The maturation of c-type cytochromes requires the covalent attachment of the heme cofactor to the apoprotein. For this process, plant mitochondria follow a pathway distinct from that of animal or yeast mitochondria, closer to that found in α- and γ-proteobacteria. We report the first characterization of a nuclear-encoded component, namely AtCCME, the Arabidopsis thaliana orthologue of CcmE, a periplasmic heme chaperone in bacteria. AtCCME is targeted to mitochondria, and its N-terminal signal peptide is cleaved upon import. AtCCME is a peripheral protein of the mitochondrial inner membrane, and its major hydrophilic domain is oriented toward the intermembrane space. Although a AtCCME (Met79-Ser256) is not fully able to complement an ccmA–ccmH mutant strain for bacterial holo-cytochrome c production, it is able to bind heme covalently through a conserved histidine, a feature previously shown for E. coli CcmE. Our results suggest that AtCCME is important for cytochrome c maturation in A. thaliana mitochondria and that its heme-binding function has been conserved evolutionarily between land plant mitochondria and α-proteobacteria.
phate carboxylase (a gift from B. Camara, IBMP-CNRS, Strasbourg, France) and α-tubulin (Amersham Pharmacia Biotech) were used as control. Antibodies directed against potato porin (25) (provided by H.-P. Braun, Hannover University, Hannover, Germany), tobacco marja-rose-superoxide dismutase (26) (obtained from F. van Breugel, Gatersleben, Germany), wheat subunit 9 of NADH dehydrogenase (10), and yeast cytochrome c1 (provided by G. Schatz, Basel University, Basel, Switzerland) were used as control for outer membrane, matrix, and extrinsic or intrinsic inner membrane protein fractions, respectively, of the mitochondria. The production of antibodies against \( E.\ coli \) CcmE and analysis of proteins expressed in \( E.\ coli \) with alkaline phosphatase- and secondary antibody are described elsewhere (19). The apparent molecular weight of proteins was calculated using middle range molecular weight markers (Bio-Rad) as ladder.

**Southern Hybridization—** \( A.\ thaliana \) ecotype Columbia were grown on soil in a greenhouse for \( \sim 4 \) weeks. Total DNA was extracted as described (28). 25 \( \mu \)g of DNA were digested with each restriction endonuclease and analyzed on 0.8% agarose gels. After Southern transfer to a nylon membrane, hybridization was performed under standard conditions at 65 °C with a \( ^{32}P \)-labeled AtCCME cDNA probe prepared by random hexamer extension. The membrane was washed in 2× SSC, 0.1% SDS for 30 min at 65 °C and twice in 0.2× SSC, 0.1% SDS for 30 min at 65 °C.

**Bacterial Growth Conditions and Cell Fractionation—** \( E.\ coli \) was grown aerobically in LB medium or anaerobically in minimal medium with 5 \( \mu \)m nitrate as the electron acceptor (29). For the expression of \( B.\ rhizobioum \) \( japonicum \) cytochrome \( c_{\text{cmm}} \), \( E.\ coli \) cells were grown to midexponential phase and then induced with 0.4% arabinose. Whole-cell protein analysis, isolation of periplasmic and membrane fractions, and heme staining were performed as described previously (3, 21, 22, 36).

**Purification of \( A.\ thaliana \) Mitochondria and Chloroplast—** Arabidopsis protoplasts were prepared from 3–4-day-old suspension cell cultures as described previously (31). The washed protoplasts were resuspended in an extraction buffer (400 \( \mu \)m sucrose, 50 \( \mu \)m Tris-HCl, pH 7.5, 3 \( \mu \)m EDTA, 0.1% bovine serum albumin, and 2 \( \mu \)m dithiothreitol), and disrupted by filtration through nylon membranes (32). The broken cells were diluted in a large volume of the extraction buffer, and differential centrifugations were carried out as described previously (33). The chloroplast-enriched fraction was loaded on a 40/80% Percoll step gradient, and intact chloroplast were collected as described (31). Mitochondria were layered onto a 13.5–21.45% Percoll step gradient and spun at 75,000 \( \times \) g for 45 min. The mitochondria were collected at the 21/45% interface and washed in the extraction buffer without bovine serum albumin and dithiothreitol.

