AtCCMA Interacts with AtCcmB to Form a Novel Mitochondrial ABC Transporter Involved in Cytochrome c Maturation in Arabidopsis*\[S\]

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ABC transporters make a large and diverse family of proteins found in all phyla. AtCCMA is the nucleotide binding domain of a novel Arabidopsis mitochondrial ABC transporter. It is encoded in the nucleus and imported into mitochondria. Suborganellar and topology studies find AtCCMA bound to the mitochondrial inner membrane, facing the matrix. AtCCMA exhibits an ATPase activity, and ATP/Mg\(^{2+}\) can facilitate its dissociation from membranes. Blue Native PAGE shows that it is part of a 480-kDa complex. Yeast two-hybrid assays reveal interactions between AtCCMA and domains of CcmB, the mitochondria-encoded transmembrane protein of a conserved ABC transporter. All these properties designate the protein as the ortholog in plant mitochondria of the bacterial CcmA required for cytochrome c maturation. The transporter that involves AtCCMA defines a new category of eukaryotic ABC proteins because its transmembrane and nucleotide binding domains are encoded by separate genomes.

ATP binding cassette (ABC)\[6\] transporters belong to a large family of proteins that are found in all prokaryotic and eukaryotic species. ABC proteins are composed of four domains, two nucleotide binding domains (NBD) characterized by a highly conserved ATP binding cassette, and two transmembrane domains (TMD). These four modules may be expressed into a single polypeptide (full transporter), two polypeptides (half-transporter), or four polypeptides. The substrates of these transporters are actively transported using ATP hydrolysis as energy source. Substrates include a wide variety of structurally and chemically different compounds such as ions, sugars, lipids, proteins, antibiotics, and drugs (1). Other ABC proteins regulate the activity of channels instead of being transporters themselves (2). The analysis of Arabidopsis thaliana nuclear genome has revealed 129 potential ABC proteins, a large number when compared with other eukaryotes (3). Most of the Arabidopsis ABC proteins could be assigned to known subfamilies with the exception of a heterogeneous group of ABC proteins lacking transmembrane domains that were termed non-intrinsic ABC protein (NAP). These proteins are reminiscent of ATP binding quarter molecules of prokaryotic ABC transporters. However, up to now none of these NAPs was found to be associated to TMDs, thus reconstituting a complete ABC transporter.

During evolution, distinct assembly pathways have arisen to achieve the covalent attachment of the heme prosthetic group to apocytochromes c of bacteria, mitochondria, and chloroplasts (4 – 6). System I was first described in \(\alpha\) and \(\gamma\) proteobacteria, system II in chloroplasts, and Gram-positive bacteria and system III in yeast mitochondria. Although the origin of system III could not be traced back to prokaryotes, mitochondria of plants and of some protists have inherited system I from their endosymbiotic \(\alpha\) proteobacteria ancestor. One representative for system I is Escherichia coli, where eight genes named ccm for cytochrome c maturation are essential for the periplasmic assembly of c-type cytochromes (7). In system I, CcmE is a central player of heme delivery; this so-called heme chaperon binds heme in a covalent but transient manner before its transfer to CcmF, proposed to catalyze the ligation of heme to apocytochrome c (8, 9). In E. coli, ccm genes are organized in an operon starting with ccmA that encodes the nucleotide binding domain of an ABC transporter. The two following genes which encode CcmB and CcmC both contain six transmembrane helices as found in most TMDs. The exact composition of the ABC transporter and its function have been debated for long (for review, see Ref. 10). Briefly, two models have been proposed. The first model proposed that the ABC transporter is involved in heme export and consists of CcmA, CcmB, CcmC and CcmE being the two TMDs (11, 12). In the second model CcmC delivers heme to the heme chaperon CcmE (13), and the ABC transporter consisting of CcmA, CcmB, and CcmC is not involved in heme export (for review, see Ref. 14). It was recently shown that the ATPase activity of CCMMA is required for the release of holo-CmcE from CmcC (15, 16). Feissner et al. (15) proposed that
ATP-driven conformational changes result in the liberation of holo-CcmE from CcmA2BC that would act as a chaperon releaser rather than as true transporter. For Christensen et al. (16) the need of ATPase activity for heme transfer from holo-CcmE to apocytochrome c could also be interpreted as the requirement of a compound supplied by the ABC transporter to form or break with the unusual histidine–heme bond in CCME.

