A Novel Pathway of Cytochrome c Biogenesis Is Involved in the Assembly of the Cytochrome b₆f Complex in Arabidopsis Chloroplasts

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We recently characterized a novel heme biogenesis pathway required for heme $c'$-covalent binding to cytochrome $b_6$ in Chlamydomonas named system IV or CCB (cofactor assembly, complex C ($b'_6f$), subunit B (PetB)). To find out whether this CCB pathway also operates in higher plants and extend the knowledge of the $c$-type cytochrome biogenesis, we studied Arabidopsis insertion mutants in the orthologs of the CCB genes. The ccb1, ccb2, and ccb4 mutants show a phenotype characterized by a deficiency in the accumulation of the subunits of the cytochrome $b'_6f$ complex and lack covalent heme binding to cytochrome $b_6$. These mutants were functionally complemented with the corresponding wild type cDNAs. Using fluorescent protein reporters, we demonstrated that the CCB1, CCB2, CCB3, and CCB4 proteins are targeted to the chloroplast compartment of Arabidopsis. We have extended our study to the YGGT family, to which CCB3 belongs, by studying insertion mutants of two additional members of this family for which no mutants were previously characterized, and we showed that they are not functionally involved in the CCB system. Thus, we demonstrate the ubiquity of the CCB proteins in chloroplast heme $c'$-binding.

The cytochrome $b'_6f$ complex is a large multisubunit pigment-protein complex located in the photosynthetic membranes of cyanobacteria, algae, and vascular plants. It has a plastocyanin/cytochrome $c$$_6$ oxidoreductase activity and mediates the electron transfer between photosystems II and I. The electron flow through the cytochrome $b'_6f$ complex is coupled to the translocation of protons, thereby contributing to ATP synthesis (reviewed in Refs. 1 and 2). The cytochrome $b'_6f$ complex also regulates the use of light energy by playing a role in the processes of state transition and redox regulation (3–7), cyclic electron flow, and photoprotection (8, 9).

The crystal structure of the cytochrome $b'_6f$ complex in a cyanobacterium and a green alga revealed its highly conserved organization (10, 11). It forms a functional dimer comprising four large and four smaller subunits and binding several cofactors. Two heme prosthetic groups are covalently bound to the protein moieties: the heme $c$ of cytochrome $f$ and the newly discovered heme $c'$ attached to cytochrome $b_6$ in the quinone-binding site Q$_i$ (referred as $c_i$ in Refs. 10 and 11). This additional heme was first identified by in vivo spectroscopy as a redox center in equilibrium with heme $b_6$, named “G” and proposed to be located near thestromal side of the membrane (12). G was later characterized as a cytochrome $c'$ (13) and is hereafter referred to as heme $c'_i$. Typical members of the $c$-type cytochrome family, to which cytochrome $f$ belongs, are characterized by (i) covalent ligation via two thioether bonds of the heme vinyl groups to two cysteinyl residues located in a highly conserved CXXCH motif of the protein and (ii) a hexacoordinated heme iron with two amino acid residues of the protein providing the heme axial ligands, one of them being the histidinyl residue of the CXXCH motif.

Interestingly, the covalently bound heme of cytochrome $b_6$ differs from the majority of the $c$-type cytochrome family hemes (10, 11, 14). In cytochrome $b_6$, this heme is pentacoordinated and therefore high spin, hence the designation of $c'_i$, and covalently attached by a single thioether bond to the protein backbone (15–19). The unique heme iron axial ligand is not provided by the side chain of amino acid residue but by a water or hydroxyl molecule that bridges the heme iron of heme $c'_i$ to the carboxyl group of one of the propionate of heme $b_6$.

Hemes are hydrophobic and cytotoxic macrocycles that require specific pathways for their delivery to subcellular destinations. Three distinct pathways, comprising several protein components and referred to as systems I, II, and III described in bacteria, chloroplasts, and mitochondria are involved in the assembly of $c$-type cytochromes located on the positively charged side of the membrane, opposite to the side where membrane insertion of the protein backbone occurs (20–22). Indications that the maturation of cytochrome $b_6$ was not spontaneous and differed from other known maturation systems came from previous biochemical studies showing that at least four protein factors, encoded by the nuclear genome, were necessary to covalently attach a heme to cytochrome $b_6$. The genes