**Mitoplast Preparation and Submitochondrial Fractionation—** Mitoplasts were prepared as described (34) with some modifications (35). The mitochondria were resuspended in a swelling buffer, and the outer membrane rupture was achieved by Dounce homogenization. Mitoplast and outer membrane fractions were isolated after centrifugation through a bovine serum albumin-free discontinuous gradient of 22, 33, and 45% sucrose. Outer membrane proteins were collected at 45% sucrose interface, diluted, and recovered by centrifugation at 38,000 rpm in a Ti-75 rotor. Intact mitoplasts were collected from the 33/47% interface, washed and resuspended in 20 \( \mu \)l MOPS, pH 7.2, 1 \( \mu \)l EDTA, 1 \( \mu \)l phenylmethylsulfonyl fluoride at a protein concentration of 3 mg/ml, and broken by three freeze/thaw cycles followed by sonication (5 \( \times \) 10 s, 300 W, Sonic Vibra Cells). The membrane (P) and soluble (S) fraction of mitoplasts were separated by a 30-min centrifugation at 100,000 \( \times \) g in a Beckman TLA-100 rotor. The soluble proteins were precipitated by 10% trichloroacetic acid. The membrane fraction was subjected to alkaline treatment (0.1 \( \times \) Na2CO3, pH 11.5, for 30 min at 4 °C) to extract peripheral proteins (36). A 30-min centrifugation at 100,000 \( \times \) g in a Beckman TLA-100 rotor allows the separation of soluble (peripheral) from insoluble (integral) protein fractions. Freshly purified mitoplasts were subjected to proteinase K treatment. Mitoplasts (1 mg/ml) were incubated with 100 \( \mu \)g/ml proteinase K in 8.6% sucrose, 50 \( \mu \)m Tris-HCl (pH 7.5) for 30 min at 4 °C. Phenylmethylsulfonyl fluoride was added at the final concentration of 1 \( \mu \)m to stop the protease activity, and the mitoplasts were recovered by centrifugation through a 22% sucrose cushion at 15,000 \( \times \) g for 15 min.

**Preparation of Radiolabeled Proteins from Isolated Mitochondria—** Mitochondria were isolated from potato tubers (\( S.\ tuberosum \), var. Bintje) with a juice extractor as described (37). Proteins were synthesized from the corresponding cDNA clones in pBluescript vector, by coupled transcription/translation in the presence of \( ^{35}S \) methionine according to the supplier's instruction (Promega). Import assays were carried out as described (38).

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1 The abbreviations used are: PCR, polymerase chain reaction; AtCCME, Arabidopsis thaliana orthologue of CcmE; MOPS, 4-morpholinepropanesulfonic acid.
RESULTS

AtCCME Is a Single-copy Gene in A. thaliana—The physical and genetic analysis of a nuclear gene cluster on A. thaliana chromosome 3 (39, 40), revealed the presence of AtCCME, a gene encoding a protein showing sequence similarity with the E. coli CcmE (3) and its orthologues for Gram-negative bacteria (4, 41). All cDNAs, isolated from a library prepared from growing cell suspensions, share the same 5′-end at only 5 nucleotides from the initiation codon, whereas two different polyadenylated ends were found at 226 and 268 nucleotides from the initiation codon, which is in agreement with the shift observed in import assays. A radiolabeled probe generated from an AtCCME cDNA probe labeled by random priming. The molecular weight ladder is indicated in kilobase pairs. Restriction enzymes are the same as EcoRI; PstI. After Southern blotting, the membrane was hybridized with an AtCCME cDNA probe labeled by random priming. The molecular weight ladder is indicated in kilobase pairs, shown above. The physical map of the AtCCME locus on A. thaliana chromosome 3 derived from the nucleotide sequence (GenBank™ accession number AFO49236). The restriction fragments that hybridize with AtCCME are represented above the physical map, and their sizes are indicated in kilobase pairs. Restriction enzymes are the same as used for panel A.

