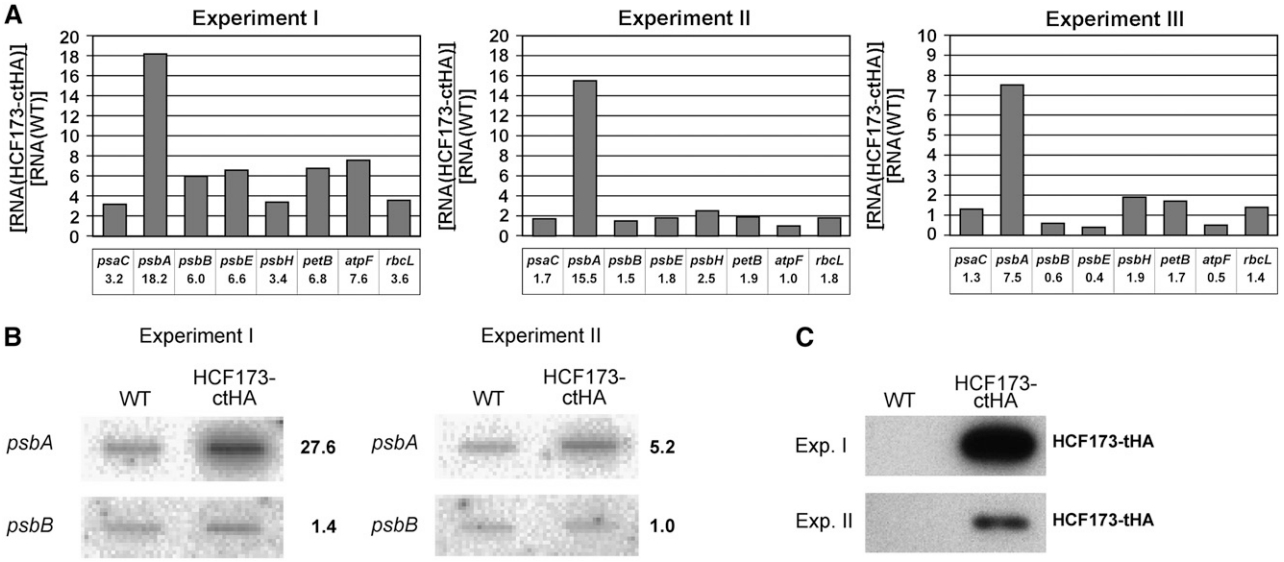

Figure 13. RT-PCR and Slot Blot Hybridization Analysis of Affinity Chromatography Eluates.

Anti-HA affinity chromatography was performed with extracts from wild-type and HCF173-ctHA plants. RNA was purified from the elution samples.
(A) Relative amounts of distinct plastidic transcripts were determined by RT-PCR, DNA gel blot analysis, and phosphor imaging. The ratios of transcript amounts purified from HCF173-ctHA plant extracts and wild-type plant extracts were calculated. Results from three independent experiments are shown.
(B) Purified RNA was submitted to slot blot hybridization with probes for *psbA* or *psbB*, respectively. After phosphor imaging, the ratios of transcript amounts purified from HCF173-ctHA and wild-type plant extracts were calculated and indicated adjacent to the signal. Results from two independent experiments are shown.
(C) HCF173-tHA protein that was purified in parallel to the RNA, which was isolated for slot blot hybridization.

experiments indicate that membrane association of HCF173 is a genuine feature of this protein. Salt washes with sodium chloride or sodium carbonate indicated peripheral membrane binding of HCF173.