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encoding these proteins were referred to as the CCB genes for cofactor assembly of complex C (cytochrome \( b_{5}f \)) targeting subunit \( b \) (cytochrome \( b_{5} \) encoded by the petB gene) (23). Recently, four CCB genes were characterized in *Chlamydomonas*, and their analysis revealed not only that they described a new maturation pathway for \( c \)-type cytochromes located on the negatively charged side (n-side) of the membrane but also that the CCB pathway should be conserved among organisms performing oxygenic photosynthesis (24). These four CCB proteins had no previously identified conserved domains except CCB3, which belongs to the YGGT protein family. The YGGT protein family (European Molecular Biology Laboratory InterPro accession number IPR003425) was named after the *Escherichia coli* yggT gene. The YGGT repeat is found in conserved hypothetical integral membrane proteins present in bacteria and chloroplasts and has an unknown function. Photosynthetic eukaryotes contain up to four YGGT members (three in *Chlamydomonas* and four in *Arabidopsis*), all predicted to be chloroplast-localized.

To extend knowledge on the biogenesis of \( c \)-type cytochromes and explore the role of the CCB proteins in higher plants, *Arabidopsis* mutants of three *Chlamydomonas* CCB gene orthologs were analyzed (no *Arabidopsis* mutants altered in the CCB3 gene were available). Using protein fluorescent reporters, we show that the four CCB proteins are targeted to chloroplasts. We have also studied insertion mutants in other members of the YGGT protein family for which no *Chlamydomonas* mutants were available. Our results clearly indicate that the function of the CCB proteins is conserved in *Arabidopsis*, demonstrating that the CCB pathway can be regarded as generalized for holocytochrome \( b_{5} \) assembly in chloroplasts.

**EXPERIMENTAL PROCEDURES**

*Plant Growth and Selection*—The mutant ccb and ygg-t-\( b \) lines (see supplemental Table S1), ecotype Columbia, were from the collection of the Salk Institute, (La Jolla, CA). The ygg-t-\( a \) line, ecotype Columbia, was from the collection of the University of Wisconsin (Madison, WI). Seeds were obtained from the Nottingham *Arabidopsis* Stock Centre. Seed sterilization and growth conditions for wild type and mutant plants were described in Ref. 25. Plants were grown under continuous light at a photon flux density of 40–50 \( \mu \)E m\(^{-2} \) s\(^{-1} \) for 20 days on sterile medium containing 1X muriarge and skog salts (26), 1.5% (w/v) sucrose, 2.5 mm MES-NaOH, \( \text{pH} \) 5.7, and 0.3% (w/v) Gelrite. Mutants were selected according to fluorescence induction kinetics measured with an in-house built set-up described in Ref. 27. To have the same genetic background, phenotypically wild type plants of progenies of heterozygous lines grown under the same conditions were compared with mutants in all experiments. Propagation of the seedling-lethal *ccc* mutants occurred via heterozygous offspring grown on soil.

To prove the T-DNA insertion sites, PCR analyses were performed using primers specific for the T-DNA, LB, and the gene of interest, ccb1-f, ccb2-r2, and ccb4-f1 for the *CCB1*, *CCB2*, and *CCB4* genes, respectively (supplemental Table S2 and Fig. 1). To select homozygous mutants, gene-specific primer combinations ccb1-f/ccb1-r, ccb2-f2/ccb2-r2, and ccb4-f1/ccb4-r1 were used for *CCB1*, *CCB2*, and *CCB4* genes, respectively (supplemental Table S2 and Fig. 1). The T-DNA insertion prevented PCR amplification of the corresponding locus in the homozygous lines. Actin-f and actin-r primers (supplemental Table S2), which amplify the At2g37620 actin 1 gene locus, were used in combination with the gene-specific primers as an internal PCR control.

**cDNA Clones**—The cDNAs of *CCB1* (RAFL09-81-B07), *CCB2* (RAFL21-80-A07), *CCB3* (RAFL06-10-D06), and *CCB4* (RAFL21-69-K09 and RAFL25-07-B10) were obtained from the RIKEN BioResource Center (28, 29). The RAFL25-07-B10 *CCB4* cDNA arose as a result of alternative splicing and carried the unspliced intron 6 and rearrangements in the 3' untranslated region.