AtCCME Is a Mitochondrial Protein and Its Precursor Is Imported into Mitochondria through an N-terminal Cleavable Targeting Sequence—AtCCME cDNA encodes a putative protein of 256 amino acids, which presents an N-terminal extension of about 70–80 amino acids when compared with bacterial proteins (Fig. 2). An extension of a similar size is also present in the putative protein encoded by a CCME homologous gene of Oryza sativa (AC025783). In AtCCME, this extension is enriched in positively charged (Arg) and hydroxylated (Ser) residues and contains few acidic residues. Its first 20 amino acids could form an amphiphilic α-helix. These features are characteristic of the import domain of mitochondrial targeting sequences (42, 43). The localization of AtCCME was checked first by immunodetection using purified polyclonal antibodies generated against an overexpressed fusion protein (from Phe111 to Ser256). The anti-AtCCME antibodies recognized a 27-kDa protein in mitochondria but not in chloroplast protein fractions prepared from A. thaliana protoplasts (Fig. 3A). CCME could also be detected in cauliflower, turnip, rapeseed, and radish mitochondria, but not in potato, pea, sunflower, wheat, and maize mitochondria, indicating that anti-AtCCME antibodies are rather specific to Brassica species (data not shown). To test whether AtCCME encodes a precursor processed after import, we tried to import the radiolabeled protein in vitro into purified mitochondria. A major 32-kDa protein was obtained by in vitro coupled transcription/translation of AtCCME cDNA (Fig. 3B). After its incubation with mitochondria, a signal corresponding to a 27-kDa protein resistant to added proteinase K appeared. The protection was abolished when mitochondrial membrane proteins were extracted by Triton X-100 before proteinase K treatment. AtCCME import was inhibited in the presence of valinomycin, indicating the requirement of an electrochemical membrane potential, Δψ, to achieve AtCCME translocation (44). A 5-kDa reduction was observed between the apparent molecular weights of the precursor and the mature protein. For the mitochondrial processing peptidase domain, only a weak consensus has been found, mainly a conserved Arg in position-2 or-3 from the cleavage site. For plant mitochondrial precursors, two cleavage motifs were proposed (45), of which one, an “R-2” motif (RXA/S)(T/S), is present at position 47–50 (RLSS, Fig. 2). Although the actual processing site has not been determined experimentally, the R-2 motif described above is a good candidate. A cleavage at this position would shorten the precursor protein by 48 amino acids, corresponding to a 5.4-kDa peptide, which is in agreement with the shift observed in import assays. The radioactive band of the in vitro processed protein and the immunodetected A. thaliana endogenous mitochondrial protein migrate at the same position (Fig. 3C), which strongly suggests that in vitro processing in potato mitochondria reflects in vivo maturation of A. thaliana protein.

AtCCME Is a Peripheral Inner Membrane Protein—Bacterial CcmE are mainly hydrophilic proteins except for a short hydrophobic region at the N-terminal domain, which is predicted to act as a noncleavable signal sequence and to anchor the protein in the membrane. Although its amino acid sequence is not conserved, such a hydrophobic domain is present in AtCCME (Fig. 2), which could, as for its bacterial counterparts, attach the protein in a membrane. AtCCME antibody recognized a protein of 27 kDa in the mitochondria (M) and mitoplasts fraction (MP), whereas no signal could be detected in the outer membrane fraction (OM), characterized by the presence of porin, a major protein of the mitochondrial outer membrane (25) and the absence of cytochrome c1, a subunit of the mitochondrial inner membrane cytochrome bc1 complex (Fig. 4A). The presence of porin in the mitoplasts fraction indicated that, in the experimental conditions used, part of the outer membrane remains attached to the inner membrane, most likely at contact points between the two membranes. The mitoplasts were broken and further separated into soluble (S) and membrane (P) protein fractions; AtCCME was present in the membrane fraction (Fig. 4B). No contamination between matrix and membrane proteins could be detected, which was verified using antibodies directed against a matrix protein, manganese-superoxide dismutase, and antibodies against cytochrome c1.

2 F. Grellet, personal communication.
AtCCME amino acid sequence.

The amount of precursor included in each import reaction. Mature polypeptides are indicated by Val. The precursor and theabolished in the presence of valinomycin (TX). The import process isbranes are disrupted by Triton X-100 (PK) in intact mitochondria but is degraded when mitochondria mem-
appears at 27 kDa. This mature protein is resistant to proteinase K, a smaller protein corresponding to the mature formmitochondria (M). In the presence of Pre, p corresponds to a 32-kDa band (lane 1). In the presence of mitochondria mem-

FIG. 2. Amino acid sequence comparison of CCME proteins. CCME proteins from different organisms are aligned: AtCCME (39); O. sativa (OsCCME) (GenBank accession number AC025783); α-proteobacteria B. japonicum (BjCcmE) (18); and γ-proteobacteria E. coli (EcCcmE) (GenBank accession number U00008). The four protein sequences were aligned using the GCG PileUp algorithm (57). Residues identical in more than two sequences are highlighted in black, and the functionally conserved residues are highlighted in gray. The conserved motif 1 and motif 2 regions are indicated. The consensus R-2 plant processing site in AtCCME is underlined. The conserved histidine residue, which binds heme covalently in E. coli, is marked with an asterisk. The numbering is that of the AtCCME amino acid sequence.

