Running head: Involvement of HCF243 in PSII D1 Stability

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Title: HCF243 Encodes a Chloroplast-localized Protein Involved in the D1 Protein Stability of the Arabidopsis PSII Complex

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Numerous auxiliary nuclear factors have been identified to be involved in the dynamics of the photosystem II (PSII) complex. In this study, we characterized the high chlorophyll fluorescence 243 (hcf243) mutant of Arabidopsis, which shows higher chlorophyll fluorescence and is severely deficient in the accumulation of PSII supercomplexes compared to wild type. The amount of core subunits was greatly decreased while the outer antenna subunits and other subunits were hardly affected in hcf243. In vivo protein-labeling experiments indicated that the synthesis rate of both D1 and D2 proteins decreased severely in hcf243, whereas no change was found in the rate of other plastid-encoded proteins. Furthermore, the degradation rate of the PSII core subunit D1 protein is higher in hcf243 than the wild-type and the assembly of PSII is retarded significantly in the hcf243 mutant. HCF243, a nuclear gene, encodes a chloroplast protein that interacts with the D1 protein. HCF243 homologs were identified in angiosperms with one or two copies, but lack in lower plants and prokaryotes. The results suggest that HCF243, which arose after the origin of the higher plants, may act as a cofactor to maintain the stability of D1 protein and promote the subsequent assembly of the PSII complex.
INTRODUCTION

Photosystem II (PSII) is a multisubunit protein-pigment complex embedded in the thylakoid membrane, which harnesses light energy to split H₂O into O₂, protons and electrons. It comprises >20 different subunits, most of which are integral membrane proteins, and bind numerous cofactors (Wollman et al., 1999; Iwata and Barber, 2004; Nelson and Yocum, 2006). The PSII reaction center complex is composed of the D1 and D2 proteins, the α- and β-subunits of cytochrome b₅₅₉, and the PsbI protein. The D1 and D2 heterodimer binds all the essential redox components of PSII required for primary charge separation and subsequent electron transfer (Nanba and Satoh 1987). In addition, PSII core complexes also contain the intrinsic chlorophyll a binding proteins (CP43 and CP47), the extrinsic oxygen-evolving complex (33-, 23-, and 17-kD proteins), and the other low molecular mass proteins (Bricker and Ghanotakis, 1996). The functional form of PSII in the thylakoid membrane consists of the PSII core and the associated light-harvesting complex (Nelson and Yocum, 2006).

Despite considerable advances in the elucidation of the structure and function of PSII, knowledge of the assembly of this multiprotein complex is only in its infancy. A number of studies have proved that this assembly is likely to involve multistep processes (Rokka et al., 2005). The first step is formation of the PSII reaction center, in which the D1 protein is incorporated into a precomplex, probably consisting of the D2, cytochrome b₅₅₉, and PsbI proteins (Adir et al., 1990; van Wijk et al., 1997; Müller and Eichacker, 1999; Zhang et al., 1999). Subsequently, CP47 and CP43, core antenna subunits that bind chlorophyll a, are recruited to form the PSII core complex, which allows further binding of the oxygen-evolving enhancer (OEE) proteins, including PsbO, P and Q. D1, D2 and CP47 appear to accumulate in a coordinated manner (Jensen et al., 1986; de Vitry et al., 1989; Yu and Vermaas, 1990). CP43 is synthesized independently and is a dynamic component of PSII, with dissociation and reassociation constantly cycling (de Vitry et al., 1989; Zhang et al., 2000). Integration of the low molecular mass proteins into PSII has been found to occur at different stages of the PSII assembly process (Hager et al., 2002; Suorsa et al., 2004; Rokka et...
al., 2005). The final establishment of a functional PSII involves the dimerization of PSII monomers and the association of LHCII trimers (Rokka et al., 2005).

Because of the structural complexity of PSII, the functional assembly of this highly complex oligomeric protein should require precisely controlled mechanisms. Specific mechanisms should operate to allow the stoichiometric accumulation of the various subunits encoded by the two genetic compartments required for the PSII assembly (Choquet and Vallon, 2000). So far, a number of nucleus-encoded auxiliary and regulatory proteins have been identified which should be involved in this dynamic process (Goldschmidt-Clermont, 1998; Barkan and Goldschmidt-Clermont, 2000; Leister, 2003). For example, although plastid-encoded proteins are normally synthesized in the hcf136 mutant of Arabidopsis, the assembly of PSII reaction centers is blocked and no stable PSII complexes appear to accumulate because of the lack of the HCF136 protein (Meurer et al., 1998; Plücken et al., 2002). Similarly, accumulation of PSII supercomplexes is defective in the lpa2 mutant of Arabidopsis, the LPA2 protein directly interacts with ALB3 and may form a complex that is specifically involved in the efficient assembly of CP43 within PSII (Ma et al., 2007).

Furthermore, photosynthetic water splitting is inevitable coupled with the formation of reactive oxygen species followed by photooxidative damage and repair cycle of PSII (Anderson et al., 1997; Keren et al., 2005). During this progress, D1 protein is the main target for light-induced damage among PSII proteins (for reviews, see Prasil et al., 1992; Aro and Andersson, 1993), and it undergoes a very high light-dependent turnover, and shows very high rates of both synthesis and degradation (Prazil and Ohad, 1992). The damaged D1 protein depletion should be considered as the first phase in the repair cycle of PSII, much effort has been made to identify the proteases responsible for the degradation of the D1 protein (Adam and Clarke, 2002; Yoshioka and Yamamoto, 2011). The photodamaged D1 protein is replaced constantly with newly synthesized D1 protein which has been described as co-translational event to maintain PSII in a functional state (Aro and Andersson, 1993). Therefore, many researches were focused on the D1 turnover and numerous regulation factors have been reported. For example, PAM68, a conserved integral
membrane protein found in cyanobacterial and eukaryotic thylakoids, has also been suggested to play a role in the stability and maturation of D1 (Armbruster et al., 2010).