Transcription studies of the mitochondrial genome of wheat reveal the existence of the \(ccmB\) (17), \(ccmC\) (18), and \(ccmF\) genes (19, 20). Orthologs of \(ccmABCF\) were identified as well on the mitochondrial genomes of \(Reclinomonas americana\), a jakobid flagellate with an ancestral mitochondrial genome (21), and of \(Cyanidioschyzon merolae\), a primitive unicellular red alga (22). However, although \(ccmBCF\) orthologs are conserved on all mitochondrial genomes of land plant, \(ccmA\) could not be found on any of them. The sequence similarity between CcmA and the nuclear encoded NAP10 strongly suggests that ccmA has been transferred to the nucleus as shown in \(A. thaliana\) for genes encoding two other plant mitochondrial CCM proteins, the heme chaperone AtCcmE (23), and the thiol-disulfide oxidoreductase AtCcmH (24). In this work we show that NAP10 is a mitochondrial protein, which has kept the topology of its bacterial counterpart CcmA and has characteristics of the NBD of an ABC transporter. Interaction between AtCcmA and domains of AtCcmbB were revealed by yeast two-hybrid assays. Therefore, we propose that NAP10 is the CcmA counterpart of the mitochondrial CCM ABC transporter. Contrary to the mitochondrial ABC transporters described so far, which belong to the full or half-transporter types (25), the plant ABC transporter involved in c-type cytochrome maturation makes a novel class of organellar ABC transporter since its TMDs are encoded on the mitochondrial genome and its NBDs are encoded by the nuclear gene NAP10.

**EXPERIMENTAL PROCEDURES**

**Cloning and Expression of AtCCMAs, Production of Antibodies**—Total RNA was prepared from the aerial parts of 4-week-old \(A. thaliana\) plants. cDNA was synthesized using random hexamers as primers for reverse transcription. The coding region of \(AtCCMA\) was amplified by PCR using primers P1 and P2 (supplemental Table S1) and cloned into the HindIII/EcoRI sites of pSK, resulting in pNG22, the plasmid used for in vitro transcription/translation. The coding sequence of \(AtCCMA\) was amplified using primer P3 and P4 and cloned in pQE60 (Qiagen), generating pNG31. The recombinant CCMA protein expressed in \(E. coli\) BL21D3 possesses an additional Val at position +2 and a His\(_6\) tag at the C-terminal end. CCMA–His was expressed after induction with 1 mM isopropyl-1-thio-\(\beta\)-galactopyranoside, affinity-purified under denaturing conditions on a nickel-nitrilotriacetic acid-Sepharose column (Novagen), resolved on preparative SDS-PAGE, and electroduted. The purified protein was used to immunize rabbits. Anti-AtCCMA–His antibodies were purified by immunoadfinity for the overexpressed protein coupled with CNBr-activated Sepharose (Amersham Biosciences).

Using pNG31 as a template, the PCR product obtained with P5 and P6 was digested by SmaI and introduced in the EcoRV site of pACYC184, generating pNG34 used in complementation experiments. Similarly, the \(AtCCMA\) coding region was amplified with P7 and P8 using pNG31 as template. The PCR fragment obtained was digested by HindIII and EcoRI and cloned in the corresponding sites of pMALc2 (New England Biolabs) generating pNG35. The fusion protein maltose binding protein (MBP)-CCMA was expressed in Rosetta strain \(E. coli\) cells (Novagen). After induction by isopropyl-1-thio-\(\beta\)-galactopyranoside, it was purified by affinity chromatography under native conditions using an amylose resin column.

**Subcellular Fractionation of A. thaliana Protoplasts and Sub-mitochondrial Fractionation—Cytosolic, chloroplast, and mitochondrial fractions from \(A. thaliana\) protoplasts were obtained as described previously (26). Mitoplasts were prepared as described (27). Mitoplasts were resuspended at a protein concentration of 1 mg/ml, and trypsin was added at 25 \(\mu\)g/mg of mitochondrial proteins and incubated for 30 min on ice. Trypsin inhibitor was added to stop the reaction, and the mitoplasts were recovered after centrifugation through a 27% (w/v) sucrose cushion in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA pH 8.0, 100 mM K\(_2\)HPO\(_4\), and 1 mg/ml bovine serum albumin) at 15,000 \(\times\) g for 10 min. Membrane and soluble fractions of mitochondria and mitoplast were prepared as described previously (23). Extrinsic membrane proteins were solubilized with 0.1 M Na\(_2\)CO\(_3\), pH 11.5, for 30 min at 4 °C. Mitochondrial membranes were treated with varying concentrations of urea (3.6 and 7.3 M) and with 6.6 M urea in the presence of 15 mM ATP and 15 mM MgSO\(_4\) on ice for 60 min. Centrifugation at 100,000 \(\times\) g for 30 min at 4 °C in a Beckman TLA-100 rotor allowed the separation of soluble proteins from insoluble ones.