FIG. 3. AtCCME is imported into mitochondria. A, Western blot analysis of Arabidopsis protein fractions (30 µg) of protoplast (P), chloroplast (C), and mitochondria (M), probed with antibodies directed against AtCCME, NAD9, LSU (large subunit of ribulose bisphosphate carboxylase), and α-tubulin. B, in vitro translated AtCCME precursor (Pre, p) corresponds to a 32-kDa band (lane 1). In the presence of mitochondria (M), a smaller protein corresponding to the mature form appears at 27 kDa. This mature protein is resistant to proteinase K (PK) in intact mitochondria but is degraded when mitochondria mem-

These results strongly suggest that AtCCME is located in the mitochondrial inner membrane. To further characterize the nature of the membrane interaction, extreme pH treatment was used to extract extrinsic proteins by disruption of electro-

static interactions (46). After alkali treatment of mitoplast membranes, AtCCME was found in the soluble fraction (Fig. 4C). AtCCME protein behaves like NAD9, a protein located in the peripheral arm (iron-sulfur protein fraction) of the L-shaped complex I (27, 47), and not like cytochrome c1, a protein with a C-terminal membrane helix responsible for its intrinsic behavior (Fig. 4C). Our results suggest that AtCCME is attached to the mitochondrial inner membrane by electrostatic interactions such as protein-protein interactions rather than direct contact with the lipid bilayer.

AtCCME Is Oriented toward the Intermembrane Space—PhoA fusion analyses of B. japonicum CcmE (BjCcmE) and E. coli CcmE (EcCcmE) proteins have shown that the hydrophilic part of the protein is exposed to the periplasm (18, 19). By analogy, AtCCME is predicted to be located at the outer face of the inner membrane. To assess this hypothesis, intact mitoplasts were treated with proteinase K to strip inner membrane proteins, which are exposed to the intermembrane space. Mitochondria and mitoplasts incubated in the same conditions in the absence of protease treatment were used as control (Fig. 4D). When proteinase K was added, the immunodetection of AtCCME was lost, whereas it was still observed in untreated mitoplast. The intactness of the inner membrane after protease K treatment was assayed with NAD9 antibody (27). NAD9 is located in the iron-sulfur protein fraction of complex I, which is facing the matrix (47), and therefore protected from proteinase K. The hydrophilic domain of AtCCME is most likely completely digested in treated mitoplasts because no signal corresponding to a smaller partially protected protein could be detected. Therefore in AtCCME, as in its bacterial counterpart, the main conserved motifs are localized on the external side of the inner membrane.

Assaying Complementation of a ccmE E. coli Strain for Holocytochrome c Production—In addition to its location and
AtCCME, constructed the plasmid pAT1, which expresses a truncated prokaryotic CcmE proteins share the motif 1 and motif 2, are present in AtCCME, although prokaryotic CcmE proteins share from 26 to 81% identity. Two major conserved motifs in bacteria, motif 1 and motif 2, are present in AtCCME, although in A. thaliana and in O. sativum, the insertion of a sequence rich in charged residues increases the distance between them (Fig. 2). In motif 2, the histidine residue, which was shown to complement the organisms. To test whether the eukaryotic protein could complement the AtCCME, potato porin, wheat NAD9, yeast cytochrome c, and tobacco manganese-superoxide dismutase (Mn-SOD).