The nuclear-encoded factor HCF173 is related to the SDR protein family. SDR proteins are enzymes, which use NAD(P)(H) as coenzymes, function as dehydrogenases, dehydratases, epimerases, and isomerases of a variety of substrates, and consist of 250 to 350 amino acids. To date, >3000 primary protein sequences are known, which typically show 15 to 30% identical residues in pairwise comparisons. Although the sequence similarity among SDR enzymes is low, their three-dimensional structures show highly similar α/β-folding patterns with a central β-sheet (Kallberg et al., 2002; Oppermann et al., 2003). A secondary structure prediction revealed the same pattern for HCF173 in the SDR-like regions. Hence, the typical three-dimensional structure might be maintained in this protein despite the separation of the SDR-like domains by 200–amino acid residues. Classical SDR proteins show a Gly-rich motif (TGXXXGXG) and a conserved Asp residue in the N terminus, which are positioned in a $\beta\alpha\beta$-turn and are needed for coenzyme binding (Filling et al., 2002). Additional residues are conserved in the $\beta\alpha\beta$-turn (Filling et al., 2002) possibly due to structural reasons. Those residues are conserved in HCF173 as well; however, the Thr and the second Gly residue of the Gly-rich motif are changed (Figure 9). These findings suggest that HCF173 possesses no NAD(P)(H) binding activity. However, an alcohol dehydrogenase from *Drosophila melanogaster* was shown to have NAD^+ binding activity, although the Gly-rich motif is not strictly conserved (Sofer and Ursprung, 1968). The enzymatic activity of SDR enzymes is associated with a YXXXK motif (Jörnvall et al., 1995), which forms a catalytic tetrad with a conserved Asn and a conserved Ser residue (Filling et al., 2002). This sequence motif is not conserved in *Arabidopsis* HCF173 and its homologs. From these observations, we predict that HCF173 does not exhibit its original dehydrogenase activity but has possibly gained other functions during evolution.

It has been proposed that several SDR-related proteins have lost their dehydrogenase activity but are adapted to other cellular functions. An interesting example with distant relationship to the SDR family is CSP41, which exhibits endonucleolytic activity (Baker et al., 1998). CSP41 is able to bind to specific RNAs. The protein has been proposed to initiate degradation of plastidic mRNAs and is able to cleave efficiently within the 3′ stem-loop structures of *psbA*, *rbcL*, and *petD* mRNA in vitro (Yang and Stern, 1997). RNA binding is realized by the dinucleotide binding domain of the protein (Bollenbach and Stern, 2003), indicating that the domain has gained a function in chloroplast RNA metabolism. RNA binding via this domain has been shown for several NAD^+-dependent dehydrogenases and (di)nucleotide binding metabolic enzymes that play an additional role in RNA metabolism (Hentze, 1994; Nagy et al., 2000). Therefore, HCF173 might also be an RNA binding protein, which possibly interacts with the *psbA* mRNA, affecting its translational activity and possibly also its stability.

HCF173 Is Part of a High Molecular Weight Complex That Contains *psbA* RNA

Blue native gel electrophoresis indicated that HCF173 is part of a high molecular weight complex, as are several other proteins, which are involved in chloroplast RNA metabolism and/or translation (Fisk et al., 1999; Vaistij et al., 2000; Ostheimer et al., 2003; Sane et al., 2005; Schmitz-Linneweber et al., 2005). Affinity purification of this HCF173-containing complex revealed that the *psbA* mRNA is specifically bound to this complex. What could be the functional significance of this binding to the *psbA* mRNA?

It is known that several proteins are enhancers of RNA translation and/or are necessary for their stability (Barkan and Goldschmidt-Clermont, 2000; Monde et al., 2000; Choquet and Wollman, 2002). Binding occurs via the UTRs of the RNAs mainly in adenine- and/or uracil-enriched sequences and in dependence of stem-loop structures (Klaff et al., 1997; Yang and Stern, 1997; Yohn et al., 1998a; Nickelsen et al., 1999; Ossenbühl and Nickelsen, 2000; Shen et al., 2001; Nakamura et al., 2003; Barnes et al., 2004). Possibly, the binding of the HCF173 complex to the *psbA* mRNA is a prerequisite for successful initiation of translation. In an in vitro translation system of tobacco (*Nicotiana tabacum*), it has been shown that *psbA* mRNA translation is dependent on several *cis*-elements, including RBS1 and RBS2, which are thought to function like a bipartite Shine-Dalgarno sequence for ribosome binding (Hirose and Sugiura, 1996). A third *cis*-element, the so-called AU-box (UAAAUAAA), is located between RBS1 and RBS2. This sequence is essential for translation of the tobacco *psbA* mRNA, and it is thought to bind one or more *trans*-acting factors (Hirose and Sugiura, 1996). The AU-box is conserved in the 5′-UTR of the *psbA* mRNA of *Arabidopsis*, and it is conceivable that HCF173 is able to bind this sequence motif via its dinucleotide binding site (see above) acting as an enhancer of translation initiation. Moreover, this protein binding might protect the *psbA* mRNA against exonucleolytic degradation and/or masks potential endonucleolytic cleavage sites of CSP41 or RNase E/G-like proteins that are thought to initiate chloroplast mRNA decay (Bollenbach et al., 2004).