**Complementation Experiments**—The \(\Delta ccmA\) \(E. coli\) strain EC21 (28) and the plasmids pRJ3291 containing \(Bradyrhizobium japonicum\) cycA coding for the soluble periplasmic cytochrome \(c_{550}\) (13) and pEC71 (EccmA cloned in pACYC184) were provided by Prof. L. Thöny-Meyer (ETH, Zurich). EC21 was transformed with pNG34 or pEC71 containing \(AtCCMA\) or EccmA, respectively, and pRJ3291 containing the reporter cycA gene. \(E. coli\) were grown in anaerobic conditions in minimal salt medium in the presence of 5 mM nitrate as the electron acceptor (29). Expression of the reporter cytochrome \(c_{550}\) was induced with 0.4% (w/v) arabinose. Preparation of total bacterial and periplasmic protein extracts and heme staining were performed as described (7, 30, 31).

**Immunodetection Assays**—Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred on to polyvinylidene difluoride membranes (Immobilon-P, Millipore). Immunodetection with purified anti-AtCCMA antibodies was done at a dilution of 1/1000. Antibodies used as control were directed against the cytosolic \(Arabidopsis\) thioredoxin \(h_3\), the chloroplastic \(Chlamydomonas\) light harvesting complex II, and mitochondrial yeast cytochrome \(c_1\) as an intrinsic inner membrane protein, tobacco manganese-superoxide dismutase as a soluble matrix protein, wheat subunit 9 of NADH dehydrogenase as an extrinsic inner membrane protein (32), and AtCCME (23) for a mitochondrial inner membrane protein oriented toward the intermembrane space.

**In Vitro Import of Radiolabeled Proteins into Isolated Mitochondria**—Mitochondria were purified from cauliflower heads using a juice extractor (33). Synthesis of the radiolabeled
proteins was done using the coupled reticulocyte transcription/translation system (TNT system, Promega) in the presence of \[^{35}\text{S}\]methionine. In vitro import assays were performed as described previously (26).

**Blue Native PAGE and In-gel ATPase Activity**—Blue native PAGE and BN-SDS-PAGE of mitochondrial membrane proteins were carried out as described (20). Total mitochondrial proteins solubilized with digitonin, 5/1 detergent/protein (w/w), and native MBP-CCMA recombinant protein were loaded on a BN-PAGE. The ATPase activity was visualized by histochemical staining based on the precipitation of lead nitrate by the inorganic phosphate produced by ATP hydrolysis. The BN gel was incubated in 50 mM glycine, pH 8.6, 5 mM MgCl\(_2\), 5 mM ATP, and Pb(NO\(_3\))\(_2\) reagent was added stepwise to a final concentration of 0.1% (w/v) as described (34).

**Yeast Two-hybrid Assays**—cDNA of \textit{AtCcmB} were cloned in pSK after reverse transcription-PCR using primers P9 and P10. A fully edited version (pNG36) was selected after sequencing. cDNA fragments coding for the full-length AtCCMA protein and for seven domains of AtCcmB (corresponding to following regions: B1, Met-1—Ile-19; B2, Pro-33—Trp-52; B3, Ser-61—Gln-99; B4, Leu-110—Gly-129; B5, Gly-138—Pro-167; B6, Leu-168—Tyr-188; B7, Phe-186—Asp-206) were amplified with primers listed in supplemental Table S1. The PCR products obtained were amplified with primers attB1 and attB2 to introduce phage \(\lambda\) att recombination sites. Final products were recombined with pDONR207 (Invitrogen) to obtain entry vectors that were recombined with Clontech pGBKT7- and pGADT7-modified vector (35) to obtain constructs expressing all the combinations of AtCCMA or part of AtCcmB fused to the activation (AD) or binding (BD) domains of Gal4. Constructs were transformed in yeast strain p69-4A by heat shock. Two-hybrid assays were performed according to standard methods (36). Transformation in yeast was controlled by the growth on media lacking leucine and tryptophan. The expression of reporter genes ADE2 and HIS3 was monitored by the growth on media lacking both adenine and histidine. The expression of lacZ was followed by measuring at \(A_{420}\) the accumulation of the product metabolized by \(\beta\)-galactosidase with 2.2 mM 2-nitrophenyl \(\beta\)-D-galactopyranoside (Sigma) as substrate.