**TABLE I**

| Strains and plasmids used in this work | Relevant characteristics |
|---------------------------------------|--------------------------|
| Strain                               | Reference                |
| MC1061                               | hsdR merB araD139 Δ(araABC-leu) 7679 ΔlacX74 galU galK rpsL thi (58) |
| EC06                                 | Δcem derivative of MC 1061; Km<sup>R</sup> (3) |
| EC65                                 | Δcem<sup>E</sup>; Δ3–95, derivative of MC 1061; Km<sup>R</sup> (3) |
| Plasmid                               |                           |
| pAT1                                 | A. thaliana CCME (Met<sup>79</sup>–Ser<sup>256</sup>) cloned into pISC-2; Ap<sup>R</sup> This work |
| pAT2                                 | A. thaliana CCME (Met<sup>79</sup>–Ser<sup>256</sup>) His<sup>222</sup> Ala mutant cloned into pISC-2; Ap<sup>R</sup> This work |
| pEC412                                | E. coli ccmE cloned into pISC-2; Ap<sup>R</sup> This work |
| pEC101                                | E. coli ccmABCD cloned into pACYC184; Cm<sup>R</sup> This work |
| pBH2921                               | B. japonicum cya cloned into pISC-2; Km<sup>R</sup> (21) |

topology in mitochondria, the AtCCME sequence similarity with bacterial proteins suggests that the plant protein could fulfill similar functions for cytochrome c biogenesis in mitochondria. Indeed, AtCCME shares 35.5 and 40.6% identical amino acids with *E. coli* CcmE and *B. japonicum* CcmE proteins, respectively, whereas prokaryotic CcmE proteins share from 26 to 81% identity. The two major conserved motifs in bacteria, motif 1 and motif 2, are present in AtCCME, although in *A. thaliana* and in *O. sativum*, the insertion of a sequence rich in charged residues increases the distance between them (Fig. 2). In motif 2, the histidine residue, which was shown to bind heme covalently in *E. coli* (19), is strictly conserved in all organisms. To test whether the eukaryotic protein could complement the *E. coli* ΔcemE mutant strain (EC65, Table I), we constructed the plasmid pAT1, which expresses a truncated form of AtCCME deleted from its first 78 amino acids. AtCCME (Met<sup>79</sup>–Ser<sup>256</sup>) best corresponds to the EcmE size, pI, and hydrophobicity profiles. EC65 strain was transformed with a vector carrying various *cem* genes together with pBH2921 (Table I), carrying cya, the soluble periplasmic cytochrome c<sub>550</sub> gene from *B. japonicum* used as a reporter for holocytochrome c maturation (3). The peroxidase activity associated with covalently bound heme was used to check the presence of holocytochromes c in periplasmic protein extract (22). The transformants were grown anaerobically to induce expression of the chromosomal genes *ccmA*–*ccmH* and promote cytochrome c production. Both CcmE and cytochrome c<sub>550</sub> were expressed from an arabinose-inducible promoter. When EcmE was expressed in EC65 background, three cytochromes c were detected by heme stain: NrfA and NapA, two endogenous cytochromes c, and the reporter cytochrome c<sub>550</sub> of *B. japonicum* (Fig. 5, lane 1). When the complementation was tried with pAT1, no holocytochrome c could be detected (Fig. 5, lane 2).

*AtCCME Covalently Binds Heme in E. coli*—The cytochrome c maturation pathway was impaired when trying to complement a ΔcemE *E. coli* strain with AtCCME in anaerobic respiration. We wanted to know at which step heme trafficking was blocked. For this determination, we tested whether heme transfer to CcmE was possible. Heme stain was used to check the formation of holo-CCME, i.e. a protein binding heme in a covalent way. We transformed EC06, an *E. coli* strain deleted in all *cem* genes (3) with pAT1 alone or with pEC101 (Table I). The assays were performed under aerobic growth conditions in the presence of the CcmA–D proteins, a condition that is sufficient for heme incorporation into CcmE. We first checked whether AtCCME was correctly expressed and inserted in *E. coli* membranes. After induction by arabinose, a protein corresponding to the truncated form of AtCCME was expressed and detected in the membrane fraction (Fig. 6A, lane 1). Immuno-detection using antibodies directed against AtCCME or EcCme suggests that the level of expression of AtCCME is reduced compared with that of EcCcmE (Fig. 6, A and B). In a Δcem background, AtCCME, as its *E. coli* counterpart, did not bind heme (Fig. 6C, lane 1). When the *E. coli* CcmABCD proteins were expressed with AtCCME, the mitochondrial protein was able to bind heme in a covalent way (Fig. 6C, lane 2). This shows that the truncated mitochondrial protein is correctly inserted in the bacterial membrane and that its conserved heme-binding domain is oriented toward the periplasm, thus allowing heme attachment. In *E. coli*, CcmC is the only Ccm protein that is strictly required for heme transfer and binding to EcCcmE (19). Our results suggest that heme transfer is possible from *E. coli* CcmC to *A. thaliana* CCME. To check whether, in AtCCME, heme is attached to the conserved histidine of motif 2, we changed the histidine 222 to an alanine by site-directed mutagenesis. EC06, the Δcem strain, was cotransformed with pAT2 expressing the His<sup>222</sup>Ala truncated AtCCME and pEC101 expressing CcmABCD. Although the mutant protein was expressed, no heme-binding AtCCME could be detected (Fig. 6, A and C, lane 3). A positive control was done.