Several investigations have shown that D1 protein synthesis is coupled to chloroplast membrane systems (Aro et al., 1993; Zhang et al., 2000). Therefore, the observed membrane binding of HCF173 gives rise to the hypothesis that the HCF173 complex could be necessary for membrane targeting of *psbA* mRNA translation. Possibly, the HCF173 complex is required for association of the *psbA* mRNA with chloroplast membranes, and this might be a prerequisite for efficient translation of the transcript. A similar tethering function was proposed for the RNA binding protein RBP63 from *C. reinhardtii* that binds to *psbA* mRNA and stroma thylakoid membranes (Ossenbühl et al., 2002). The existence of HCF173 in the stroma fraction does not contradict this hypothesis. Rather, this protein pool might be involved in binding and trafficking of the stroma-localized *psbA* transcripts to the chloroplast membranes. A detailed study of HCF173 localization inside the chloroplast would contribute to test these assumptions. Moreover, for a functional analysis, it will be of great importance to identify the components of the HCF173 complex. To this end, specific protein tags have been developed to isolate interacting partners of HCF173 by affinity purification.

Taken together, we were able to show that HCF173 is weakly related to the SDR superfamily and is mainly attached to chloroplast membranes where it acts as a part of a high molecular mass complex. Our investigations characterized HCF173 as a nuclear-encoded factor of higher plants that is essential for translation of the D1 encoding *psbA* mRNA. Whether the protein is involved in basic aspects of D1 synthesis or instead is part of the regulatory apparatus adjusting *psbA* mRNA translation remains to be determined in future studies.

METHODS

Growth Conditions

Wild-type and mutant seedlings were grown on sucrose-supplemented Gelrite medium under a 16-h photoperiod at a photon flux density (PFD) of ~50 to 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Meurer et al., 1998). The mutant *hcf173* was selected from a collection of EMS-induced mutants. Mutant plants exhibiting the high chlorophyll fluorescence phenotype were identified under UV light as described (Meurer et al., 1996b). For blue native PAGE and affinity chromatography experiments, plants were grown on soil (Floraton-I; Floragard) in a growth chamber at a PFD of 50 to 70 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a temperature of 21 to 23°C. Plants were illuminated for 8 h (short-day conditions) or 16 h (long-day conditions) per day, respectively.

Spectroscopic Analyses

Chlorophyll fluorescence induction and P700 absorption measurements were performed as described by Meurer et al. (1996b) with the following modifications. Saturating light pulses had a length of 1 s, and actinic light had a PFD of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For the P700 absorbance kinetics, the pulse amplitude–modified fluorometer was modified with the dual-wavelength emitter detector unit ED-P700DW-E (Walz).

SDS-PAGE and Immunoblotting

Crude leaf proteins (Meurer et al., 1996b) from wild-type and mutant plants were separated by SDS-PAGE according to Schägger and von Jagow (1987) and transferred onto nitrocellulose membranes (PH79, 0.1 μm; Schleicher and Schüll). Blotted proteins were immunodecorated with specific antibodies as listed by Meurer et al. (1996b) and visualized by chemiluminescence (ECL; Amersham Biosciences). The fusion protein HCF173-TAP was detected by peroxidase-antiperoxidase soluble complex (Sigma-Aldrich). The protein HCF173-tHA could be identified by two monoclonal antibodies (HA.11 [BabCO] or anti-HA high affinity antibody peroxidase conjugate [Roche]).

In Vivo Labeling of Chloroplast Proteins and Immunoprecipitation

The in vivo labeling and immunoprecipitation reactions were performed as described by Lennartz et al. (2001) with the following modifications. Instead of 5 μL of specific antibody, 10 μL were used for immunoprecipitation to ensure quantitative immunoprecipitation. To solubilize membrane proteins for immunoprecipitation, 2% (w/v) SDS was used. The antibody-antigen-protein A complex was dissociated by incubation in gel loading buffer (100 mM Na_2CO_3, 10% [w/v] sucrose, 50 mM DTE, and 2% SDS). After SDS-PAGE, radiolabeled proteins were analyzed by autoradiography.

