AtCCMH, an essential component of the *c*-type cytochrome maturation pathway in *Arabidopsis* mitochondria, interacts with apocytochrome *c*

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The maturation of *c*-type cytochromes requires the covalent ligation of the heme cofactor to reduced cysteines of the CXXCH motif of apocytochromes. In contrast to mitochondria of other eukaryotes, plant mitochondria follow a pathway close to that found in α- and γ-proteobacteria. We identified a nuclear-encoded protein, AtCCMH, the *Arabidopsis thaliana* ortholog of bacterial CcmH/CycL proteins. In bacteria, CcmH and the thioredoxin CcmG are components of a periplasmic thio-reduction pathway proposed to maintain the apocytochrome *c* cysteines in a reduced state. AtCCMH is located exclusively in mitochondria. AtCCMH is an integral protein of the inner membrane with the conserved RCXXC motif facing the intermembrane space. Reduction assays show that the cysteine thiols in the RCXXC motif of AtCCMH can form a disulfide bond that can be reduced by enzymatic thiol reductants. A reduced form of AtCCMH can reduce the intra-disulfide bridge of a model peptide of apocytochrome *c*. When expressed in *Escherichia coli*, AtCCMH coimmunoprecipitates with the bacterial CcmF, a proposed component of the heme lyase. Blue-native PAGE of mitochondrial membrane complexes reveals the colocalization of AtCCMH and AtCcmF$_{N2}$ in a 500-kDa complex. Yeast two-hybrid assays show an interaction between the AtCCMH intermembrane space domain and *A. thaliana* apocytochrome *c*. *A. thaliana ccmh*/*ccmh* knockout plants show lethality at the torpedo stage of embryogenesis. Our results show that AtCCMH is an essential mitochondrial protein with characteristics consistent with its proposed apocytochrome *c*-reducing and heme lyase function.

plant mitochondria | cytochrome *c* | disulfide reductase | heme lyase | embryo-lethal

Type *c* cytochromes are hemoproteins that play an essential role in respiratory and photosynthetic electron transport. In prokaryotes and eukaryote organelles, they function on the so-called *p*-side of the energy-transducing membranes. They are characterized by the covalent ligation of two vinyl groups of the prosthetic heme to the two cysteines within the CXXCH motif of apocytochromes. The genetic analysis of *c*-type cytochrome maturation in bacteria, mitochondria, and chloroplasts has led to the identification of three different biogenesis pathways, referred to as systems I, II, and III (1–3). The transport of both heme and apocytochrome *c* to their site of activity and their maintenance in reduced state are common requirements for the assembly of holocytochromes *c* in the three systems.

Earlier studies in yeast (system III) showed cytochrome *c* and cytochrome c_1 heme lyases (CCHL and CCHL$_1$) to be the central players in the formation of mitochondrial cytochrome *c* and c_1 (4, 5). Orthologs of CCHL genes are found in nuclear genomes of vertebrates, invertebrates, and some green algae (i.e., *Chlamydomonas reinhardtii*), suggesting that their mitochondrial *c*-type cytochromes are assembled through system III.

In contrast, system I seems more complex. In *Escherichia coli*, eight *ccm* genes (for cytochrome *c* maturation) arranged in an operon (6) and three *dsb* genes (for disulfide bond formation) are essential for the production of holocytochrome *c* (7, 8). CcmA and CcmB are components of an ATP-binding cassette transporter whose substrate is not known (9, 10). CcmCDE are involved in heme delivery. CcmC is required for the covalent attachment of heme to the heme chaperone CcmE (10–12). CcmF has been proposed to form a bacterial cytochrome *c* heme lyase complex together with CcmH (13). Cysteine thiols of the heme-binding motif of apocytochromes *c* are oxidized by the periplasmic DsbA. CcmG, a thioredoxin-like protein, and CcmH, a putative thiol-disulfide oxidoreductase, reduce intramolecular disulfide bonds in apocytochromes before heme attachment by transferring electrons from the transmembrane disulfide oxidoreductase DsbD (DipZ) (14, 15). In bacteria, the loss of CcmH (CycL/Ccl2) results in either the absence of any detectable *c*-type cytochromes (2, 6) or reduced levels of *c*-type cytochromes (16, 17). CcmH proteins possess an RCXXC motif exposed to the periplasm. This motif is believed to be active in the reduction of the cysteines of the CXXCH motif on apocytochromes *c* (18, 19). Because in fungal and animal mitochondria system III is used for cytochromes *c* maturation, the occurrence of bacterial system I *ccm* orthologs in plant mitochondrial genomes was unexpected. Orthologs of *ccmB*, *ccmC*, and *ccmF* have been found in all embryophyte mitochondrial genomes studied so far. In *Arabidopsis thaliana*, three genes encode proteins similar to different domains of CcmF (20). The study of the *A. thaliana* genomic sequence has led to the identification of nuclear-encoded CCM components: the heme chaperone AtCCME (21) and the ATP-binding domain of the ABC transporter AtCCMA (N.R., unpublished data). Here, we identify AtCCMH and investigate its function as a mitochondrial thiol-disulfide oxidoreductase and a component of a heme lyase complex involved in cytochrome *c* maturation.