**RESULTS**

**Identification and Expression of AtCCMA**—In \textit{A. thaliana}, five mitochondrial genes encode proteins showing sequence similarities with bacterial CcmB-C proteins and with three domains of CcmF. No ortholog to the bacterial \textit{ccmA} was found in land plant mitochondrial genomes. The conservation of genes coding for the TMDs of a potential CCM transporter prompted us to search for a nuclear gene encoding the NBD \textit{CcmA} ortholog. Because of the high sequence similarity between non-homologue ABC transporters and the limited information concerning \textit{CcmA}-specific conserved patterns, it was essential to search in a fully sequenced plant genome. Using the tblastn program, we identified the \textit{Arabidopsis} At1g63270 gene as the best candidate for AtCCMA. The coding region is 690-bp long and codes for a protein of 229 amino acids showing around 30% identity with its bacterial counterparts. This unique gene was registered as NAP10 in the complete inventory of the \textit{A. thaliana} ABC protein superfamily.
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(3). NAP10 belongs to a heterogeneous group of soluble proteins containing a single NBD. AtNAP10 protein sequence is aligned with NAP10 orthologs from other plant species, a subset of bacterial CcmA, and the mitochondrion encoded CcmA of the protist \textit{Reclinomonas} (Fig. 1). AtNAP10 possesses the typical Walker A \textbf{(P-loop)} and Walker B motifs and the ABC signature. In the bacterial CcmA family a conserved charged residue lysine or arginine is found at the 7th position within the ABC signature LSXGQX(K/R)R, whereas a glutamine is found at this position in bacterial importers (11, 37). This feature is conserved in AtNAP10 and its plant orthologs. Both bacterial and mitochondrial CcmA subgroups possess a conserved histidine but located at a different position (Fig. 1). The analysis of a full-length AtNAP10 cDNA reveals the presence of an intron in the 5'-untranslated region. No organ-specific expression data were available for AtCCMA. Reverse transcription-PCR analysis revealed the presence of AtCCMA transcripts in \textit{A. thaliana} stems, leaves, and flowers (data not shown). In \textit{Arabidopsis}, the protein is detected in cell culture, root, leaf, and flower mitochondrial extracts (data not shown). AtCCMA is expressed in all organs of the plant but at low levels, suggesting that this protein has a housekeeping function.

\textbf{Complementation Assay}—The bacterial protein CcmA has been shown to be essential for the maturation of \textit{c}-type cytochromes (28, 38, 39). The complementation of \textit{ΔccmA} \textit{E. coli} strain EC21 lacking the chromosomal copy of \textit{ccmA} with \textit{AtCCMA} for the production of holocytochrome \textit{c} was tested in anaerobic conditions that induce the expression of the chromosomal \textit{ccm} operon. \textit{ΔccmA} \textit{E. coli} were transformed with a plasmid carrying the reporter cytochrome \textit{c}_{550} gene (pR3291) and the vector alone pACYC184 or the vector carrying either the bacterial \textit{ccmA} (pEC71) or \textit{AtCCMA} (pNG34). Although the bacterial \textit{ccmA} gene could fully complement the absence of the chromosomal \textit{ccmA}, resulting in the formation of holocytochrome \textit{c}_{550} and two endogenous \textit{c}-type cytochromes NrfA and NapB, no \textit{c}-type cytochromes were detected in bacteria transformed with \textit{AtCCMA} (supplemental Fig. S1). This was not because of the absence of expression of \textit{AtCCMA}, because the protein is detected in total extract. The inability of \textit{AtCCMA} to functionally complement \textit{ΔccmA} \textit{E. coli} could be due to its failure to associate with the bacterial transmembrane domains to form a functional ABC transporter.