**Complementation of the ccb Mutants**—The full-size *CCB1*, *CCB2*, and *CCB4* cDNAs were amplified using the Expand High Fidelity\(^{\text{PLUS}}\) PCR System (Roche Applied Science). For the amplification of the *CCB1* cDNA, the ccb1-Bam-f and ccb1-Xba-r oligonucleotide primers (supplemental Table S2) were used, introducing BamHI (for the former) and XbaI (for the latter) restriction sites. After digestion with BamHI and XbaI and purification, the PCR product was ligated into the BamHI/XbaI sites of the plant binary expression vector pSEX001-VS (30). The result of cloning was verified by sequencing. The construct for the *ccc2* complementation was done in the same way using the ccb2-Bam-f and ccb2-Xba-r primers (supplemental Table S2). The two *CCB4* cDNAs were amplified using the ccb4-Bam-f/ccb4-Bam-r1 and ccb4-Bam-f/ccb4-Bam-r2 primer combinations. The resulting fragments were cloned into the BamHI site of the vector pSEX001-VS. The obtained constructs were introduced into *Agrobacterium tumefaciens* GV3101 (pMP90RK) (31) and transformed into progenies of heterozygous plants using the floral dip method (32). Selection of transformants was performed on rock wool (Grodan, Hobro, Denmark) soaked in one-quarter strength Murashige and Skoog medium (26) supplemented with 10 mg/liter sulfadiazine (33). Homozygosity and the T-DNA insertion in resistant complemented lines was confirmed as described above. The presence of the cDNA was analyzed by PCR using exon-specific primers ccb1-f/ccb1-r1 for *CCB1*, ccb2-f1/ccb2-r1 for *CCB2*, ccb4-f2/ccb4-r2, and ccb4-f3/ccb4-r3 for *CCB4.1* and *CCB4.2*, respectively.

**Subcellular Localization**—cDNA sequences encoding full-length *CCB1* and *CCB3* proteins and putative transit peptides of the *CCB2* and *CCB4* proteins were amplified using the Expand High Fidelity\(^{\text{PLUS}}\) PCR System (Roche Applied Science) and ccb1-Kpn-f/ccb1-Kpn-r, ccb2-Kpn-f/ccb2-Kpn-r, ccb3-Sal-f/ccb3-Sal-r, and ccb4-Sal-f/ccb4-Sal-r primer combinations (supplemental Table S2) for the *CCB1*, *CCB2*, *CCB3*, and *CCB4* genes, respectively. The amplified *CCB1* and *CCB2* fragments were digested with KpnI, and the *CCB3* and *CCB4* fragments were cut with SalI. After purification, the *CCB1*, *CCB3*, and *CCB4* products were cloned in-frame into the KpnI or SalI site of the GFP expression vector pOL-LT (34). The *CCB2* was introduced in-frame into the RFP expression
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vector pOL-DsRed (34). Transient expression was performed in polyethylene glycol-treated protoplasts of *Arabidopsis* cell suspension (35).

Fluorescence was visualized 16 h after transformation, using a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Wetzlar, Germany). GFP was excited at 488 nm, and the fluorescence emission signal was detected between 500 and 535 nm. The RFP fusion construct was excited at 543 nm, and the emission signal was recovered between 570 and 637 nm. Chlorophyll autofluorescence was recorded between 675 and 750 nm.

**Protein Analyses**—Membrane proteins of wild type and mutant plants were isolated as described in Ref. 25. Protein separation on 12–18% SDS-polyacrylamide gels, electrophoresis, immunoblotting, and heme staining on blots using chemiluminescence were performed as described in Refs. 15 and 24. After transfer, membrane-bound proteins were stained using Coomassie Brilliant Blue according to the manufacturer’s instructions (WESTRAN® Clear Signal protein transfer blotting membrane; Whatman®; Schleicher & Schuell). Protein amounts of mutants were adjusted to protein amounts in wild type having 8.5 μg of chlorophyll. Chlorophyll concentrations were measured according to Ref. 36. For Western blot analyses antisera raised against the whole higher plant cytochrome *b*, *AtpC* and *PsaC* proteins (at a dilution of 1:5000), *PsbA* antisera raised against the whole higher plant cytochrome *f* (antiserum raised against the entire polypeptide at a 1:10,000 dilution) were used.