Materials and Methods

Overexpression of AtCCMH and Antibody Production. The 5′ part of AtCCMH cDNA (corresponding to M1-Q84) was amplified by PCR by using P1 and P2 primers (for primer sequences, see List 1, which is published as supporting information on the PNAS web site) and cloned in pQE60 (Qiagen, Valencia, CA). The His-tag fusion protein (D1-His) was expressed in *E. coli*, purified under denaturing conditions, and injected into rabbits. Sera were purified against D1-His coupled to CnBr-activated Sepharose according to the supplier's instructions (Amersham Pharmacia Biotech). *A. thaliana*

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Abbreviations: ccm, cytochrome *c* maturation; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; NTR, NADPH thioredoxin reductase.

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$CcmF_{N2}$-specific antibodies were raised against the peptide MKQQASVRRTYKKEM coupled with ovalbumin (N.R., unpublished data).

Coexpression of AtCCMH and EcCcm Proteins. We have constructed the plasmid pEM19, which encodes *E. coli* CcmA-G and a chimeric CcmH consisting of EcCcmH (M1-T19)::AtCCMH (D5-R159 + His-tag) for complementation experiment in *E. coli* Δ*ccm* strain EC06 (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site). For coimmunoprecipitation experiments, 500 μg of *E. coli* membrane proteins were solubilized and precipitated with 5 μl of anti-CcmF antibodies (13) according to previous work (12). *E. coli* spheroplasts were prepared by treatment with 8 μg/ml lysozyme in 100 mM Tris·HCl (pH 8.0) containing 20% sucrose.

Purification of *A. thaliana* Mitochondria and Chloroplasts, Mitoplast Preparation, and Submitochondrial Fractionation. *A. thaliana* protoplasts were prepared from 5-day-old suspension cell culture (22). Mitochondria and chloroplast purification on Percoll step gradient, mitoplast preparation, submitochondrial fractionation, proteinase K, and alkali treatments were performed as described (21).

Solubilization of Mitochondrial Complexes and Separation on Two-Dimensional Blue-Native-SDS/PAGE. Four hundred micrograms of mitochondrial membrane proteins were solubilized with digitonin, 5:1 detergent:protein (wt/wt) for 30 min on ice. Mitochondrial membrane complexes were resolved by blue-native PAGE in the first dimension followed by SDS/PAGE in the second dimension as described (23).

***Arabidopsis* Lines.** The *A. thaliana ccmh* mutant was obtained from the SALK T-DNA (transferred DNA) insertion lines collection (24). Seeds from WT (Columbia) or mutant (SALK_046872) were sown on Murashige and Skoog medium or soil and grown under 16-h light/8-h dark cycles at 22°C/20°C.

Microscopy. Seeds were removed from siliques, fixed in 2% glutaraldehyde, and treated with Hoyer's solution (25). Embryos were observed by differential interference contrast (DIC) microscopy with a Nikon E800 microscope. Isolated embryos were fixed in 2% glutaraldehyde, stained, dehydrated, and included in Epon 812 as described (26). Observations of 90-μm-thick sections were performed with a Hitachi H600 transmission electron microscope at a tension of 75,000 volts.

Thiol Titration and *in Vitro* Redox Assay. D1-His was expressed in *E. coli* and purified under native conditions. The thiol content of D1-His was measured by using a 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay as follows. D1-His (100 μmol) was reduced with 10 mM DTT for 10 min at room temperature. DTT was removed by buffer exchange by using a 10-kDa cutoff filtration membrane. Resulting thiols were labeled with 1 mM DTNB for 30 min. DTNB in excess was removed by using centrifugal filter devices. Procedures for the expression and purification of *A. thaliana* NADPH thioredoxin reductase B (AtNTRB), poplar thioredoxin h_1 and h_2 (Pt-$TRXh_1$, $PtTRXh_2$), and glutaredoxin (PtGRX) are described elsewhere (27–30). AtNTRB and $PtTRXh_2$ are mitochondrial proteins (29, 31). Interactions between labeled D1-His (6 μM) and disulfide reductants were performed in 30 mM Tris·HCl buffer (pH 8.0) by measuring the release of thionitrobenzoic acid (TNB^-) after 30 min at 412 nm (ε_{412} = 13,600 $M^{-1}\cdot cm^{-1}$). Thioredoxins (50 μM) and glutathione (35 μM) were reduced by using catalytic amounts of NTR (0.8 μM) and glutathione reductase (0.5 UI, Sigma), respectively, in the presence of 250 μM NADPH. Glutaredoxin was used at a concentration of 50 μM. The peptide FRTKCAQCHTVE used as a model for *A. thaliana* apocytochrome *c*-a (At1g22840) was synthesized and oxidized with DMSO (NeoMPS, Strasbourg, France). The predicted molecular mass of the peptide with an intra-disulfide bridge (1,420 Da) was verified by mass spectrometry. Equimolar amounts (85 μM) of oxidized peptide and reduced D1-His were incubated for 1 hr at 20°C and separated on a C4 column by using a Waters HPLC system. Mass measurement was carried out on a Biflex III (Bruker-Daltonik, Ettlingen, Germany) MALDI-TOF mass spectrometer used in reflector-positive mode.