LPA1 appears to be an integral membrane chaperone that assists efficient PSII assembly through direct interaction with D1 (Peng et al., 2006) and REP27 is essential for D1 protein turnover, permitting completion of the translation process, maturation, and activation of D1 into a functional PSII reaction center complex (Park et al., 2007). Ossenbühl et al. (2006) further found that Srl1471p, a homolog of ALB3, is essential for integrating the D1 precursor into the thylakoid membrane, leading to accumulation of the D1 precursor in the membrane phase.

Despite these advances, the assembly and stabilization of the D1 protein in PSII and its regulation mechanism remain poorly understood (Aro et al., 1993; Yokthongwattana and Melis, 2006). Screening mutants with altered chlorophyll fluorescence has been proved to be a specific and efficient way to dissect the molecular mechanisms underlying the biogenesis of photosystem (Peng et al., 2006), since alterations in chlorophyll fluorescence indicate defects in the photosynthetic electron transport chain, which may result from changes in the structure or function of the thylakoid membrane (Miles, 1994). In this study, we characterized the high chlorophyll fluorescence 243 (hcf243) mutant of Arabidopsis. RNA gel-blot and immunoblotting analyses revealed that plastid-encoded mRNAs for PSII core subunits were present in the mutant, but the corresponding subunits were dramatically reduced. Protein-labeling studies revealed that the accumulation of D1 protein was significantly reduced in hcf243 mutant. The results indicate that the HCF243 gene encodes a cofactor that is involved in the D1 dynamics and subsequently the stability and assembly of the PSII complex.

RESULTS

Phenotype of the hcf243 Mutant

To investigate genes involved in the biogenesis of the PSII complex, we screened the T-DNA mutant collection from the Arabidopsis Biological Resource Center (Weigel et
for the high chlorophyll fluorescence phenotype, which was reported previously (Meurer et al., 1996; Peng et al., 2006), and isolated hcf243, a previously unidentified mutant. Genetic analysis showed that the hcf243 mutation is recessive. The phosphinothricin resistance marker carried by the T-DNA and the hcf243 mutant phenotype cosegregated, indicating that the mutation was due to the T-DNA insertion (data not shown).

In addition to the high chlorophyll fluorescence phenotype, we found that plant growth was also affected in the hcf243 mutant (Fig. 1A). The inflorescence stems of the hcf243 mutant were shorter in height, and its rosette leaves were paler and smaller in size (Fig. 1A). The leaf areas of 6-week-old hcf243 mutant were approximately 70% smaller than the wild type (WT) (Fig. 1B). The high chlorophyll fluorescence phenotype in hcf243 indicates the impaired photosynthesis, which in turn results in the phenotypes of pale leaf and the reduced plant growth.

PSII Activity is Dramatically Reduced in the hcf243 Mutant

Noninvasive fluorometric analyses were performed to investigate the photosynthetic characteristics of the hcf243 mutant. Chlorophyll fluorescence induction experiments revealed that the ratio of variable fluorescence to maximum fluorescence (Fv/Fm) (indicating the maximum potential of photochemical reactions of PSII) was dramatically reduced in the hcf243 mutant (0.42 ± 0.02) compared with that of WT plants (0.82 ± 0.03) (Fig. 2A), indicating that the hcf243 mutant has defects in energy transfer within PSII. Furthermore, it is noteworthy that P700 content was lower in the hcf243 mutant than in WT plants (Fig. 2B), suggesting that P700 might be partially oxidized, but PSI is functional in the hcf243 mutant, as observed in both lpa1 and lpa2 mutants (Peng et al., 2006; Ma et al., 2007). Clearly, these findings demonstrate that hcf243 mutation causes a dramatic decrease in PSII activity.

HCF243 is Involved in the Induction Kinetics of Chlorophyll Fluorescence

To determine the genetic basis of the hcf243 phenotype, the genomic regions flanking the left border of the T-DNA were isolated by TAIL PCR. Sequence analysis showed
that the T-DNA was inserted in the 5' untranslated region of At3g15095. There are three different gene models in the TAIR database about At3g15095. The cloned sequence of HCF243 gene is consistent with At3g15095.1 and has been submitted to Genbank (Accession No. HM748832). To evaluate the effect of the T-DNA insertion on gene expression, RT-PCR and Northern blot analysis revealed that expression of the HCF243 gene in the isolated mutant was barely detectable compared with that in WT plants (Figs. 3A and 3B). Further immunoblot analyses with the HCF243 polyclonal antibody, which was raised against recombinant HCF243 protein (amino acids 221 to 408), also showed that no signal was detected in the total protein preparations (Fig. 3C).

We recovered homologous genes or protein sequences from all angiosperm species with complete genome sequences. However, no corresponding homologous genes were identified in lower plants and prokaryotes, for example, Selaginella moellendorffii, Physcomitrella patens and Cyanobacteria. The predicted protein sequences of all homologous genes are highly variable and no distinct domain was identified (Fig. S1). These aligned proteins are 36% to 86% (averaged 48%) identity to one another. Two copies were found for four species, namely Zea mays, Populus trichocarpa, Glycine max and Manihot esculenta, while only one was recovered for the others. It remains unknown whether the homologous genes exist in the gymnosperms because the complete genome sequence of this group is not reported. The comparison suggests that HCF243 is a novel gene and may be only found in higher plants. Phylogenetic analysis further suggested that two copies in a few angiosperm species due to duplication occurred independently after the origin of each separate lineage (Fig. S2). In addition, in some such lineage (for example, the Sorghum-Zea lineage), one duplicated copy seem to has lost again (Fig. S2).

To prove that the disruption of At3g15095.1 gene was responsible for the phenotypes observed in the hcf243 mutant, the isolated full-length cDNA of HCF243 was fused to the CaMV 35S promoter in the plant transformation vector pSN1301, and introduced into a homozygous hcf243 mutant using the floral dip method by means of Agrobacterium tumefaciens. Twenty-five independent transgenic plants
were regenerated. The protein levels of HCF243 in the complemented plants were comparable with that in WT plants (Fig. 3C), and their chlorophyll fluorescence induction kinetics were also indistinguishable from those of WT plants (Fig. 2A). These results unequivocally demonstrate that the disruption of the *At3g15095.1* gene is responsible for the phenotypes of *hcf243* mutant.