**RESULTS**

The Conservation of the CCB Genes in Arabidopsis—The four CCB proteins, which were recently implicated in heme c biosynthesis in the unicellular green alga *Chlamydomonas* are conserved in all organisms performing oxygenic photosynthesis whose genomic sequences are available (24). The *Chlamydomonas* CCB2 and CCB4 proteins are paralogous proteins with an amino acid identity of 30% using BLAST 2 sequences algorithm (37). The *Arabidopsis* genome comprises orthologs for *CCB1* (AT3G26710) and *CCB3* (AT5G36120) as well as for the paralogous *CCB2* (AT5G52110) and *CCB4* (AT1G59840) genes (genes in Fig. 1 and proteins in Fig. 2A). The two alternative spliced gene models for *CCB2* are translated in exactly the same protein. In contrast, the second gene model for *CCB4* corresponds to a shorter cDNA and is translated as a protein lacking the last 57 amino acids (indicated in *CCB4* protein sequence in italic in Fig. 2A). The encoded *Arabidopsis* proteins share high similarity with their *Chlamydomonas* counterparts. Amino acid identity of 37, 30, 52, and 42% for the *CCB1*, *CCB2*, *CCB3*, and *CCB4* full-length proteins could be identified using a BLAST 2 sequences algorithm (37).

The CCB proteins are encoded in the nucleus genome of *Arabidopsis* and have chloroplast transit peptides indicated in Fig. 2A as predicted by the ChloroP (38). Experimental evidence sustaining the chloroplast membrane localization of the CCB proteins is their immunodetection in chloroplast membranes of *Chlamydomonas* (24), their targeting to *Arabidopsis* chloroplasts using fluorescence protein reporters.
(see below in this study), and the presence of CCB proteins in Arabidopsis chloroplast proteome studies as that of CCB3 in thylakoids (39) and of CCB4 in total chloroplast preparations (40). The topological arrangement of the CCB proteins in the thylakoid membrane (shown in Fig. 2B) was predicted based on in silico analysis using TMAP (41). CCB1 has three transmembrane domains, whereas CCB2, CCB3, and CCB4 have only two. The distribution of positive charges at the border of the putative transmembrane domains according to the “positive inside rule” (42) suggests the respective location of the N and C termini of each protein (Fig. 2B). The transmembrane topology predicted for the Arabidopsis CCB proteins was found to be similar to that predicted for the Chlamydomonas proteins.

Disruption of the CCB Genes in Arabidopsis Leads to the Impairment of Photosynthesis—To analyze the functions of the CCB proteins in Arabidopsis, we applied a reverse genetics approach and characterized Arabidopsis T-DNA lines available in public collections (see “Experimental Procedures”) carrying insertions in the CCB1, CCB2, and CCB4 genes (Fig. 1 and supplemental Table S1). No mutants altered in the CCB3 gene were available. The T-DNA insertion sites and genotypes were verified by PCR amplification followed by sequencing of the flanking regions (Fig. 1 and “Experimental Procedures”). The plants, homozygous for the T-DNA insertion, were nonphototrophic and seedling-lethal on a medium lacking a reduced carbon source; therefore, they were grown on sucrose-supplemented medium. Under these conditions mutant plants looked pale green and smaller compared with the wild type (Fig. 3C). Using a fluorescence imaging system, the ccb mutants were characterized by their lower fluorescence yields and shorter half-times of fluorescence rise (as seen by the fluorescence rise kinetics (Fig. 3A) and the fluorescence ratios \( F_{200 \text{ ms}}/F_{1200 \text{ ms}} \) shown in the fluorescence imaging panels (Fig. 3B)).

The CCB Proteins Are Targeted to the Chloroplasts—To verify the intracellular localization of the CCB proteins, we constructed chimeras where either transit peptides or full-length CCB proteins were fused to the N terminus of a fluorescent reporter protein. Full-length protein sequences of CCB1 and CCB3 were fused to the GFP. In the case of CCB2 and CCB4, only the transit peptide sequences were used and respectively fused to the RFP or to GFP. The constructs were then transiently expressed in cell suspension of Arabidopsis protoplasts, and the fluorescence was recorded. As shown on Fig. 4, GFP and RFP fluorescence perfectly overlapped

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**FIGURE 2.** Sequence and topology of the CCB proteins of Arabidopsis in the thylakoid membrane. A, CCB protein sequences. Chloroplast transit peptides as predicted by ChloroP (38) are shown in gray, and transmembrane domains predicted by TMAP (39) are underlined. The last 57 amino acid residues missing in the short cDNA of CCB4 are shown in italics. B, topology of the CCB proteins based on the “positive inside rule” predictions.