Yeast Two-Hybrid Assays. cDNA fragments coding for D1 (M1-Q84) and D2 (Y105-R159) domains of AtCCMH, as well as for the full-length cytochrome *c*-a gene (C), were amplified with primers P7 and P8 for D1, P9 and P10 for D2, and P11 and P12 for C, then reamplified with universal primers P13 and P14 to introduce phage lambda *att* recombination sites. The products obtained were recombined with pDONR207 donor vectors by using Gateway technology (Invitrogen) to obtain entry vectors. These vectors were recombined with Clontech pGBKT7- and pGADT7-modified vectors (32) to obtain vectors expressing all of the combinations of fusion proteins. Constructs were transformed in yeast strain pJ69-4A by heat shock. Two-hybrid assays were performed according to standard methods (33). Transformation in yeast was controlled by the growth on media lacking leucine and tryptophan. The expression of the reporter genes ADE2 and HIS3 was monitored by the growth on media lacking adenine, histidine, and both. The expression of *lacZ* was followed by measuring at OD_{420} the accumulation of the product metabolized by β-galactosidase with 2.2 mM 2-nitrophenyl β-D-galactopyranoside (*o*-NPG, Sigma) as substrate.

Results

Identification and Expression of *AtCCMH*. We have identified a single-copy gene present on *A. thaliana* chromosome 1 (At1g15220) encodes a protein showing 20–25% identity with *Bradyrhizobium japonicum* CycL, *Rhodobacter capsulatus* Ccl2, *Rhodobacter sphaeroides* CcmH, and the N-terminal half of *E. coli* CcmH. In eukaryotes, orthologs of *ccmH* are found in nuclear genomes of angiosperms and gymnosperms and of the unicellular primitive red alga *Cyanidioschyzon merolae*. Up to now, no *ccmH* ortholog is found in protists, green algae, or mosses. EST data revealed high conservation of CCMH proteins among the plant species (60–90% identity). The reduced number of ESTs found and the absence of detection of *AtCCMH* transcripts by Northern experiments reflect a low level of expression. By RT-PCR experiments, we found that the gene is expressed in all *A. thaliana* organs and that its three introns are spliced (data not shown). The mature transcript encodes a protein of 159 aa composed of two hydrophilic domains, D1 and D2, separated by a hydrophobic domain (Fig. 1). The N-terminal signal sequence responsible for targeting the bacterial CycL to the periplasm is not conserved in plant proteins. Instead, AtCCMH has a C-terminal extension not found in bacterial proteins. The potential thiol-disulfide oxidoreductase motif (RCXXC) is located in the N-terminal D1 domain.

AtCCMH Is Localized in the Mitochondrial Inner Membrane. The cellular localization of AtCCMH could not be predicted because no organellar N-terminal targeting sequence could be identified. The anti-AtCCMH antibodies generated against D1 recognized a unique protein in total protoplast extract prepared from *A. thaliana* cells, with an apparent molecular mass of 20 kDa, close to the calculated 17.9 kDa (Fig. 2*A*). The protein was found in the purified mitochondrial fraction and was absent in both cytosolic and chloroplastic protein fractions.

Bacterial CcmH proteins are inserted into the cytoplasmic membrane with their N-terminal domain localized in the periplasm (18, 19). Mitochondrial subfractions were analyzed to check AtCCMH location and topology. No signal was detected with the outer membrane fraction, whereas AtCCMH was immunodetected in the inner membrane fraction (Fig. 2 *B* and *C*). The nature of AtCCMH