**Thylakoid Membrane Protein Composition in the *hcf243* Mutant**

A block in energy transfer within PSII found in the *hcf243* mutant could be the result of a defect in the PSII complex. To address this possibility, we assayed putative structural alterations of photosynthetic protein complexes in the mutant, and the chlorophyll–protein complexes were subjected to BN/SDS-PAGE analysis. After the first-dimensional separation in the presence of Coomassie blue G-250 dye, six major chlorophyll–protein complexes, marked I–VI, were revealed (Fig. 4A). Those positions of numerous thylakoid membrane complexes were identified from similar gels by immunoblot analysis with distinct antibodies, and apparently represented PSII supercomplexes (band I), monomeric PSI and dimeric PSII (band II), monomeric PSII (band III), CP43 minus PSII (band IV), trimeric (band V), and monomeric LHCII (band VI) (Li *et al.*, 2003; Guo *et al.*, 2005). As clearly shown in Figure 4A, PSII supercomplexes were almost absent, and both band II (monomeric PSI and dimeric PSII) and band III (monomeric PSII) were dramatically reduced in the mutant compared with WT plants. Analyses of the two-dimensional SDS-urea-PAGE gels after Coomassie blue staining also confirmed that the relative amounts of subunits of PSII protein complexes were greatly reduced in the mutant (Fig. 4B). In addition, to verify the steady-state levels of the thylakoid protein change, immunoblot analysis with specific antibodies against the subunits of photosynthetic protein complexes were performed. Our results showed that levels of the plastid-encoded PSII core subunits D1, D2, CP47 and CP43 were dramatically reduced in the *hcf243* mutant compared with those in WT plants, interestingly, alerted accumulation of the D1 precursor was absolutely detected in the mutant (Fig. 4C), whereas no significant changes were found in either plastid-encoded proteins (PsaA/B, Cytf, Cytb6) or
nuclear-encoded proteins (LHCII, PsbO) (Fig. 4C), which is consistent with the results of two-dimensional SDS-urea-PAGE gels (Fig. 4B). Altogether, the accumulation of PSII and its core subunits is significantly affected due to interruption of HCF243, which should confer for partially inefficient PSII activity proved above by the spectroscopical analyses (Fig. 2).

Steady-state mRNA Levels and Polysome Association of the hcf243 PSII Core Subunits

To test whether the significant reductions observed in the mutant’s PSII complexes resulted from one or more of impaired transcription of PSII core subunits, the effect of the hcf243 mutation on transcription of PSII core subunits was investigated by northern blot analysis. The results clearly showed that the amounts of psbA, psbB, psbC, and psbD (encoding the D1, CP47, CP43 and D2 subunits of PSII, respectively) were almost identical in the mutant and WT plants (Fig. 5A). The protein synthesis capacity of chloroplasts were further studied by analyzing changes in the polysome association of psbA, psbB, psbC, and psbD transcripts following sucrose gradient fractionation. Interestingly, no obvious differences were observed in the association of these transcripts with polysomes between the mutant and WT plants (Fig. 5B).

In vivo Synthesis and Stabilization of the PSII Core Subunits in the hcf243 Mutant

To determine whether the impaired accumulation of the subunits of the PSII complex is caused by decreased translation or accelerated degradation, 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. As shown in Figure 6A and 6B, the rates of synthesis of subunits CP43 and CP47 of PSII, proteins A / B of PSI reaction center, and the subunits of the chloroplast ATP synthase (CF1$\alpha$/$\beta$) in the the 12-day-old and 4-week-old mutant were comparable to those in their WT counterparts. In contrast, the incorporation of $^{35}$S-methionine label into the D1 and D2 polypeptide was reduced
significantly, especially D1 protein. When the time of pulse labeling was extended to 30 min, the amount of radioactivity incorporated into D1 in the hcf243 mutant was obviously increased compared with that occurring after 15-min labeling (Fig. 6C). Pulse labeling for 30 min was followed by a chase with unlabeled methionine to monitor the turnover rates of plastid-encoded proteins in young seedlings. The results showed that the turnover rate of D1 protein is much more strongly affected than that of other subunits in the hcf243 mutant (Fig. 6C). To study the assembly of photosynthetic protein complexes, the $^{35}$S-methionine labeled thylakoid membrane proteins were separated by BN gel electrophoresis. After a 15-min pulse, most of the radiolabeling was found in protein complexes as shown in Figure 6D. Compared with WT plants, the incorporation of radioactivity into monomeric PSI superimposed on the PSII dimer (band II), monomeric PSII (band III), CP43-free PSII monomer (band IV) were dramatically reduced in the hcf243 mutant. During the chase period, those protein complexes significantly increased in the autoradiogram obtained after a 60-min chase in the WT plants. However, as the chase time increased, the accumulation of radioactivity in those complexes is barely increased in the hcf243 mutant. These results indicated that assembly of PSII is partially inefficient in the hcf243 mutant because of the lack of HCF243 protein.

**The HCF243 Protein is Targeted to Chloroplast and Interacts with the D1 Protein* in vivo*

Sequence analyses predicted that the N-terminal sequence of HCF243 protein is rich in positive and hydroxylated amino acid residues, which is characteristic of chloroplast transit peptides. Further sequence analysis predicted HCF243 to be a chloroplast-localized protein using the ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/) (data not shown). To determine the actual subcellular location of HCF243, yellow fluorescent protein (YFP)-tagged HCF243 construct was transformed into *Nicotiana benthamiana* leaf epidermal cells. It was demonstrated that HCF243-YFP signals were co-localized with the chloroplast autofluorescence signals in the same cell (Fig. 7A). Therefore, our results demonstrate that HCF243 is a
chloroplast-localized protein.