\textit{AtCCMA Is a Mitochondrial Protein}—\textit{AtCCMA} only possesses a short N-terminal extension of 4–8 amino acids when compared with most bacterial proteins. The amino acids at the N terminus do not form a typical amphiphilic \textalpha{} helix, a characteristic feature of N-terminal mitochondrial targeting sequences. However, the prediction programs unanimously predicted a mitochondrial location for \textit{AtCCMA}. Mitochondria from cauliflower, a Cruciferae as is \textit{A. thaliana}, were used for \textit{in vitro} import assays. The calculated molecular weight of \textit{AtCCMA} is 25.9 kDa. When \textit{AtCCMA} is translated \textit{in vitro}, 2 products are obtained with apparent molecular masses of 28 and 23 kDa (Fig. 2A, lane 1). Both proteins could be immunoprecipitated by anti-CCMA antibodies, indicating that these bands correspond to in-frame translation products of \textit{AtCCMA} (data not shown). After import, a processed band of about 25 kDa was detected (Fig. 2A, lane 2) but disappeared with proteinase K treatment of the mitochondria after import (Fig. 2A, lane 3). Only the 28-kDa protein was resistant to proteinase K (Fig. 2A, compare lanes 2 and 3). This protection to proteinase K was lost when the mitochondrial membranes were solubilized with Triton X-100 (Fig. 2A, lane 4). The import of \textit{AtCCMA} requires an electrochemical membrane potential as evidenced by the inhibition of import after treatment by the ionophore valinomycin (Fig. 2A, compare lanes 3 and 6). \textit{AtTRX-\textalpha{}}, a thioredoxin of the mitochondrial matrix, was used as a positive control (26) to validate the import competence of the mitochondria and the efficiency of the protease treatment. The pre-
cursor form of thioredoxin is still detectable after proteinase K treatment. This band corresponds to an imported protein not processed, as often observed in import assays rather than resulting from an incomplete proteolysis of precursor. In the later case such undigested protein should also be detected in the import assay with valinomycin followed by proteinase K treatment. No signal was observed in this assay for both AtTRX-o1 precursor form and AtCCMA 28-kDa band, indicating that, when import is inhibited, the bound precursors are fully digested by proteinase K treatment. We, therefore, conclude that AtCCMA is imported into mitochondria in a membrane potential-dependent manner without apparent processing.

To confirm the localization of AtCCMA, an analysis of subcellular protein fractions of A. thaliana protoplasts was carried out by Western blotting and immunodetection with anti-AtCCMA antibodies (Fig. 2B). A 28-kDa signal was detected in mitochondria, which corresponded to the size of the imported AtCCMA in the in vitro import experiments. This band was absent in both cytosolic and chloroplastic extracts, indicating that AtCCMA is exclusively located in mitochondria. Anti-AtCCMA antibodies also recognized unidentified proteins of 66 and 40 kDa in the mitochondrial protein fraction. In addition, a 42-kDa cytosolic protein and a 40-kDa chloroplastic protein cross-react with anti-AtCCMA antibodies. The results obtained with antibodies directed against a cytosolic protein (AtTRX-h3), a chloroplastic protein (LHCII), and a mitochondrial protein (Cyt c1) show no detectable cross-contamination between the different protein fractions obtained. The in vitro import, the immunoprecipitation assays, and the immunolocalization experiments clearly show that the 28-kDa protein corresponds to AtCCMA and is localized exclusively in mitochondria.

AtCCMA Is Associated with the Inner Membrane and Oriented toward the Mitochondrial Matrix—The sub-mitochondrial location of AtCCMA was further investigated by the immunodetection of various sub-mitochondrial protein fractions with anti-AtCCMA antibodies. The 28-kDa AtCCMA protein is detected in the membrane protein fraction prepared from mitoplasts but not in the soluble matrix protein fraction (Fig. 3A). Although it is a hydrophilic protein, AtCCMA is present in or associated with the mitochondrial inner membrane. Mitoplasts were treated with trypsin to digest proteins located on the intermembrane space side of the inner membrane. Anti-AtCCMA antibodies detected AtCCMA in mitoplasts that had undergone this treatment, indicating that it is oriented toward the matrix (Fig. 3B). Antibodies against Nad9, a subunit of the mitochondrial respiratory complex I facing the matrix (32), were used as a control for the intactness of the inner membrane. Nad9 is indeed protected against protease treatment. Conversely, the efficiency of the digestion was ascertained by antibodies directed against the hydrophilic domain of AtCCME oriented toward the intermembrane space (23). AtCCME is not detected in mitoplasts treated with trypsin. AtCCMA is oriented toward the mitochondrial matrix, a topology analogous to that of its bacterial counterpart.