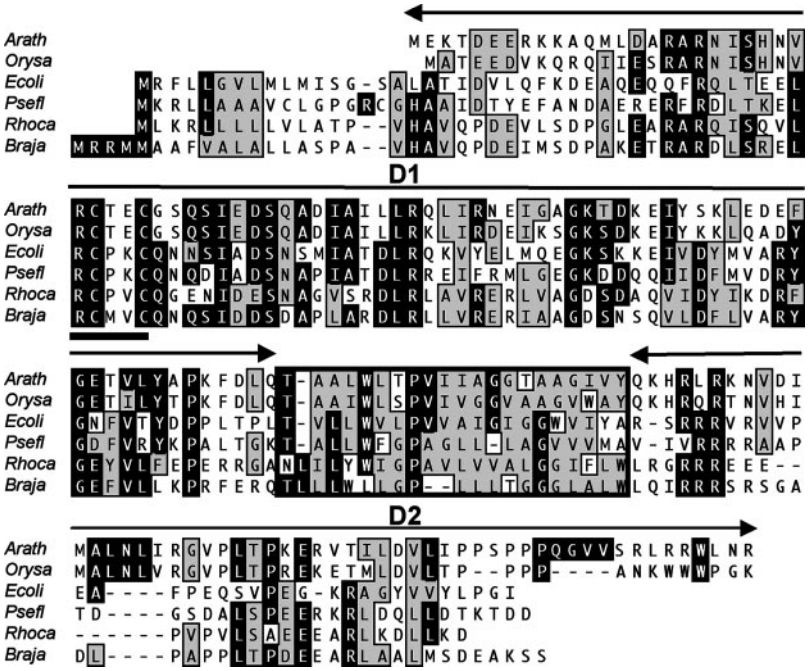

Fig. 1. Amino acid sequence alignment of CCMH/CycL/Ccl2 proteins from different organisms: *Arabidopsis thaliana* (Arath), *Orysa sativum* (Orysa), *Escherichia coli* (*Ecoli*, CcmH partial sequence, M1-I154), *Pseudomonas fluorescens* (Psefl), *Rhodobacter capsulatus* (Rhoca), *Bradyrhizobium japonicum* (Braja). Residues identical in more than three sequences are highlighted in black whereas similar residues are highlighted in light gray. The hydrophobic domain is boxed. Arrows indicate D1 and D2 hydrophilic domains. The conserved RCXXC motif is underlined.

interaction with the inner membrane was investigated by alkali solubilization. Like CYTc_1, a protein with a transmembrane helix, AtCCMH remained in the insoluble fraction whereas Nad9 was found in the extrinsic protein fraction (Fig. 2*D*). We propose that the hydrophobic domain present in AtCCMH is a transmembrane domain. The orientation of the protein predicted to follow the N-out and C-in model was investigated by treating intact mitoplasts with proteinase K. The inner membrane integrity was controlled by Nad9 detection. After the protease treatment, the signal obtained with antibodies raised against D1 was strongly reduced, thus confirming the localization of that domain (containing the RCXXC motif) in the mitochondrial intermembrane space (Fig. 2*E*).

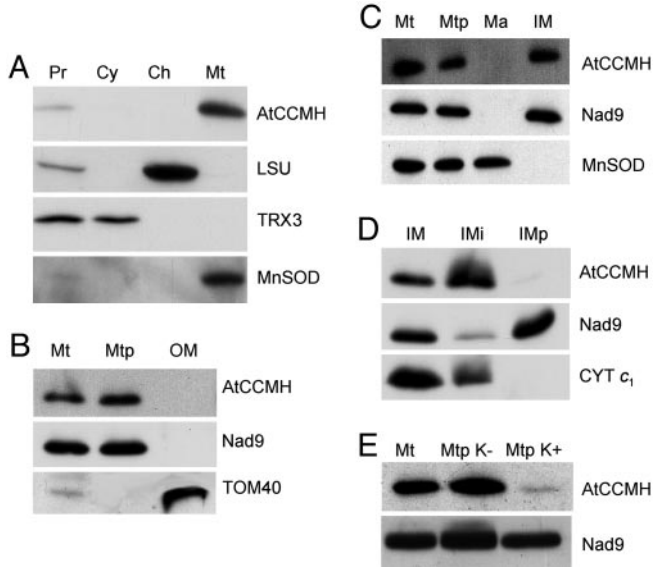

Fig. 2. Localization of AtCCMH. Protein fractions from *A. thaliana* were analyzed by Western blots probed with antibodies directed against AtCCMH. (*A*) Protein extracts (20 μg) of protoplasts (Pr), cytosol (Cy), chloroplasts (Ch), and mitochondria (Mt). (*B*) Mitochondrial (Mt), mitoplast (Mtp), and outer membrane (OM) fractions were purified on gradients. (*C*) Matrix (Ma) and inner membrane (IM) fractions were prepared from broken mitoplasts. (*D*) Peripheral proteins (IMp) and integral membrane proteins (IMi) were obtained after alkali treatment of IM. (*E*) Mitoplasts were treated with proteinase K (Mtp K^+). Controls were performed by using antibodies directed against the chloroplast large subunit of RuBisCo (LSU), a cytosolic thioredoxin (TRX3), a peripheral protein of complex I oriented toward the matrix (Nad9), a subunit of the translocase of the outer membrane (TOM40), the matrix manganese superoxide dismutase (MnSOD), and cytochrome c_1 (CYTc_1), an integral protein of complex III.

***AtCCMH* Is Essential for Embryo Development.** To gain insights into the function of AtCCMH, we analyzed an *A. thaliana* insertion line (SALK_046872) carrying a transferred DNA (T-DNA) in the second intron. No homozygous plants for the insertion could be identified by PCR analysis, suggesting that this mutation is lethal in its homozygote state. No phenotype could be evidenced after careful observation of four individual heterozygous *ccmh/CCMH* plants at different stages of development. The observed kan^R segregation ratio of 2:1 of their progeny indicated that there is only one insertion site and that lethality of *ccmh/ccmh* homozygotes is embryonic. We dissected siliques of self-fertilized *ccmh/CCMH* and WT plants at different stages of development. Although, in WT siliques, seeds were of uniform color, size, and shape, *ccmh/CCMH* immature siliques observed 7 days post-fecundation contained 25% of smaller sized pale seeds that aborted and shrank at later developmental stages (Fig. 3*A*). The embryos from these abnormal seeds were dissected out of the mother seed tissue. PCR analysis showed that these embryos were homozygous (*ccmh/ccmh*) for the insertion in *AtCCMH* (Fig. 6, which is published as supporting information on the PNAS web site). The effect of AtCCMH deficiency on *A. thaliana* embryo development was examined by comparing embryos isolated from the two types of seeds obtained from the same silique. Although green seeds contained embryos at the cotyledon stage, *ccmh/ccmh* embryos were arrested at the torpedo and occasionally heart stages without visible abnormality in the morphogenesis (Fig. 3 *B* and *C*). Embryos at the torpedo stage were examined by transmission electron microscopy to investigate cell differentiation and ultrastructure. Cotyledon cells of WT or heterozygote mature embryo had a regular shape and started to differentiate (Fig. 3*D*). Nuclei with a nucleolus, chloroplasts, and mitochondria were clearly identified. Storage bodies had a round or