To test whether HCF243 interacts with the D1 protein in plant cells in vivo, bimolecular fluorescence complementation (BiFC) analysis was performed by using *Nicotiana benthamiana* leaf epidermal cells as a transient expression system. In this approach, the yellow fluorescent protein (YFP) molecule is split into amino-terminal (nYFP) and carboxy-terminal (cYFP) non-fluorescent portions. Restoration of the YFP fluorescence signal is observed when nYFP and cYFP are brought together as fusions with interacting proteins (Tzfira *et al*., 2004). Coexpression of both nYFP-tagged HCF243 and cYFP-tagged D1 resulted in significant fluorescence in *Nicotiana benthamiana* leaf epidermal cells (Fig. 7B). This result suggests that the HCF243 protein interacts with the D1 protein. Moreover, coexpression of nYFP-HCF243 with cYFP-D1 further verified the chloroplast localization of the HCF243 protein. No fluorescence was found in *Nicotiana benthamiana* leaf epidermal cells with transformation of either nYFP-HCF243, cYFP-D1, or both nYFP and cYFP respectively (Fig. 7C–7E). Collectively, these results demonstrate that HCF243 is a chloroplast-localized protein and interacts with the D1 protein.

To further investigate whether HCF243 is a transmembrane protein, the WT membrane fractions were isolated, and then the protein was subjected to immunoblot analysis. HCF243 protein was still retained in the membranes when membrane preparations were sonicated in the presence of various salts described in method (Fig. 8A). During these treatments, PsbO (the 33-kD luminal protein of PSII) and CP47 (the PSII core protein) were used as controls. Above results showed that HCF243 is an intrinsic membrane protein.

To provide further evidence for such interaction, pull-down experiments were performed with recombinant HCF243 protein fused with N-terminal His tags. The purified His-HCF243 fusion proteins were incubated with DM-solubilized thylakoid membranes, and then Ni-NTA resin-bound proteins after washing with the buffer described in methods were separated by SDS-PAGE and examined by immunoblot analysis. As shown in Figure 8B, D1 protein was detected with D1 antibody when His-HCF243 fusion proteins were used in the assay, but no signal was detected when
only the solubilized thylakoid membrane and the resin were used. However, PSII core
protein CP47 was not detected when His-HCF243 fusion proteins were used. These
results indicated a direct interaction between HCF243 and D1 proteins.

DISCUSSION

Considerable progress has been made in recent years in identifying and defining
nuclear genes that regulate the biogenesis and assembly of both the chloroplast- and
nucleus-encoded proteins into the PSII of Arabidopsis. These nuclear genes act as
additional auxiliary and regulatory factors to assist multistep processing in the
biogenesis and assembly of the PSII complex (Goldschmidt-Clermont, 1998; Barkan
and Goldschmidt-Clermont, 2000; Rochaix, 2001; Leister, 2003). The identification
and molecular characterization of these factors are essential for our understanding of
the ways in which photosynthetically active protein complexes are assembled and
functionally maintained. Here we present evidence that HCF243, a novel
nuclear-encoded gene, is also critical for biogenesis and assembly of the Arabidopsis
PSII, which marks an important step toward further understanding this process.

Sequence analyses predict that HCF243 is localized to chloroplast, which then
was confirmed by the actual subcellular location (Fig. 7A and 8A). This location
further suggested that the chloroplast-localized HCF243 protein might have a
biological function involved in the biogenesis of the PSII complex. This implication is
in fact substantiated by the T-DNA mutation. Mutation of the HCF243 gene resulted
in a severe defect in the leaf size and growth of plants (Fig. 1A). In addition, this
mutation caused a dramatic reduction in the PSII activity, implying a deficiency in
PSII function (Fig. 2A). Thorough analyses of the thylakoid membrane complexes
isolated from leaves of hcf243 mutant indicate a dramatic reduction in the content of
both the PSII complex and its reaction core subunits (D1, D2, CP43 and CP47)
compared with WT plants (Fig. 4). Interestingly, a dramatic increase in accumulation
of the D1 precursor was detected in the mutant (Fig. 4C), which has also been
observed in pam68 (Armbruster et al., 2010) and hcf136 plants (Meurer et al., 1998).
In contrast, no significant difference in the contents of either plastid-encoded proteins
(PsaA, Cytf and Cytb6) or nuclear-encoded proteins (LHCII and PsbO) was observed between the mutant and WT plants (Fig. 4C). Taken together, these results indicate that HCF243 should have been involved in the biogenesis of the PSII complex. This is consistent with previous observations that LPA1 and LPA2 are specifically localized in the chloroplast and are required for the biogenesis of the PSII complex (Peng et al., 2006, Ma et al., 2007).

To gain insights into the molecular function of HCF243, it was critical to pinpoint the malfunctioning in accumulation of the PSII core subunits in the hcf243 mutant. There are several possible explanations for the reduced content of the PSII core subunits. The similar abundance and patterns of PSII core subunit gene transcripts in both the hcf243 mutant and WT plants were detected, indicating that the reduced PSII core subunits are not due to the absence of transcripts encoding one or more of these PSII proteins. Further polysome analyses indicated that initiation of the translation of PsbA, PsbB, PsbC and PsbD mRNA was not altered in the hcf243 mutant (Fig. 5B). Therefore, the reduced accumulation of the PSII core subunits may result from impaired translation or, alternatively, accelerated degradation of the PSII subunits once they have been synthesized.