RNA Isolation and Gel Blot Analysis

Isolation of total leaf RNA was performed according to Westhoff et al. (1991). For small amounts of RNA or RNA from seedlings, the RNeasy

plant mini kit (Qiagen) was used. Poly(A)$^+$ RNA was obtained by incubation of total leaf RNA from wild-type and mutant plants with OligoTex slurry (Qiagen).

Electrophoresis, gel blot analysis, and hybridization were performed as described before (Westhoff et al., 1991). Hybridization was done with specific RNA probes listed by Westhoff and Herrmann (1988) and Meurer et al. (1996b). Gene-specific probes for *At1g16610*, *At1g16630*, and *At1g16720* were obtained by first-strand cDNA synthesis with total leaf RNA from wild-type plants as template and subsequent amplification by PCR with the following primer pairs: 16610-H2 (5′-CTCGTTCCAGTTCCAGATCTCG-3′) and 16610-R1 (5′-CGATGGGGAGAATCAGGTGATCGC-3′); 16630-H2 (5′-CTTGGTGTGGCGTATCTGTTGTTGG-3′) and 16630-R1 (5′-GGCTACTCCGGTTTGTTCTTGCTGG-3′); 16720-H1 (5′-CTACTGTGCGACTGCTCGGTCTACG-3′) and 16720-R1 (5′-CTCCCGAACCAGTACACGATACCAG-3′).

Polysome Purification

Polysome fractions were prepared as described previously (Barkan, 1993). To disrupt polysomes, EDTA was added to the extraction buffer according to Lu and Hanson (1996).

Measurement of mRNA Stability after Inhibition of Transcription with Actinomycin D

Arabidopsis thaliana wild-type and mutant plants were harvested under water and incubated for 0, 2, 6, 12, 18, and 24 h in 200 μM actinomycin D (Sigma-Aldrich). Prior to this treatment, plants were preincubated in actinomycin D for 30 min to allow proper distribution of the antibiotic. Plants were preilluminated for 4 h before actinomycin D treatment and were kept in the light during incubation. Plants incubated with water served as a control. Total RNA was isolated and quantified after gel blot analysis and hybridization with radiolabeled probes specific for *psbA* and *rbcL. PsbA* and *rbcL* mRNA amounts were normalized using the 16S rRNA levels as internal standard.

Mapping of the *hcf173* Mutation

A mapping population was obtained by crossing wild-type plants of the ecotype Landsberg *erecta* (L*er*) with heterozygous *hcf173* plants, ecotype Columbia (Col). Plants of the F2 and the F3 generation were examined. The linkage group of the *hcf173* mutation was determined with a set of microsatellite and CAPS markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). For fine-mapping, the mapping population was examined with markers listed in The Arabidopsis Information Resource database and markers designed with the information of the Cereon Arabidopsis Polymorphism Collection (www.arabidopsis.org.cereon; Jander et al., 2002). The region of the mutation locus was narrowed down between the two markers SNP711/918 (SNP711/918-H, 5′-CATACAGAACACATACAGCAGCCCCG-3′; SNP711/918-R, 5′-AGGATGGTTCTGCTTCATTCCTGAGC-3′; polymorphism, *Ssp*I restriction site in Col) and SNP872 (SNP872-H, 5′-CTTGAGGCTTTGCGAGATGGTGAGC-3′; SNP872-R, 5′-ACATGTGAGCACACTCAAAGGGAGG-3′; polymorphism, *Rsa*I restriction site in L*er*).