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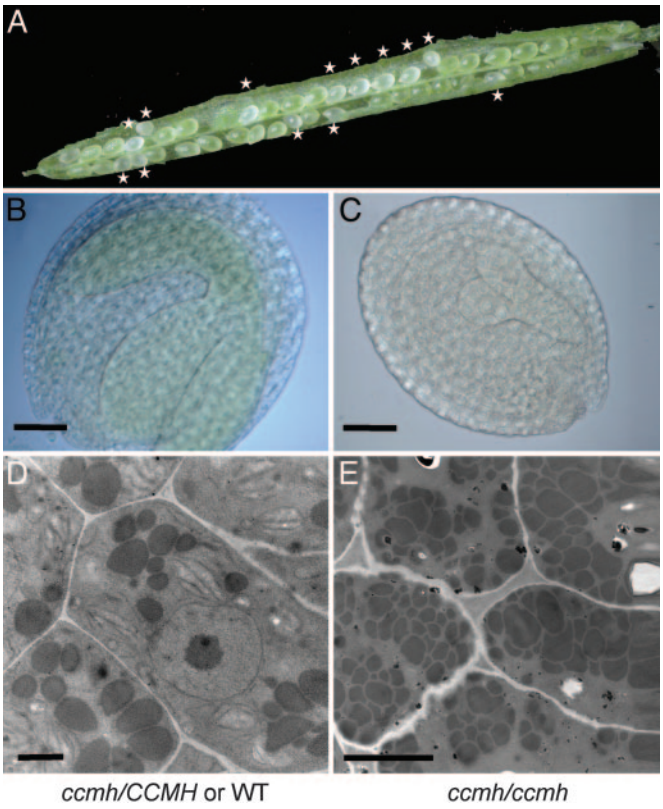

Fig. 3. Phenotype of *ccmh*/*ccmh* seeds and embryos. (*A*) Immature silique from a self-fertilized *CCMH*/*ccmh* plant. Asterisks indicate white seeds with *ccmh*/*ccmh* embryos. (*B* and *C*) Microscopical images of seeds distained with Hoyer's solution. Shown are phenotypic WT embryo at mature cotyledon stage (*B*) and white immature seeds containing a *ccmh* embryo arrested at torpedo stage from the same silique as *B* (*C*). (*D* and *E*) Electron micrograph of cotyledon cells from green embryo (*D*) and white embryo (*E*). (Scale bars, 200 μm in *B* and *C* and 2 μm in *D* and *E*.)

ovoid shape. On the other hand, mutant cotyledon cells had an irregular shape, with abnormal cell wall and an enlarged intercellular space (Fig. 3*E*). The cell content was completely disorganized, with no organelles, a denser cytosol, and smaller and more abundant storage bodies. The defect in CCMH resulted in the arrest of embryo development due to severe cellular disorder, which strongly suggests that *AtCCMH* is an essential gene encoding a housekeeping function.

AtCCMH Expressed in *E. coli* Interacts with EcCcmF. In *E. coli*, CcmH is a bipartite protein with domains corresponding to *B. japonicum* CycL and CycH proteins (1). The CycL domain containing the conserved RCXXC motif is required to synthesize *c*-type cytochromes whereas the CycH domain is not (19). To check whether AtCCMH could replace EcCcmH, an *E. coli* Δ*ccm* strain (EC06) was transformed with pEM19, which contains a modified *E. coli ccm* operon in which *AtCCMH* was transitionally fused with the sequence coding for the periplasm-targeting peptide of EcCcmH and a C-terminal His-tag. The expression of AtCCMH in bacteria, as well as the insertion into the cytoplasmic membrane with a correct topology, was observed (Fig. 7, which is published as supporting information on the PNAS web site). No holocytochrome c_{550} could be detected by heme staining of periplasmic proteins, whereas the WT *ccm* operon allowed the formation of mature *c*-type cytochromes (data not shown) as reported for the *E. coli* operon containing a truncated CycL-like EcCcmH (19). Thus, AtCCMH is unable to complement the lack of its *E. coli* counterpart. Because *E. coli* CcmH forms a complex with CcmF (13), we