As shown in Figure 6, the accumulation rate of both D1 and D2 proteins decreased severely in the hcf243 mutant, especially the significantly reduced content of the D1 protein. However, the synthesis rate of the other plastid-encoded proteins was barely affected (Fig. 6A and 6B). A possible explanation for the decreased accumulation of the D1 and D2 proteins in the hcf243 mutant may be that their degradation rates were higher than those in WT plants. Similar phenomenon was found in cyanobacteria that inactivation of the PSII genes has no severe effects on the translation of their respective proteins, but instead appears to accelerate degradation of the close assembly partners (Yu and Vermaas, 1990, 1993). To monitor the degradation rates of D1 and D2 proteins in hcf243 mutants, chase experiment with unlabeled methionine was performed after pulse labeling for 30 min (Fig. 6C). The results showed that a significantly increased degradation rate of D1 protein in the hcf243 mutant was detected compared with that of WT plants. Interestingly, previous
researches showed that deficiency of some special proteins results in the increased
degradation of D1, suggesting that these proteins may be required for the D1 stability
(Choquet and Vallon, 2002; Choquet and Wollman, 2002). Similar results have also
been observed in the Arabidopsis lpa1 mutant, in which synthesis of D1 is indicated to
be considerably reduced owing to accumulation of the unassembled D1 protein in the
membrane (Peng et al., 2006). Furthermore, the increased degradation of D1 protein
should impact the assembly of PSII. Indeed, as shown in Figure 6D, the assembly of
PSII in the hcf243 mutant is retarded significantly. Taken together, it can be
speculated that the rapid degradation of the D1 protein may result from the impaired
stability of the protein in hcf243 mutant, which is further supported by HCF243
localization and its interaction with D1 protein (Fig. 7 and 8). Our bioinformatics
study of the known homologs of HCF243 in plants suggested that homologous genes
were found only in angiosperms with one or two copies existing in each species, but
no such homologous genes in lower plants or prokaryotes (Fig. S2). Our results
suggest that HCF243, which arose after the origin of the high plants, may act as an
important cofactor to maintain the stability of D1 protein and promote the subsequent
assembly of the PSII complex.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (ecotype Columbia) was grown under short-day conditions (10-h
light/14-h dark cycle) with a photon flux density of 120 µmol m⁻² s⁻¹ in soil at a
constant temperature of 20°C. To ensure synchronized germination, the seeds were
sown in darkness for 48 h at 4°C. The hcf243 mutant was isolated as a recessive high
chlorophyll fluorescence plant in a collection of pSKI015 T-DNA–mutagenized
Arabidopsis lines from the Arabidopsis Biological Resource Center (Weigel et al.,
2000). Mutants exhibiting the high chlorophyll fluorescence phenotype were initially
selected in the dark under strong long-wavelength UV light as described elsewhere
(Miles, 1994; Meurer et al., 1996). Measurement of leaf area was performed with LI-
Measurements of Chlorophyll Fluorescence

Fluorescence measurements were performed as described by Peng et al. (2006) using a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany). Before measurement, leaves were dark-adapted for 30 min. The minimum fluorescence yield (F₀) was measured under measuring light (650 nm) with very low intensity (0.8 µmol m⁻² sec⁻¹). To estimate the maximum fluorescence yield (Fₘ), a saturating pulse of white light (3000 µmol m⁻² sec⁻¹ for 1 sec) was applied. The maximum photochemical efficiency of PSII was determined from the ratio of variable (Fᵥ) to maximum (Fₘ) fluorescence (Fᵥ/Fₘ = (Fₘ - F₀)/Fₘ). All the above measurements were performed in a dark room with stable ambient conditions. For recording P₇₀₀ absorbance changes, the PAM fluorometer was equipped with an ED 800 T emitter detector unit (Walz, Effeltrich, Germany) and the measurements were performed according to Meurer et al. (1996). The relative amount of photo-oxidizable P₇₀₀ was reflected from the absorbance changes induced by saturating far-red light.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), SDS-PAGE and Immunoblot Analysis

The thylakoid membrane was prepared in accordance with standard methods (Zhang et al., 1999). BN-PAGE was performed as described previously (Schägger et al., 1994) with some modifications (Cline and More, 2001; Li et al., 2003). The thylakoid membrane was solubilized with 1% (w/v) dodecyl-β-D-maltoside in 20% glycerol, 25 mM BisTris-HCl, pH 7.0 at 1.0 mg chlorophyll ml⁻¹. After incubation at 4°C for 5 min, insoluble material was removed by centrifugation at 13000×g for 10 min. The supernatant was combined with 1/10 volume of 5% Serva blue G in 100 mM BisTris-HCl, pH 7.0, 0.5 M 6-amino-n-caproic acid, 30% (w/v) glycerol and applied to 0.75-mm-thick 6-12% acrylamide gradient gels in a Hoefer Mighty Small vertical electrophoresis unit connected to a cooling circulator. For two-dimensional analysis,
excised BN-PAGE lanes were soaked in SDS sample buffer with 5% β-mercaptoethanol for 30 min and were layered onto 1-mm-thick 15% SDS polyacrylamide gels containing 6 M urea (Laemmli, 1970).

Thylakoid proteins were solubilized and separated by SDS-PAGE (Laemmli, 1970) on 15% (w/v) acrylamide gels with 6 M urea. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and probed with specific antibodies.

Nucleic Acid and Polysome Analysis

Genomic DNA was extracted from Arabidopsis leaves as described by Liu et al. (1995). Total RNA was extracted from 0.5 g leaves of WT and hcf243 mutant plants using an RNA Isolation Kit (U-gene) according to the manufacturer’s protocol. After separation of 15 µg total RNA in a formaldehyde denaturing 1.5% agarose gel, RNA was transferred onto Hybond-N+ nylon membranes (Sambrook and Russel, 2001). The membranes were probed with 32P-labelled cDNA probes specific for HCF243, PsbA, PsbB, PsbC and PsbD. Following high-stringency hybridization and washing, all the blots were exposed to X-ray film for 1–3 days.

RT-PCR of HCF243 and two genes located around the insertion site expression were performed using the following primers: At3g15095, 5'-TTTGGATATTGTTCATGCTTCGC -3' and 5'-AATTACCCAGGTAGAGTTCGAG-3'; At3g15090, 5'-GACTGCTTGGCGTGCTTT-3' and 5'-CCCGGAATCTGTTTCTTCTC-3'; At3g15110, 5'-ACTCCGATAGAAGGTGGT-3' and 5'-TGATGAGTCTCCAGGTTGT-3'. To ensure equal amounts of RNA in each sample, RT-PCR analysis of actin cDNA was carried out using the following primers: sense (5'-AACTGGGATGATATTGGAGAA-3') and antisense (5'-CCTCCAATCCAGACACTGTA-3').