Cloning of *HCF173* cDNA

Full-length cDNA of *At1g16720* was obtained by the use of the SMART RACE cDNA amplification kit (Clontech). Poly(A)$^+$ RNA was used as template in 5′-rapid amplification of cDNA ends (RACE) and 3′-RACE reactions producing overlapping cDNA fragments. Full-length cDNA was constructed via a *Kpn*I restriction site in the overlapping regions. The complete cDNA was subcloned into the vector pCR2.1-TOPO (Invitrogen) and into the binary Ti-vector pPEX001 (kindly provided by B. Reiss, MPIZ Cologne) and renamed pPEXAtHCF173-1. Nucleic acid and protein sequences were analyzed with the programs MacMolly Tetra 3.8 (Softgene) and ClustalW (Thompson et al., 1994). For localization predictions, the internet tools ChloroP (Emanuelsson et al., 1999) and WolfPSORT (Horton et al., 2006) were used. The BLAST server (Altschul et al., 1994), the conserved domain database (Marchler-Bauer et al., 2005), the Pfam database (Bateman et al., 2004), and the SMART database (Schultz et al., 1998; Letunic et al., 2004) were used for protein domain predictions.

Complementation of the Mutant *hcf173*

For complementation of the *hcf173* phenotype, three different constructs were produced. Construct At-HCF173-2 consisted of the *HCF173* cDNA (beginning with the start codon), which was amplified by PCR with the primers 16720-5′-SmaI-H (5′-TCATCCCGGGATGGTGGGTAGTATTGTTGG-3′; the *Sma*I restriction site is underlined), 16720-3′-BamHI-R (5′-CACGGATCCATGCGTATCAGAGCTTGAG-3′; the *Bam*HI restriction site is underlined), and pPEXAtHCF173-1 as template.

The construct At-HCF173-TAP consisted of the *HCF173* cDNA beginning with the start codon fused to the nucleic acid sequence for a C-terminal TAP tag (Puig et al., 2001). The TAP tag comprises a calmodulin binding domain, a TEV protease cleavage site, and two IgG binding domains of the *Staphylococcus aureus* protein A. The TAP tag fragment was obtained by restriction of the pBS1479 vector (Puig et al., 2001) with the enzymes *Nco*I and *Bam*HI. The *HCF173* fragment was amplified by PCR with the primers 16720-5′-SmaI-H and 16720-3′-NcoI-R (5′-CTGACCATGGTGTGTTCTTCTCAAGTACAG-3′; the *Nco*I restriction site is underlined) and pPEXAtHCF173-1 as template.

The fusion gene At-HCF173-tHA was composed of the *HCF173* cDNA beginning with the start codon and the nucleic acid sequence for a C-terminal triple HA tag. The HA tag is an epitope tag, which is derived from the influenza hemagglutinin protein. The triple HA fragment was amplified with the oligonucleotides 3xHANcoI-H (5′-GACTGCCATGGTACCCATATGACGTTCCAGAC-3′; the *Nco*I restriction site is underlined) and 3xHABamHI-R (5′-CAGTGGATCCTCAAGCGTAGTCAGGTACGTCG-3′; the *Bam*HI restriction site is underlined). The plasmid Spa1g3xHA-pBS served as the template, which was kindly provided by U. Hoecker (University Cologne, Germany). This vector contains the triple HA sequence according to Sato and Wada (1997). The *HCF173* fragment was obtained as described for At-HCF173-TAP.

All three cDNA constructs were cloned into the binary Ti vector pPEX001 via the *Sma*I and *Bam*HI restriction sites (pPEXAtHCF173-2, pPEXAtHCF173-TAP, and pPEXAtHCF173-tHA) and thereby fused to the 35S promoter of *Cauliflower mosaic virus*. Subsequently, they were transferred to heterozygous *hcf173* plants by *Agrobacterium tumefaciens*–mediated transformation according to the floral dip method (Clough and Bent, 1998). Complemented transgenic plants were named HCF173-c, HCF173-cTAP, and HCF173-ctHA.

Immunolocalization Studies

For a total leaf protein extract, HCF173-ctHA and wild-type plants were homogenized in protein isolation buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 2 mM EGTA, and 10 mM DTE). Cell debris were separated by Miracloth filtration.

For chloroplast isolation, leaves of 3- to 4-week-old *Arabidopsis* wild-type, HCF173-cTAP, and HCF173-ctHA plants were homogenized in homogenization buffer (330 mM sorbitol, 50 mM HEPES/KOH, pH 7.8, 1 mM $MgCl_2$, 2 mM EDTA, 0.1% [w/v] sodium ascorbate, and 0.25% BSA) with a razor blade on ice. The suspension was centrifugated at 4°C and immediately stopped when a speed of 5900*g* was reached. The pellet was resuspended in homogenization buffer. Chloroplasts were obtained by Percoll gradient centrifugation and lysed in ice-cold lysis buffer (10 mM HEPES/KOH, pH 7.8, 10 mM $MgCl_2$, and 25 mM KCl) for 10 min.