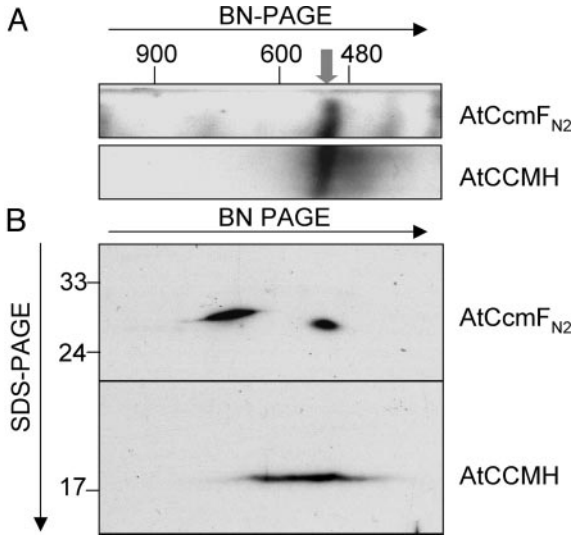

Fig. 4. Association of CCMH with CcmF$_{N2}$ investigated by blue-native (BN)-PAGE. The BN gel of mitochondrial protein complexes solubilized with digitonin (*A*) and the corresponding denaturing second dimension gel (*B*) were blotted and reacted with antibodies directed against CcmF$_{N2}$ and CCMH. The molecular mass standard is given in kDa. Position and size in kDa of respiratory complexes I, V, and III are shown above the BN-PAGE. The gray arrow indicates the 500-kDa protein complex.

used the complementation assay to check whether a heterologous interaction occurs *in vivo* between AtCCMH and EcCcmF. A membrane extract was solubilized and immunoprecipitated by using antibodies directed against EcCcmF, and indeed AtCCMH was found to coimmunoprecipitate with EcCcmF (Fig. 7).

AtCCMH and AtCcmF$_{N2}$ Are Found in a 500-kDa Complex. In *A. thaliana*, the main conserved domain of CcmF proteins, including the "WWD" motif (2), is encoded by the mitochondrial *ccmF$_{N2}$* gene. The association of CCMH with CcmF$_{N2}$ was investigated by immunodetection of mitochondrial membrane complexes solubilized with digitonin and separated by blue-native PAGE (34). In the first dimension, both anti-CCMH and anti-CcmF$_{N2}$ antibodies detected a 500-kDa complex (Fig. 4*A*). The analysis of the second denaturing dimension, reveals that the 30-kDa CcmF$_{N2}$ is present in an additional complex of 700 kDa. The CCMH antibody detected a continuous signal between 450 and 650 kDa, with a main signal of 500 kDa (Fig. 4*B*). These results strongly suggest that CCMH and CcmF$_{N2}$ can be associated in a same protein complex, although this association does not seem to occur systematically.

AtCCMH Interacts with AtCYT*c*-a. The proposed roles of CcmH, i.e., the reduction of apocytochrome cysteines and heme ligation (together with CcmF), imply an interaction with apocytochromes. So far, *c*-type apocytochromes had never been reported to interact with any Ccm proteins. We used the yeast two-hybrid system to investigate whether an interaction between AtCCMH and apocytochrome *c* could take place. For this purpose, the sequences coding for *AtCYTc-a*, an *A. thaliana* cytochrome *c* (C), and the sequences coding for D1 and D2 domains of AtCCMH (Fig. 1) were cloned in two-hybrid vectors in fusion with the activation domain (AD) and the DNA binding domain (BD) of the transcription factor Gal4. Protein interactions were assayed by growth on selective medium due to the activation of the nutritional reporter genes *ADE2* and *HIS3*. Growth was observed for both D1-C combinations, but only background growth (on medium lacking adenine or histidine) or no growth at all (on medium lacking both adenine and histidine) was observed for the D2-C combinations (Fig. 5*A*). This result shows that AtCCMH can indeed interact with cytochrome *c* and that, as