![Fig. 4. Submitochondrial localization and topology of AtCCME](http://www.jbc.org/)

**FIG. 4.** Submitochondrial localization and topology of AtCCME. A, mitochondria (M), outer membrane (OM), and mitoplast (MP) protein extracts were analyzed for AtCCME, porin, and cytochrome c (cyt c). B, mitoplasts were subjected to freeze/thaw cycles and sonication. Soluble and membrane proteins were collected after ultracentrifugation. Total mitoplast (MP), supernatant (S), and pellet (P) fractions were analyzed with the indicated antibodies. C, The mitoplast pellet fraction was treated with Na<sub>2</sub>CO<sub>3</sub>, pH 11.5, to extract peripheral proteins (S), whereas intrinsic membrane proteins remain in a 100,000-g pellet (P). D, mitoplasts were treated with 100 µg/ml proteinase K and analyzed for AtCCME and NAD9.

NAD9, which is exposed to the matrix, is shown as the control for inner membrane integrity. The Western blots were probed with antibodies directed against the following mitochondrial proteins: AtCCME, potato porin, wheat NAD9, yeast cytochrome c, and tobacco manganese-superoxide dismutase (Mn-SOD).
Heme from CcmC.

rather than by the low efficiency with which AtCCME gets heme release to the following proteins of the pathway (CcmF, -G, or strain by AtCCME is most probably impaired by the absence of motifs to bind heme, most likely through the conserved histidine of motif part of cytochrome complementation assays, these results show that AtCCME is par-

E. coli

2

E. coli

2

EcCcmE; lane 2, pAT1 expressing AtCCME(Met79-Ser256); lane 3, pISC-2, the empty vector, as control. NrfA and NapB are two endoge-

mitsochondria and immunodetection into Arabidopsis protein extracts, we have demonstrated clearly that AtCCME is a mito-

chondrial protein. In addition, in vivo experiments were performed using transient expression of different AtCCME-GFP fusion proteins in tobacco cells. All these fusion proteins were detected exclusively in mitochondria. We can exclude a dual targeting to both organelles, which has been reported for other proteins such as glutathione reductase (49), ferrochelatase (50), and aminoaacyl-tRNA synthetases (51, 52).

We have shown that AtCCME is associated with the mitochondrial inner membrane. AtCCME has a typical mitochondrial targeting sequence at its N terminus, which is able to target a reporter protein to mitochondria and which is cleaved upon in vitro import. Our attempts to purify the mature protein for N-terminal sequencing have been unsuccessful, mainly because of its instability when extracted. The exact N-terminal sequence of the mature protein remains unknown. Nevertheless, we propose that a domain of about 16 to 30 residues, conserved in several plant CCME proteins and rich in charged amino acids, is present at the N-terminal end of the mature mitochondrial protein, preceding the hydrophobic domain. This region would constitute a plant-specific motif not found in bacterial CcmE proteins.

In a number of plants, mitochondrial genes encoding counterparts of CcmB, CcmC, and CcmF are transcribed and their mRNAs are edited. In some cases, the putative corresponding proteins could be immunodetected in the membrane protein fraction of mitochondria. However, no topology of any of these proteins has been described, and no functional analysis of the corresponding mitochondrial genes has been successful up to now. AtCCME, the product of a nuclear gene, comprises the relevant features that play a role in mitochondrial cytochrome c biogenesis and appears a better candidate for functional analysis based on complementation of E. coli mutant strains.