Polysomes were isolated from leaf tissues of WT and hcf243 mutant plants under conditions that maintain polysome integrity according to Barkan (1988). RNA was isolated, fractionated, and transferred onto nylon membranes. The filters were hybridized with the same 32P-labeled cDNA probes described above.

In vivo Labeling of Chloroplast Proteins
In vivo protein labeling was performed essentially according to Meurer et al. (1998). Primary leaves of twelve-day-old and four-week-old mutant and WT seedlings were preincubated for 30 min in 50 μl double distilled water containing 100 μg ml\(^{-1}\) cycloheximide and radiolabeled with 1 μCi μl\(^{-1}\) \(^{35}\)S-methionine (specific activity >1000 Ci mmol\(^{-1}\); Amersham Pharmacia Biotech) in the presence of 50 μg ml\(^{-1}\) cycloheximide, and radioactive methionine was allowed to incorporate for varying periods at 25°C in ambient light. Pulse labeling of the leaves was followed by a chase in the presence of 10 mM unlabeled methionine. After labeling, the thylakoid membranes were isolated and subjected to BN-PAGE and SDS-PAGE analyses by the method described by Ma et al. (2007). For autoradiography, gels were stained, dried, and exposed to x-ray films. The relative amounts of \(^{35}\)S-methionine in proteins were quantified by scanning the x-ray films and analyzing the acquired data, using the AlphalImager 2200 system.

Antiserum Production

The nucleotide sequences encoding amino acids 221 to 408 of HCF243 protein (nucleotide positions 661 to 1224 of the HCF243 gene) were amplified by RT-PCR using the primers 5’-GGATCCAGTAATTCGTGTGGTGCG-3’ and 5’-CTCGAGTCTTCCAAACTTTTGAC-3’. The resulting DNA fragment was cleaved with BamHI and XhoI, and fused in frame to the N-terminal His affinity tag of pET28a vector. The construct was transformed into E. coli strain BL21, and harvested after the application of 0.4 mM isopropylthio-β-D-galactoside for 4 h and resuspended in 500 mM NaCl and 20 mM NaH\(_2\)PO\(_4\), pH 8.0. After incubation for 30 min at 4°C in the presence of lysozyme at a final concentration of 1 mg ml\(^{-1}\), the bacterial lysate was sonicated for about 30 min (10 seconds sonication with an interval of 10 seconds) at 4°C. The overexpressed proteins in inclusion bodies were centrifuged at 3000×g for 30 min, and the pellet was solubilized in 500 mM NaCl, 8 M urea, and 20 mM NaH\(_2\)PO\(_4\), pH 8.0. The fusion protein was purified on a Ni–NTA resin matrix, and a polyclonal antibody was raised in rabbit with purified antigen.
Cloning of HCF243

To clone the T-DNA–tagged gene in the hcf243 mutant, T-DNA left border sequences were isolated using a TAIL PCR strategy essentially according to Liu et al. (1995). The amplified products were cloned into a pMD18-T vector (Takara) in accordance with the manufacturer’s instructions and sequenced with universal M13 primers. Briefly, the preamplification was performed using the SK1 primer together with the AP1 primer, and then the amplification products were diluted 600-fold and 5 μl aliquots were used for the second step amplifications using the SK2 primer together with the AP2 primer. The specific primers SK1 and SK2 were designed according to the T-DNA left border sequences. The two-step PCR conditions were as described by Liu et al. (1995). The PCR products were cloned into a pMD18-T vector (Takara) in accordance with the manufacturer’s instructions, and propagated in the Top10 strain of E. coli. Positive clones were harvested and cultured. Plasmid DNA was extracted using a U-gene Plasmid Moni Kit (U-gene) and sequenced using universal M13 primers. The primers used were as follows: AP1 (5'-GTAATACGACTCACTATA GGGC-3'); AP2 (5'-ACTATAGGGCACGCGTGGT-3'); SK1 (5'-GACTCTAGCTA GAGTCAAGCAGATCGT-3'); and SK2 (5'-GATCGACCGGCATGCAAG-3'). The full length of this gene was deposited in the GenBank under the accession number (HM748832).

Complementation of the hcf243 Mutant

For complementation of the hcf243 mutation, the cDNA containing the coding region was amplified by PCR with the following specific primers, which include SalI and XhoI restriction sites at their 5’ end to facilitate cloning: sense (5'-CTAGTCGAC TCTCAATATCTCTCCAATGGCT-3') and antisense (5'-GATCTCGAGTCCTTTAT TCCCACTCACGTCT-3'). The PCR product was subcloned into the plant expression vector of pSN1301 under the control of the CaMV 35S promoter. The construct pSN1301-HCF243 was transformed into Agrobacterium tumefaciens strain C58 and introduced into hcf243 mutant plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected with 0.1% hygromycin. The successful
complementation was confirmed by immunoblotting analysis with the specific antibody against the HCF243 protein.

### BiFC Assay and Subcellular Localization of HCF243

Full-length CDS of HCF243 and D1 were amplified by PCR with specific primers (HCF243 sense: 5'-TGGCGGAAACTGAGAGACC-3', HCF243 antisense: 5'-CTAGAACCCTACTCCGCGG-3'; D1 sense: 5'-ATGACTGCAATTTTAGAGACGC-3', D1 antisense: 5'-TTATCCATTGATGGAGCCTC-3') and were fused in frame with a YFP at the N terminus or the N terminus of either the 5' half (nYFP) or the 3' half (cYFP) of the YFP open reading frame dissected between codons 154 and 155 (Hu et al., 2002). YFP-tagged HCF243, nYFP–tagged HCF243 and cYFP–tagged D1 were constructed using the GATEWAY recombination system. All vectors were transformed into A. tumefaciens strain GV3101. Different combinations of A. tumefaciens containing each construct were prepared and mixed to an OD_{600} of 0.5:0.5. Bacterial infiltration of Nicotiana benthamiana leaf epidermal cells was performed as described by Lavy et al. (2002). YFP fluorescence and chloroplast autofluorescence were visualized with an Olympus Fluoview Fv1000 spectral confocal scanning microscope after 48 h of incubation.