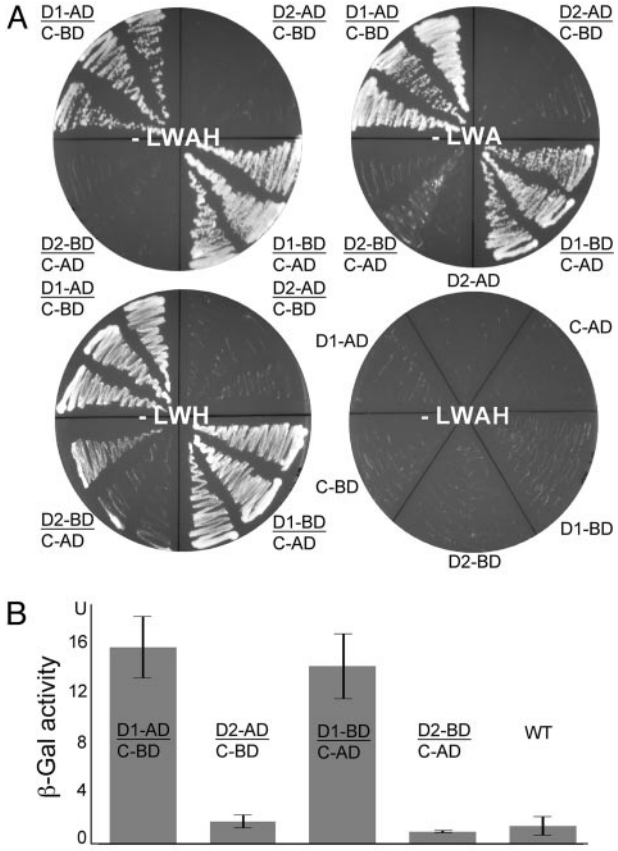

Fig. 5. Yeast two-hybrid assays with AtCCMH N-terminal domain (D1), AtCCMH C-terminal domain (D2), and AtCYTc-a (C). (*A*) The domains or proteins were fused to the activating domain (AD) or the DNA binding domain (BD) of GAL4, cotransformed in yeast, and plated on different combinations of drop-out medium-selective plates, i.e., minus leucine (L), tryptophane (W), adenine (A), and histidine (H). (*B*) Liquid β-gal assay using 2-nitrophenyl β-D-galactopyranoside (*o*-NPG) as a substrate. β-Gal activity is expressed in U (U = nmol/min). WT stands for WT pJ69-4A yeast cells. The values displayed are the average β-Gal activities for three individual double transformant or WT cell colonies, with standard deviations indicated by error bars.

predicted, D1 located in the inter-membrane space can interact with apocytochrome *c*, whereas D2, oriented toward the matrix, cannot. As a control, the six single constructs were cotransformed in yeast with the corresponding empty vectors (e.g., D1-AD with empty BD). The absence of growth showed that the activation of reporter genes was a true reflection of D1 interaction with apocytochrome *c* (Fig. 5*A*). The interaction was quantified by the activation of *lacZ*, a third reporter gene. Background levels were estimated in WT yeast cells. The signal resulting from the interaction of cytochrome *c* with D1 was found to be 10 times higher than background levels, whereas, with D2, β-gal activity never raised above background levels (Fig. 5*B*).

***In Vitro* Redox Properties of AtCCMH.** The recombinant D1-His polypeptide contains the only two cysteines of AtCCMH located in the presumed catalytic RCXXC motif. When D1-His, expressed in *E. coli* and purified under native conditions, was reduced by a DTT excess (100-fold), ≈2 mol of thiol per mol of protein are found by using DTNB, whereas only ≈0.6 mol of thiol per mol has been titrated on untreated protein. On nonreducing PAGE, recombinant D1-His appeared mainly as monomers; this result suggests that the cysteinyl residues can form an intramolecular disulfide bridge in AtCCMH. DTT-reduced D1-His has been labeled with DTNB and then submitted to different disulfide reductases (Table 1). NTR-reduced thioredoxins *h*1 and *h*2, which differ at least by their catalytic site (WCPPC and WCGPC, respectively), are able to interact with the labeled AtCCMH cysteines releasing thionitrobenzoic acid (TNB^-). Addition of glutaredoxin enhanced the TNB^- release observed in the presence of both glutathione and glutathione reductase.

Table 1. *In vitro* reduction of DTNB-labeled D1-His

	Reduction, %				
	NADPH + AtNTRB			NADPH + GR	
DTT	—	PtTRXh1	PtTRXh2	GSH	GSH + PtGRX
100	6	74	90	35	60

The N-terminal domain of AtCCMH containing the DTNB-oxidized RCXXC motif was reduced by DTT, AtNTRB, NTR-reduced thioredoxin (PtTRXh1 or PtTRXh2), GR-reduced glutathione (GSH), or GSH-reduced glutaredoxin (PtGRX). The amount of TNB^- obtained after reduction with DTT was chosen as 100%.

The ability of AtCCMH to reduce apocytochrome *c* was tested in an experiment involving the reduced D1-His and a 12-aa synthetic peptide identical to AtCYT*c*-a F19-E30 containing an intramolecular disulfide. After incubation and reverse phase chromatography with a water/acetonitrile gradient, two peaks corresponding to the model peptide were eluted. MALDI mass measurement in reflector-positive mode detected two peptides of 1,420.747 Da and 1,422.766 Da, corresponding, respectively, to the oxidized and reduced form of the peptide (Fig. 8, which is published as supporting information on the PNAS web site). We tested the reducing activity of AtCCMH in the insulin reduction assay (35). Similar to its bacterial homologue RcCcl2 (18), AtCCMH (in excess) was unable to reduce insulin *in vitro* (data not shown).

Taken together, these data show that D1-His cysteines could interact with different biological disulfide reductants and the heme-binding motif of an apocytochrome *c* model peptide, suggesting that the RCXXC motif of CCMH can be functionally active in redox processes needed for cytochrome *c* maturation.

Discussion

We report here the identification of *AtCCMH*, a plant nuclear gene ortholog of the *ccmH/cycL/ccl2* bacterial gene family of system I. We have demonstrated that AtCCMH is a monotopic type I membrane protein and that the conserved RCXXC motif is located in the intermembrane space where the assembly of *c*-type cytochromes occurs. These characteristics are consistent with the conservation of CCMH function in *c*-type cytochrome maturation from bacteria to plant mitochondria.