**FIGURE 3.** Phenotype and spectroscopic analyses of the ccb mutants and WT. Fluorescence induction kinetics (A), chlorophyll fluorescence imaging (B), and phenotype (C) of the 20 days old ccb1, ccb2, and ccb4 mutant plants grown heterotrophically under continuous light of 40–50 \( \mu \text{E m}^{-2} \text{s}^{-1} \) intensity were compared with those of WT plants grown for the same time under the same conditions. Fluorescence measurements were performed after a dark period of several minutes. Pictures in B are computed from the ratio of fluorescence pictures recorded at two times (200 and 1200 ms) shown by dashed lines in A during the fluorescence rise.
with the auto-fluorescence of the chlorophyll, demonstrating that the CCB proteins were indeed targeted to the chloroplasts.

Heme c\textsuperscript{i} Binding to Cytochrome b\textsubscript{6} Is Impaired in the ccb Mutants of Arabidopsis—We studied how the photosynthetic deficiency of the mutant plants was reflected on the protein level. The levels of accumulated cytochromes b\textsubscript{6} and f, determined by immunodetection using specific antibodies, were dramatically reduced to 5–10% of those in the wild type (Fig. 5, A and B, upper panels). In contrast, the representative subunits of the ATP synthase (AtpC), photosystem I (Psac), and photosystem II (PsbA) accumulated with no obvious differences between the mutants and the wild type (Fig. 5B) and can be considered as controls to indicate equal loadings across the different lanes. We have previously shown that the typical biochemical signature of c\textsuperscript{-}type cytochrome lacking its covalently bound heme cofactor is faster migration of the protein on a denaturing SDS-PAGE gel and the inability to detect the protein by the peroxidase activity of the heme (24). As shown on Fig. 5A, immunodetection of cytochrome b\textsubscript{6} in the ccb mutants shows a band that runs slightly ahead of the band in the WT or in an unrelated b\textsubscript{6}f mutant used as a control. Moreover, the heme peroxidase activity associated with the cytochrome b\textsubscript{6} was lost for all of the ccb mutants (Fig. 5A). To exclude the possibility that the lack of peroxidase staining was due to the lower protein accumulation in the ccb mutant, we used additional controls consisting either of underloaded WT proteins (Fig. 5A, lane 4) or of an unrelated b\textsubscript{6}f mutant (Fig. 5A, lane 8). Our results clearly show that reduced amounts of protein should still be sufficient to allow detection of the peroxidase activity of cytochrome b\textsubscript{6} in the ccb mutants if it had retained its c\textsuperscript{i} heme. Fig. 5C shows that the peroxidase activity associated with cytochrome f was not altered in the ccb mutants, indicating that the mutations affected neither the general heme biosynthetic pathway nor the covalent heme binding to cytochrome f. Thus, the CCB mutations specifically prevented binding of heme c\textsuperscript{i} to cytochrome b\textsubscript{6}.

Functions of the CCB Proteins Are Conserved in Green Algae and Higher Plants—To confirm that the T-DNA insertions in the CCB genes were indeed responsible for the observed phenotypes, the mutants were functionally complemented by the corresponding wild type cDNAs constitutively expressed under the control of the cauliflower mosaic virus 3\textsuperscript{5}S RNA promoter (see “Experimental Procedures” and Fig. 6). The resulting transformants were able to grow photoautotrophically on soil and displayed a restored accumulation of cytochrome b\textsubscript{6} and heme c\textsuperscript{i} binding (Fig. 5A). A second CCB4 cDNA (RAFL25-07-B10) resulting from alternative splicing was also able to complement the ccb4 mutant (Fig. 6). Surprisingly, this cDNA encodes a shorter protein devoid of the last 57 amino acid residues as compared with the best conserved Chlamydomonas CCB4 ortholog.