AtCCMA Has Properties of the ATP Binding Domain of an ABC Transporter—The association of AtCCMA with the mitochondrial membranes was further investigated. The mitochondrial membranes were subjected to alkaline treatment (Fig. 4A) to extract extrinsic membrane proteins by disrupting electrostatic bonds. AtCCMA is detected in the membrane fraction like Cyt c1, an intrinsic inner membrane protein of the respiratory complex III, whereas Nad9, a peripheral protein of the respiratory complex I, was found in the soluble fraction. The hydrophilic AtCCMA has the behavior of an intrinsic protein. This paradoxical behavior is not surprising since in ABC transporters the association of NBDS to TMDs is a lot tighter than the association of classical peripheral membrane proteins (40). The presence of ATP and Mg2+ was shown to modulate this strong association in the case of a bacterial ABC transporter, the histidine permease (41). The effect of Mg-ATP on AtCCMA solubilization by urea was investigated (Fig. 4B). AtCCMA was detected in the pellet fraction of mitochondrial membranes treated with 3.6
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AtCCMA Is Present in a Complex of 480 kDa—The biochemical properties of AtCCMA were further investigated by searching whether AtCCMA was present in a protein complex. *Arabidopsis* mitochondrial membrane complexes were separated by non-denaturing blue native gel electrophoresis and analyzed by Western blot. Anti-AtCCMA antibodies recognize a single complex of about 480 kDa (Fig. 5). Because of the cross reactivity of anti-AtCCMA antibodies with two other proteins (see above Fig. 2B), we performed a second dimension electrophoresis. The antibodies recognize a 28-kDa protein corresponding to AtCCMA at the vertical of the 480-kDa complex recognized in the first dimension. The 40- and 66-kDa cross-reacting proteins belong to complexes of sizes close to 140 kDa not immunodecorated in the first dimension most likely because their epitopes were masked in the native complexes. Therefore, the 480-kDa high molecular mass complex indeed contains AtCCMA. Whether the ABC transporter is considered to be made up of CCMA2B2 or of CCMA2BC, it would have a calculated size of about 100 kDa. None of the CCM proteins for which antibodies are available, (i.e. AtCCME, AtCCMH, AtCcmF<sub>n14</sub>, and AtCcmF<sub>n20</sub>) is detected in the 480-kDa complex. The ABC transporter could be associated to other proteins, which need to be identified. Alternatively the ABC transporter could be present in a multimeric form.

**AtCCMA Interacts with AtCcmB Domains Oriented toward the Matrix**—The absence of antibodies against the candidate TMD domains of the CCM mitochondrial ABC transporter did not allow us to test whether AtNAP10 was indeed associated with a TMD partner of the CCM pathway. We chose to test the interactions between AtCCMA and AtCcmB by yeast two-hybrid assays. The high hydrophobicity of AtCcmB protein led us to analyze protein interactions with domains predicted to be at the surface of the membrane. A topology model of AtCcmB was constructed with TopPred (43). It predicts six transmembrane helices, four matrix domains, and three intermembrane space domains (Fig. 6A). This topology is in agreement with that experimentally determined for a bacterial CcmB protein (12). The sequences coding for the full-length AtCCMA protein and seven AtCcmB domains (B1 to B7) were cloned in two-hybrid vectors in fusion with the AD and DNA 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 AtCCMA-B1, -B3, -B5, and -B7 combinations but not the others (Fig. 6B). This result shows that AtCCMA indeed interacts with AtCcmB and that the interaction only occurs with domains predicted to be oriented toward the matrix. Because we have shown that AtCCMA is attached to the matrix side of the inner mitochondrial membrane, the results obtained are in conformity both with the model deduced from bacteria and with CcmB topology. As a control, yeast cotransformed with AtCCMA or the different domains of AtCcmB plus the empty complementary two-hybrid vector (either pGADT7 or pGBK7T7) were plated on DO-LWAH-selective medium. The absence of growth showed that the activation of reporter genes was a true reflection of AtCCMA interaction with specific domains of AtCcmB. The interaction was quantified by the activation of lacZ<sub>3</sub>, a third reporter gene. Background level was estimated with blanks and/or negative control transformed yeast. The β-galactosidase activity resulting from the interacting combination is clearly higher than the non-interacting ones, some of which did not rise above background level (Fig. 6D).

and 7.3 M urea. A concentration as high as 7.3 M urea was not sufficient to disassociate AtCCMA completely from the membranes. With the addition of ATP and MgSO<sub>4</sub> in the presence of 6.6 M urea, almost all the protein was solubilized. Conversely, a true intrinsic membrane protein like cytochrome c<sub>s</sub> was still found in the membrane fraction. It has been proposed that the binding of ATP and its hydrolysis induces a conformational change in the ATP binding subunit, which in turn modulates its interaction with the transmembrane domain of the transporter (42).