The complementation of ΔccmE E. coli strains has been tested at two different steps of cytochrome c maturation: heme binding to Ccme and heme transfer to apocytochrome c. AtCCME was detected in E. coli membranes and could be heme-stained when overexpressed with EcCcmABCD. Because heme binding occurs only when Ccme is translocated to the periplasm (19), this indicates the correct location of AtCCME in E. coli. Complementation assays of a ΔccmE E. coli strain by AtCCME (Met79-Ser256) were unsuccessful for holocytochrome c production. The results of the complementation assays could be explained by the inability of the AtCCME to release heme to the following bacterial partners of the maturation pathway, although it is able to catch it from EcCcmE. Within Ccme proteins, CcmC mitochondrial orthologues are among the closest relatives of their bacterial counterparts, by their predicted topology and by the sequence conservation of the Trp-rich motif

N. Spiewewo, J. M. Grienenberger, and G. Bonnard, unpublished results.

G. Bonnard, unpublished results.

DISCUSSION

In photosynthetic eukaryotes, two cytochrome c biogenesis pathways operate in the same cell although in separated compartments, mitochondria and chloroplasts. In land plants, sys-

tem I and system II are proposed to perform cytochromes c maturation in mitochondria and chloroplast, respectively. Aside from organelle genes, several nuclear loci are most likely involved in cytochrome c biogenesis for each system. System I and system II share some common elements (heme delivery and thioreduction), and their evolutionary link appears through the conservation of a tryptophan-rich motif and some histidine residues (3, 22, 48). Therefore, the subcellular location of nuclear-encoded proteins showing similarities with bacterial proteins required for cytochromes c biogenesis must be addressed carefully. AtCCME is the first ccm orthologue found in a plant nuclear genome. In A. thaliana this gene is unique. Using different approaches, in vitro import into isolated mito-

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and the two flanking essential histidine residues (48, 54, 55). These conserved motifs were proposed to have a function in heme delivery. They are also found in the mitochondrial CcmF orthologue to the N-terminal part of CcmF. The conserved domains are interspersed by plant-specific sequences, creating greater divergences between the plant mitochondrial and their bacterial CcmF counterparts than for CcmC ones. The potential interactions of AtCCME with EcCcmF could be less efficient in a heterologous system, explaining the absence of heme release from CCME. The knowledge of the full set of proteins for the c-type cytochrome pathway in mitochondria will help in designing design different combinations of mitochondrial/bacterial genes for holocytochrome c formation in bacteria.

Because of experimental limitations, heme binding to AtCCME in mitochondria could not be tested with the heme staining methods used for the overexpressed EcCcmE. The instability of the purified mitochondrial protein and the fact that heme binding, although covalent, is proposed to be transient were the main difficulties encountered in getting direct evidence of heme binding to AtCCME in plant mitochondria.

The cytochrome c biogenesis pathway, known as system I, is followed by α- and γ-proteobacteria and Archaea. Genes encoding related proteins were identified by sequence similarities in mitochondrially encoded genes of a few prokaryotes, one red algae (56), and land plants. The maximum set of ccm genes (system I) found in mitochondrial genomes is found in protists like R. americana. A. thaliana. (chromosome 1; 28% amino acid sequence identity with the A. thaliana plant mitochondria. In this paper, for the first time, a function proposed to be involved in cytochrome c maturation. More detailed knowledge of each of the plant mitochondrial served domains are interspersed by plant-specific sequences, plant mitochondria. In this paper, for the first time, a function proposed to be involved in cytochrome c maturation. More detailed knowledge of each of the plant mitochondrial served domains are interspersed by plant-specific sequences, plant mitochondria. In this paper, for the first time, a function proposed to be involved in cytochrome c maturation. More detailed knowledge of each of the plant mitochondrial served domains are interspersed by plant-specific sequences, plant mitochondria. In this paper, for the first time, a function proposed to be involved in cytochrome c maturation. More detailed knowledge of each of the plant mitochondrial served domains are interspersed by plant-specific sequences, plant mitochondria. In this paper, for the first time, a function proposed to be involved in cytochrome c maturation. More detailed knowledge of each of the plant mitochondrial served domains are interspersed by plant-specific sequences, plant mitochondria. In this paper, for the first time, a function proposed to be involved in cytochrome c maturation. More detailed knowledge of each of the plant mitochondrial served domains are interspersed by plant-specific sequences.
CCME, a Nuclear-encoded Heme-binding Protein Involved in Cytochrome c Maturation in Plant Mitochondria
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