To study the function of AtCCMH in plants, we analyzed an *A. thaliana* insertion mutant. Homozygote *ccmh/ccmh* embryos could be obtained, indicating that the mutation is not lethal in either gametophyte. However, such *ccmh* knockout embryos stop their development at or before the torpedo stage. The absence of morphologic defects in these dead embryos, together with the fact that *AtCCMH* gene is expressed in all organs of the plant, indicates that *AtCCMH* is an essential gene encoding a housekeeping function rather than a gene needed at a specific embryo developmental step. We propose that the restriction of available holocytochromes *c* and c_1 progressively limits the flow of electrons through respiratory chain complexes III and IV, thereby decreasing ATP production, which in turn affects chloroplast development as observed for white *ccmh/ccmh* embryos.

AtCCMH was unable to replace EcCcmH for holocytochrome *c* formation even though it was expressed and properly targeted to the periplasm. In *E. coli* CcmH, an L to A mutation in the LRCXXCQ motif found in nearly all bacteria CcmH proteins abolishes the function (U.A., unpublished results). Similarly, the absence of complementation could be attributed to the sequence

divergence from bacteria to plant CCMH proteins, which share a strictly conserved VRCTECG motif. Alternatively, the lack of complementation could originate from a structural rather than an enzymatic defect. A third explanation would be that plant mitochondrial CCMH have evolved a different function.

In bacteria, CcmH was proposed to have a structural role together with CcmF in providing apocytochrome and heme in the proper conformation for their ligation to occur. AtCCMH interacts with EcCcmF in *E. coli*, although this interaction does not lead to a functional complementation. In *A. thaliana*, the analysis of plant mitochondria membrane complexes has shown that AtCCMH and $AtCCMF_{N2}$ can be associated in a 500-kDa complex of unkown composition. Two-dimensional gels show that CCMH and $CcmF_{N2}$ are also found in other complexes, not detected on the first dimension most probably because of masked epitopes. The detection of both proteins in various complexes argues in favor of transient interactions between CCM proteins. We propose that the complex containing both CCMH and $CcmF_{N2}$ in plants could hold the heme lyase activity, as already suggested by other experiments in bacteria (13).

As part of a heme lyase complex and as a reductant for apocytochrome cysteines, AtCCMH is predicted to interact with apocytochromes. In *E. coli*, neither successful coimmunoprecipitation nor detection of mixed disulfides between CcmH and apocytochromes has been reported (13). Similarly, AtCCMH and apocytochrome *c* coimmunoprecipitation was unsuccessful in our hands. Therefore, we used yeast two-hybrid assays more appropriate for the detection of transient interactions. Indeed, the D1 domain of AtCCMH containing the RCXXC motif was found to interact with AtCYT*c*-a, whereas the C-terminal domain of AtCCMH (located in the matrix) did not. Disulfide bond formation is unlikely to occur in the reducing environment of the yeast nucleus where the two-hybrid interaction takes place. This finding suggests that the interaction between CcmH and apocytochrome could rely on a non-disulfide mediated protein–protein interaction. Indeed, CCMH could have a scaffold-like function for apocytochrome *c*.

In *E. coli*, in addition to CcmA-H proteins, holocytochromes *c* formation required three periplasmic Dsb oxidoreductases (14). No orthologs of the thioredoxin CcmG or of the Dsb proteins could be identified in plant mitochondria. Therefore, AtCCMH is so far the only component of the putative reducing pathway of system I identified in plant mitochondria. In bacteria, several lines of evidence support a role of CcmH in a redox reaction (17, 18, 36). We showed that the cysteines of D1-His can form disulfide bonds accessible to different enzymatic reductants *in vitro* and that a reduced form of D1-His is able to reduce an intra-disulfide bridge in the heme-binding motif of a model peptide for apocytochrome *c in vitro*. This finding is a good indication of the redox capacity of AtCCMH.

The intermembrane space of mitochondria is believed to be a reducing compartment where the cysteines of apocytochromes should be preserved from oxidation after their import into mitochondria. This finding suggests that a reducing pathway for cytochrome *c* might not be needed in mitochondria. However, a mitochondrial FAD-dependent sulfydryl oxidase, ERV1, located in the intermembrane space, has been described in yeast and *A. thaliana* (37). It would be interesting to investigate whether ERV1 could catalyze the formation of a disulfide bond in apocytochromes, thus making the reducing activity of proteins such as CCMH necessary. Indeed, Cyc2p, a flavoprotein was recently shown to interact with system III CCHL and proposed to be involved in the redox chemistry of the heme lyase reaction in yeast mitochondria (38). Further investigations of the function of CCMH might give clues toward the understanding of the yet unknown mechanisms controlling the intermembrane space redox state in mitochondria.

Altogether, our results enable us to propose a central role of CCMH in plant mitochondrial cytochrome *c* maturation, probably as part of a heme lyase complex that holds as well the activity of reducing apocytochrome *c*.

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