AtCCMA was predicted to have an ATPase activity as the NBD of an ABC transporter. This was tested by expressing AtCCMA in its native form in fusion with the MBP. The MBP-CCMA protein was loaded on a BN gel together with digitonin-solubilized *Arabidopsis* mitochondrial membrane complexes. After staining for ATPase activity, white lead phosphate precipitates colocalized with the 70-kDa MBP-CCMA protein as shown by the Western analysis performed in a parallel experiment (Fig. 4, C and D). Both the F<sub>1</sub>-F<sub>0</sub> ATP synthase and the F<sub>1</sub> subcomplex show ATPase in-gel activities as described previously (34). However, no activity could be detected at the size of the 480-kDa AtCCMA-containing complex in the mitochondrial extract most probably because the amount of mitochondrial native AtCCMA is far below that of the recombinant MBP-CCMA used in the assay. The results obtained indicate that AtCCMA has characteristics that define the NBD of a functional transporter in regard to its association with the TMDs and its ATPase activity.

**A**. Blue Native gel of mitochondrial protein complexes solubilized with digitonin. **B**, the corresponding second dimension denaturing SDS-PAGE were blotted and reacted with antibodies directed against AtCCMA. The molecular mass standards are given in kDa.

**FIGURE 5. Presence of AtCCMA in a large size complex.**
DISCUSSION

Here we attribute a function to one of the 15 members of the “non-intrinsic ABC proteins” family found on the genome of Arabidopsis. AtNAP10 is the plant ortholog of bacterial CcmA proteins. In Gram-negative bacteria, CcmA is the ATP binding domain of an ABC transporter involved in maturation of c-type cytochromes in the periplasm. We have shown that AtNAP10, a unique gene located on A. thaliana chromosome 1, encodes the 28-kDa mitochondrial AtCCMA. This work shows an interaction with the mitochondrial encoded TMD, AtCcmB. This is the first example of an organellar ABC transporter to be jointly coded by two different genomes. Indeed, the three organellar NAP proteins studied so far (NAP1, NAP7 and NAP6, SUFB, SUFC, and SUFD homologues, respectively) are chloroplastic proteins required for [Fe-S] cluster biogenesis that are not associated to TMDs (44). Other Arabidopsis NAPs could be imported into organelles and specifically associated with unidentified transmembrane domains or be involved in other processes not requiring the typical four modules organization.

Because ATP is available on both sides of the inner mitochondrial membrane, mitochondrial ABC transporters could in theory adopt two orientations, i.e. their ATP binding domains facing the matrix or the intermembrane space. The necessity of membrane potential for import and the resistance of AtCCMA to protease treatment of mitoplast clearly indicate that AtCCMA is located on the matrix side of the inner membrane. This orientation toward the negative side of the membrane is analogous to that of its bacterial counterparts. As for other CCM proteins, the conservation of topology speaks for a functional system I in plant mitochondria.

Bacterial and mitochondrial CCMA proteins possess the highly conserved Walker A, Walker B, and ABC signature sequences found in all ATP binding domains of ABC transporters. We have shown that AtCCMA fused to the maltose-binding protein purified in a native form can hydrolyze ATP. In addition, the attachment of AtCCMA to mitochondrial inner membrane is affected in the presence of ATP/Mg. Therefore, AtCCMA can be the functional NBD of an ABC transporter. Both phylogenetic and biological data indicate that NAP10 is the ortholog of CcmA. However, AtCCMA failed to complement a deletion mutant of E. coli ccmA for the production of holocytochrome c. The complexity of transient interactions between Ccm proteins in bacteria could explain this negative result. Indeed in different complementation assays performed in E. coli with Arabidopsis proteins no functional complementation could be obtained (23, 24). The interaction between the chimeric ABC transporter and the heme chaperon CcmE could be affected. Previous complementation assays performed in

![Diagram](image_url)

**FIGURE 6.** Yeast two-hybrid assays with full-length AtCCMA protein and domains of AtCcmB. A, schematic representation of AtCcmB topology with three domains oriented toward the intermembrane space (IMS) and four domains oriented toward the mitochondrial matrix (Ma).