### Pull-Down Assays

Fragments encoding full-length HCF243 were amplified by PCR with the following primers: sense 5'-GCTAGCATGGCGGAAACTGAGAGACC-3', and antisense 5'-GGATCCGAACCTACTCCGCGCCGCGCGCCG-3'. The PCR products were cloned into the NdeI and BamHI of the pET28a vector and transformed into E. coli BL21. The expression of fusion proteins was induced 0.4 mM isopropylthio-β-D-galactoside for 6 h. The overexpressed recombinant proteins were purified on Ni-NTA agarose resin matrix. About ten micrograms of recombinant fusion protein coupled to 100 ml of a 50% suspension (v/v) Ni-NTA beads in equilibration buffer for 60 min at 4°C. The WT thylakoid membranes (100 μg chlorophyll) solubilized with 1% (w/v) DM in 20% glycerol (w/v), 25 mM...
BisTris-HCl, pH 7.0, and 1mM PMSF for 15 min at 4°C was centrifuged at 12,000×g for 10 min at 4°C, and the supernatant was incubated with His-HCF243 coupled to the Ni-NAT resin for 4 h at 4°C. After the resin was washed five times with the buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA, the bound proteins were eluted with SDS-PAGE sample buffer and resolved by SDS-PAGE followed by immunoblot analyses.

**Immunolocalization Studies**

The subcellular localization of HCF243 was determined essentially according to Lennartz et al. (2001). The WT thylakoid membranes were suspended to a final concentration of 0.1 mg chlorophyll/mL and incubated for 30 min at 4°C in the ice-cold buffer containing 10 mM HEPES-KOH, pH 8.0, 10 mM MgCl$_2$, 330 mM sorbitol, and 1 mM PMSF supplemented with 250 mM NaCl, 200 mM Na$_2$CO$_3$, 1 M CaCl$_2$, or 6 M Urea, respectively. The membrane fractions without supplements were used as a control. After treatment, the membranes were centrifuged at 100,000×g for 2 h at 4°C, washed twice with suspension buffer, and then used for immunoblot analysis.

**Phylogenetic Analysis of HCF243**

The *Arabidopsis* HCF243 (AT3G15095) protein sequence was used as queries to search for homologs against the protein and genome database of NCBI using BLASTP and TBLASTN. All significant hits with an e-value < e$^{-5}$, coverage ratio >35% and match ratio > 35% were considered as potential homologs. To study the evolutionary history of the HCF243 genes, we also searched plant species with complete genome sequences, including *Chlamydomonas reinhardtii*, *Selaginella moellendorffii*, *Physcomitrella patens*, *Populus trichocarpa*, *Cucumis sativus*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Sorghum bicolor* and *Oryza sativa*. All homologous genes and their predicted protein sequences were used for further
analyses. We re-examined the annotation of these genes based on the original
predication and the available transcriptional data (but lack of the EST information for
this gene in a few species, for example, *A. lyrata*). Multiple nucleotide and protein
sequences were aligned using the CLUSTALX 1.81 program followed by manual
adjustment. Domain structures of these potential protein sequences were analyzed by
searching the Pfam (Finn *et al*., 2010) and SMART (Letunic *et al*., 2006) protein
domain databases, with the E-value thresholds set to 0.1. However, we did not
identify any conservative domain in these protein sequences, so phylogenetic analyses
were directly conducted using full-length protein sequences. Neighbor-joining trees
were constructed using MEGA 4.0 (Tamura *et al*., 2007; Kumar *et al*., 2008) and
reliability of internal branches was assessed with 1000 bootstrap replicates.

**Accession Numbers**

Sequence data used for the alignment from this article can be found in the
Genbank/EMBL data libraries under the following accession numbers: NP_188128,
*Arabidopsis* At3g15095; XP_002882914, *Arabidopsis lyrata* e_gw1.3.6495.1;
XP_002523264, *Ricinus communis* 29705 m000578; XP_002316801, *Populus*
trichocarpa2 POPTR_0011s09990.1; NP_001057589, *Oryza sativa* Os06t0352900-01;
XP_002467967, *Sorghum bicolor* Sb01g037260.1; cassava19184_valid.m1, *Manihot*
esculenta1 and cassava46811.m1, *Manihot esculenta2* and POPTR_0011s38210.1,
*Populus trichocarpa*1; Glyma12g35610.1, *Glycine max*1; Glyma13g34820.1, *
Glycine max*2; Cucsa.032440.1, *Cucumis sativus*; GRMZM2G358238_P01, *Zea*
mays1; GRMZM2G008490_P01 *Zea mays*2 and mgf022716m , *Mimulus guttatus*
(from http://www.phytozome.net/). The accession number of *HCF243* gene is
HM748832.

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**FIGURE LEGENDS**

**Figure 1.** Phenotype of wild-type (WT), hcf243 and hcf243 complemented plants.
A, Six-week-old WT (right), hcf243 (middle) and hcf243 complemented (left) plants.
B, Growth kinetics of hcf243 mutants compared to WT plants. Values shown are means ± S.E. of three biological replicates; each replicate represents six transgenic plants.

**Figure 2.** Spectroscopic analysis of WT, hcf243 and hcf243 complemented plants.
A, Chlorophyll fluorescence induction. The minimum fluorescence yield \((F_0)\) of dark-adapted plants was measured using a pulsed measuring beam of red light. The maximum fluorescence yield \((F_{m})\) was determined after a saturating pulse of white light in dark-adapted leaves.
B, \(P_{700}\) redox kinetics. The redox kinetics were investigated by measuring absorbance changes of \(P_{700}\) at 820 nm induced by far-red light (FR: 720 nm).