Membrane and stroma proteins were separated by 30 min of centrifugation at 17,000*g* at 4°C. Total leaf, total chloroplast, chloroplast membrane, and stroma proteins of all fractions were separated by SDS-PAGE and subsequently subjected to immunoblot analysis.

Strength of the membrane association of HCF173-tHA was tested by resuspending and sonicating pelleted membranes of lysed chloroplasts in lysis buffer or lysis buffer supplemented with 200 mM NaCl or 250 mM Na_2CO_3, respectively. Membranes were pelleted again for 10 min at 17,000*g* and 4°C. Proteins of the supernatants were obtained by precipitation with 15% (w/v) trichloroacetic acid.

For chloroplast membrane flotation, chloroplasts were lysed in 600 mM sucrose, 10 mM HEPES/KOH, pH 7.8, and 2 mM EDTA and ruptured with 15 strokes in a glass homogenizer. Lysed chloroplasts (500 μL) equivalent to 125 μg of chlorophyll were mixed with 2.6 M sucrose, 10 mM HEPES/KOH, pH 7.8, and 2 mM EDTA to a final sucrose concentration of 1.8 M and placed on the bottom of a centrifugation tube. The chloroplast fraction was overlaid with 2.05 mL of 1.2 M sucrose in 10 mM HEPES/KOH, pH 7.8, 2 mM EDTA, and 1.5 mL of 0.3 M sucrose in 10 mM HEPES/KOH, pH 7.8, and 2 mM EDTA. The gradient was centrifugated in a swinging bucket rotor (SW55Ti; Beckman Coulter) for 18 h at 117,000*g* and 4°C. Fractions (340 μL) were collected starting from the bottom of the gradient. Equal volume amounts were directly subjected to SDS-PAGE and subsequent immunoblot analysis.

Two-Dimensional Blue Native/SDS-PAGE

Leaves of 4- to 6-week-old plants were homogenized in lysis buffer and centrifugated until a speed of 5900*g* was reached. The pelleted chloroplast membranes were resuspended in ACA buffer (750 mM ε-aminocapronic acid, 50 mM Bis-Tris, and 0.5 mM EDTA, pH 7) and solubilized with 1% digitonin in ACA buffer for 40 min at 4°C and a chlorophyll concentration of 1 mg/mL. Unsolubilized material was removed by 20 min of centrifugation at 4°C and 15,000*g*. After supplementation of *n*-dodecyl-β-D-maltoside to 0.5% (w/v) and one-tenth volume sample buffer (50 mM Bis-Tris, 750 mM ε-aminocapronic acid, 30% [w/v] sucrose, and 5% [w/v] Coomassie Serva G), the supernatant was analyzed on a 6 to 12% gradient gel by blue native gel electrophoresis. Depending on the gel size, 20 μg (Hoefer Mighty Small) or 70 μg (Bio-Rad xL) of chlorophyll equivalents were used. Separated protein complexes were analyzed by SDS-PAGE in the second dimension according to Schägger et al. (1994). Immunoblot analysis was performed as described above.