- **B**, AtCCMA protein and the seven hydrophilic domains of AtCcmB were fused with the AD or the BD of the Gal4 transcription factor. AD and BD constructs were cotransformed in yeast. Double transformants were plated at different dilutions (1, 10, 100 time dilutions from left to right) on DO-LWAH solid medium. **C**, liquid β-galactosidase assay using 2-nitrophenyl-β-D-galactopyranoside (o-NPG) as a substrate. β-Galactosidase activity is expressed in units (1 unit = nmol/min). The values displayed are the average β-galactosidase activities for three individual double transformants with S.D. indicated by error bars. Negative controls were obtained by transforming yeast cells with AD or BD constructs and the complementary empty vector pGAD or pGBK.
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E. coli show that holoAtCCME is formed when EcCcmC is expressed (23), suggesting that EcCcmC/AtCcmE interaction allows heme loading on AtCcmE. Another possibility is that the heterologous interactions between AtCCMA and the bacterial TMDs might have been impaired or not properly done. Indeed, protein-protein interactions between the domains of ABC transporters are essential for their correct function (42).

Contrary to easily recognizable ABC modules, the transmembrane domains of ABC transporters present little or no sequence conservation. In bacteria, ABC transporters are usually encoded by genes in the same operon, thus facilitating the identification of partner proteins. In E. coli not only the genes of the proposed ABC transporter but the full set of ccm genes are clustered. Some remnants of this operon organization are still found in the mitochondrial genome of plants, e.g. the liverwort Marchantia polymorpha where ccmB, ccmC, and three open reading frames coding for different domains of CcmF are contiguous (45). No ortholog of ccmD has been identified in plant genomes. The scattered ccm gene organization in land plant and the co-conservation of ccmB and ccmC genes in mitochondrial genome do not give new clues to discriminate between the two ABC transporter models. AtCCMA was present in a 480-kDa complex that did not contain AtCCME, AtCCMH, or AtCcmF proteins. We could not test whether CcmB and CcmC were present in the 480-kDa complex due to our failure to obtain antibodies recognizing these very hydrophobic proteins. Therefore, we used another strategy to ascertain that AtCCMA is associated with a TMD partner of the CCM pathway. We show that AtCCMA could interact with each CcmB domains oriented toward the matrix in yeast two-hybrid assays, whereas no interaction was found with intermembrane space-oriented ones. These data are in agreement with the predicted topology of CcmB and the demonstrated location of AtCCMA and strongly suggest that AtCCMA and AtCcmB are indeed part of the same ABC transporter. Therefore, AtCCMA is another system I component conserved from bacteria to plant mitochondria.

The ABC protein was proposed to act as a releaser; still, a transporter function cannot be completely excluded. The fact that the NBD and the TMD are encoded separately is a characteristic of ABC import systems. However, in phylogenetic analysis, the CcmA family cluster belongs to the exporter subfamily ABC-A2, which contains proteins having the ABC modules not fused to TMD (46). An electron donor, essential to maintain the heme iron in a reduced state after its transfer to CcmE, could be a possible substrate for the system I ABC transporter and essential for the release of heme to apocytochrome c. Indeed, in vitro and in vivo approaches showed that heme iron needs to be reduced for the covalent linkage to occur (47–49). In yeast mitochondria, a flavin-linked electron transfer was postulated for heme lyase reaction (49), and Cyc2p a flavoprotein was proposed to reduce heme iron before its attachment to apocytochrome c by cytochrome c heme lyase (50). It is not known whether a distinct system is required in plant mitochondria and whether the CCM ABC transporter could be involved.

The ABC transporter and the heme chaperon CcmE are a hallmark of system I. They are notably not found in system II, although the two systems share common features. CcmE was proposed to function as an heme reservoir conferring specific capabilities to system I, i.e. to work with lower heme concentrations (51, 52). Neither ABC transporter nor CcmE (or proteins which fulfill heme delivery function) have been identified as components of mitochondrial system III. Does distinct heme availabilities have constrained mitochondria to follow distinct cytochrome c maturation pathways? The subcellular localization of heme synthesis pathway and consequently its route to the site of holocytochrome assembly has to be addressed. Ferrochelatase, the final enzyme of heme synthesis, is located differently in eukaryotes. In yeast it is mitochondrial; in Chlamydomonas it is exclusively chloroplastic (53), whereas in plants there is a current debate concerning whether ferrochelatase is exclusively chloroplastic or dually targeted to chloroplast and mitochondria (54–56). Mitochondria of yeast and of Chlamydomonas both use system III, and plant mitochondria use system I. Consequently there is no obvious link between the cytochrome c biogenesis system used and the required heme transport to the site of cytochrome c assembly. However, the comparison of the cytochrome c maturation systems used in eukaryotes with their respective heme synthesis and delivery pathways will most likely be extremely useful to understand these biogenesis pathways.

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