**Figure 3.** Characterization of the hcf243 mutant.
A, RT-PCR analysis of HCF243 gene expression. RT-PCR was performed with actin-specific primers and the specific primers for At3g15095, At3g15090 and At3g15110.
B, Northern blot analysis of HCF243 gene expression in WT and hcf243 plants. Thirty micrograms of total RNA from WT and hcf243 plants were size-fractionated by agarose gel electrophoresis, transferred to nylon membrane and probed with \(^{32}\)P-labeled HCF243 cDNA probe. rRNAs were detected by EtBr staining.
C, Immunodetection of HCF243 with the specific HCF243 antibody.

**Figure 4.** Analysis of thylakoid proteins from WT and hcf243 plants.
A, Two-dimensional separation of protein complexes in the thylakoid membranes. BN-PAGE-separated thylakoid proteins in a single lane from a BN gel were separated in a second dimension by 15% SDS-urea-PAGE and stained with Coomassie blue.
B, Blue native gel analysis of thylakoid membrane (10 µg chlorophyll) from WT and
*hcf243* was solubilized and separated by BN gel electrophoresis. The positions of
protein complexes were identified with appropriate antibodies (see Guo et al., 2005).
C, Immunoblot analysis of thylakoid proteins from WT and *hcf243*. The thylakoid
membrane proteins were separated by SDS-urea-PAGE, and the blots were probed
with specific anti-D1, anti-D2, anti-CP47, anti-CP43, anti-CF1β, anti-LHCII,
anti-PsbO anti-Cyt f and anti-Cyt b6 antibodies.

**Figure 5.** mRNA expression in chloroplasts and polysome association of chloroplast
mRNA.
A, Northern blotting analysis of transcripts in WT and *hcf243* plants. Transcripts of
*psbA*, *psbB*, *psbC* and *psbD* genes were detected by probing the filter with the
appropriate gene-specific probes.
B, Polysome association in WT and *hcf243* plants of mRNAs encoding the
chloroplast proteins (*psbA*, *psbB*, *psbC* and *psbD*). Total extracts were prepared and
separated on sucrose gradients. Ten fractions of equal volume were collected from the
top to the bottom of the sucrose gradients, and northern blotting analysis was
performed on equal proportions of the RNA purified from each fraction. rRNAs were
detected by ethydium bromide (EtBr) staining.

**Figure 6.** *In vivo* 35S-methionine labeling of thylakoid proteins from WT and *hcf243*
plants.
A and B, Pulse labeling of thylakoid membrane proteins in 14-day-old young
seedlings (A) and four-week-old plants (B). After pulse labeling young *Arabidopsis*
seedlings in the presence of cycloheximide for 20 min, thylakoid membranes were
isolated and the proteins were separated by SDS-urea-PAGE and visualized
autoradiographically.
C, Pulse and chase labeling of thylakoid membrane proteins. After 30 min of pulse
labeling in 14-day-old young seedlings in the presence of cycloheximide followed by
1 or 2 h of chase with cold methionine, thylakoid proteins were separated by
SDS-urea-PAGE, and visualized autoradiographically.

D, BN-PAGE analysis of the incorporation of \(^{35}\)S-methionine into thylakoid membrane protein complexes. A 15-min pulse in Arabidopsis young seedlings in the presence of cycloheximide was followed by a chase of cold unlabeled methionine for 30 and 60 min respectively. The thylakoid membranes were isolated and solubilized with DM, and the protein complexes were separated by BN-PAGE and visualized by autoradiography. Bands corresponding to various PSII assembly complexes of PSII supercomplexes (band I), monomeric PSI superimposed on the PSII dimer (band II), monomeric PSII (band III), CP43-free PSII monomer (band IV), reaction center (band V), and free proteins (band VI) are indicated at right.

**Figure 7.** Bimolecular fluorescence complementation (BiFC) detection of HCF243 and D1 protein interactions and their subcellular localization. GFP-tagged HCF243, nYFP-tagged HCF243 and cYFP-tagged D1 constructs were transiently expressed in leaf epidermal cells of *Nicotiana benthamiana* plants, and their interaction and subcellular locations were examined with a laser scanning confocal microscope. Green fluorescence indicates GFP, red fluorescence shows chloroplast autofluorescence, and orange/yellow fluorescence shows images with the two types of fluorescence merged.

A, Tobacco leaf epidermal cells expressing YFP-tagged HCF243 constructs.

B, Interaction between nYFP-tagged HCF243 and cYFP-tagged D1 in chloroplast of tobacco leaf epidermal cells.

C, nYFP and cYFP constructs were coexpressed in tobacco leaf epidermal cells as a negative control, showing no signal.

D and E, nYFP-tagged HCF243 (D) and cYFP-tagged D1 (E) constructs were expressed alone in tobacco leaf epidermal cells as a negative control, showing no signal. Scale bars: 10 µm.

**Figure 8.** Immunolocalization and Pull-Down Assay of HCF243.

A, Immunolocalization of HCF243. The WT thylakoid membranes were sonicated in
the presence of 250 mM NaCl, 200 mM Na₂CO₃, 1 M CaCl₂ and 6 M urea for 30 min at 4°C. PsbO (the 33-kD luminal protein of PSII) and CP47 (the PSII core protein) were used as markers. Membranes that had not been subjected to any salt treatment were used as controls.

B. Pull-Down Assay of HCF243 and D1 protein interaction. About ten micrograms of HCF243 His-tag fusion protein coupled to Ni-NTA resin were incubated with 100 mg of DM-solubilized thylakoid membranes. Bound proteins were eluted, separated by SDS-PAGE, and subjected to immunoblot analysis with D1 and CP47 antibodies. Similar results were obtained in two additional independent experiments.

SUPPLEMENTAL MATERIAL

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

Supplemental Figure S1: Alignment of the deduced amino acid sequences of the HCF243 gene and its homolog products in Populus trichocarpa, Cucumis sativus, Manihot esculenta, Glycine max, Zea mays, Ricinus communis, Arabidopsis lyrata, Sorghum bicolor, Oryza sativa and Arabidopsis thaliana.

Supplemental Figure S2: Phylogenetic tree of the HCF243 and genic structures of all homologous genes. The tree was established based on full-length protein sequences by the neighbor-joining method with bootstrap values >50% indicated above clades.
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