Affinity Chromatography of the HCF173-tHA Complex

Leaves of wild-type and HCF173-ctHA plants were homogenized in HEBER A homogenization buffer (330 mM Sorbit, 44 mM MES, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 2 mM EDTA, and 0.1% [v/v] β-mercaptoethanol, pH 6.1). The suspension was centrifugated at 4°C and immediately stopped when a speed of 5900*g* was reached. Pelleted chloroplast membranes were resuspended in Tris-buffered lysis buffer plus protease inhibitors (Complete, mini, EDTA free; Roche) and solubilized with digitonin as described before. After supplementation of *n*-dodecyl-β-D-maltoside to 0.5% (w/v), the supernatants were rotated in the cold room for 1.5 h with a bed volume of 200 μL anti-HA affinity matrix (Roche). At room temperature the matrix was washed with 20 bed volumes of washing buffer (20 mM Tris, pH 7.8, 150 mM NaCl, 0.1 mM EDTA, 0.1% [w/v] digitonin, and 0.05% *n*-dodecyl-β-D-maltoside) plus protease inhibitors. Bound proteins were eluted by incubating the affinity matrix three times with 1 volume of elution buffer (1 mg/mL HA peptide in 20 mM Tris, pH 7.8, 150 mM NaCl, and 0.1 mM EDTA) for 15 min at 37°C and in the presence of RNase inhibitors. The eluted fractions were pooled, and nucleic acids were purified by phenol/chloroform/isoamylalcohol (25:24:1) extraction and overnight precipitation at –20°C by addition of sodium acetate to 0.3 M and 2.5 volumes ice-cold ethanol and yeast RNA as coprecipitant. Contaminations caused by DNA were removed by DNase I treatment for 30 min at 37°C in the presence of RNase inhibitors. After heat inactivation of DNase I in the presence of 0.4 mM EDTA, the samples were subjected to RT-PCR. For both reactions, gene-specific oligonucleotides were used (psaC-F, 5′-TAGGATGTACTCAATGTGTCCGAGC-3′; psaC-R, 5′-AGGCGGATTCACATCTCTTACAACC-3′; psbA-F, 5′-CAAGCTCTCAATTATCTACTTAGAG-3′; psbA-R, 5′-CAGTTACAGAAGCGACCCCATAGG-3′; psbB-F, 5′-GAATTGTTCCATTATTACTAACAG-3′; psbB-R, 5′-GTCTCCACATTGGATCAAGAACG-3′; psbE-F, 5′-AGCACAGGAGAACGTTCTTTTGC-3′; psbE-R, 5′-TATTAATGGAATGCCTTGTCGGC-3′; psbH-F, 5′-GAATACAAAATAAGATTTATGGC-3′; psbH-R, 5′-AATACCGCAAATAGAGCCATTGC-3′; petB-F, 5′-GTCTTGAGATTCAGGCGATTGC-3′; petB-R, 5′-GACCATCGATGAACTGATCGG-3′; atpF-F, 5′-TCAGAAGAACTGCGTGAAGGAGC-3′; atpF-R, 5′-GTTGGAAAACCCGTTCGCGGAC-3′; rbcL-F, 5′-GAGTAACTCCTCAACCTGGAGTTCC-3′; rbcL-R, 5′-GGCCATTATCTCGGCAATAATGAGAC-3′). After each third PCR cycle (cycle 3, 6, 9, 12, 15, 18, and 21), samples were taken. The samples were separated by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized with gene-specific radioactive probes that were produced by PCR with the primers indicated above. Radioactive signals of the 12th, 15th, 18th, and 21st PCR cycle samples were quantified by phosphor imaging and drawn toward PCR cycles in a half logarithmic manner. For PCR cycles within the exponential phase of the polymerase chain reactions, the ratios of the transcript amounts obtained by HCF173-tHA affinity chromatography to the transcript amounts purified by wild-type affinity chromatography were determined.

For slot blot hybridization, RNAs were isolated as described above with the following modifications. Glycogen was used as coprecipitant, and purified proteins were rescued from the interphase of the phenol/chloroform/isoamylalcohol extraction, separated by SDS-PAGE, and subsequently subjected to immunoblot analysis. RNA samples were divided and applied to two nylon membranes via a slot blot manifold. The filters were hybridized with strand-specific probes for *psbA* and *psbB*. Probes were synthesized using the Klenow fragment and the oligonucleotides psbA-R or psbB-R.

Accession Numbers

The accession number of the protein that is encoded by the *HCF173* gene is NP_173116. Accession numbers of proteins described in the text are as follows: BAD53796 (Os-HCF173-1), BAD10524 (Os-HCF173-2), and NP_441422 (YCF39-like, *Synechocystis* sp). The protein ID 113913 (Cr-HCF173) corresponds to the deduced amino acid sequence of the *Chlamydomonas reinhardtii* gene model e_gwW.2.317.1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. In Vivo Protein Synthesis in Wild-Type Plants and *hcf173* Mutants.

Supplemental Figure 2. Sequence Alignment of *Arabidopsis* HCF173 and UOS1 Proteins from *Arabidopsis*, *P. sativum*, and *A. variabilis*.

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