YGGT-A and YGGT-B Are Not Essential for the CCB Pathway—Among the four CCB genes, only CCB3 encodes a protein with a known conserved domain, namely, the YGGT domain. Arabidopsis contains three other members of the YGGT family in addition to CCB3: YGGT-A (AT5G21920), YGGT-B (AT4G27990), and YGGT-C (AT3G07430) (supplemental Table S1 and Fig. 7, A and B). They all possess chloroplast targeting sequences as predicted by ChloroP (38) (lettered in gray in Fig. 7A), and chloroplast proteome studies identified both YGGT-B and YGGT-C in the chloroplast envelope fraction (43, 44). The functional ortholog of Chlamydomonas CCB3 in Arabidopsis (AT5G36120) was identified on the basis of phylogenetic trees of the YGGT family because only a single YGGT member for each photosynthetic organism segregated
in the same cluster as the Chlamydomonas CCB3 (24). We decided to explore the possibility that some of the YGGT paralogs of CCB3 could participate in the CCB system because, as mentioned above, the CCB2 and CCB4 are paralog proteins probably resulting from ancestral gene duplication and both functionally involved in the CCB system. To determine whether disruption of other YGGT family members also prevented covalent binding of heme $c_{1}$ to cytochrome $b_{6}$ and lead to photosynthetic deficiencies, we analyzed the phenotypes of heterozygous T-DNA insertion mutants for both YGGT-A and YGGT-B (Fig. 7C). The $yggt$-$a$ and $yggt$-$b$ mutants were able to grow under photosynthetic conditions (not shown), and as shown in Fig. 7D, they showed no alteration in heme $c$ binding to cytochromes $b_{6}$ and $f$. That is a strong indication that neither the YGGT-A nor the YGGT-B proteins have an essential role in the CCB pathway.

DISCUSSION

Cofactor maturation pathways such as the CCB and CCS systems for c-heme attachment are conserved in all organisms performing oxygenic photosynthesis. The ease with which it is possible to generate and screen photosynthesis mutants in Chlamydomonas has been crucial in the discovery of the two c-type cytochrome maturation systems currently known in the chloroplasts. Genes encoding components of both system II (also known as the CCS system) and system IV (the CCB system) were first molecularly identified in Chlamydomonas (24, 45, 46). Studies of photosynthesis mutants in Arabidopsis led to the characterization of two additional system II factors involved in a redox relay necessary for the reduction of the two cytochromes in the heme-binding site of apo-cytochrome $c$ (47, 48). After the discovery and the initial characterization of the CCB pathway.
in Chlamydomonas (24), we extended the study using available Arabidopsis insertion mutants and the opportunity of using fluorescent fusion proteins to identify their in situ subcellular localization and to contribute to further characterization of the CCB pathway.

Phylogenetic and Functional Conservation of CCBs—The four CCB proteins are conserved among all oxygenic photosynthetic organisms based on the currently existing sequence information. CCB2 and CCB4 are paralogs derived from a unique cyanobacterial ancestor (24). CCB3 is a protein of the YGGT family (European Molecular Biology Laboratory InterPro accession number IPR003425). Except for the CCB3 involved in Chlamydomonas in c-type cytochrome maturation of heme \( c_1 \), and one YGGT member in Streptococcus suggested to be involved in some division process (49), the other proteins of this family have no assigned function. Arabidopsis has three YGGT proteins distantly related to the CCB3 branch. All of these three proteins are predicted to be targeted to the chloroplast, and two of them, YGGT-B and YGGT-C, were identified in biochemical studies to be present in the chloroplast envelope (43, 44), raising the question of their eventual participation in the CCB pathway. Publicly available Arabidopsis insertion mutant collections gave us the opportunity to test the function of YGGT-A and YGGT-B. The lack of insertion mutants for YGGT-C did not allow us to test its role. Our study indicates that neither YGGT-A nor YGGT-B are essential for the CCB maturation pathway. However, because YGGT-B and YGGT-C are very close in the phylogenetic tree (24) and could therefore have redundant functions, a double mutant (yggt-b, yggt-c) would be needed to conclude on their respective roles. This double mutant could not be generated because of the lack of insertion mutants for YGGT-C.

It was important to test whether the function of the CCB orthologs was conserved in higher plants. Indeed, the sequence similarity or the phylogenetic conservation of an open reading frame does not necessarily reflect the functional conservation of the protein. There are multiple examples of functions that were either modified or reallocated from one organism to another.
another (50–53). In addition, Arabidopsis and Chlamydomonas organelles have distinct pathways for mitochondrial cytochrome c maturation, which is performed by system I in higher plants and by system III in Chlamydomonas (reviewed in Ref. 54). The four CCBs are well conserved between green algae and plants, and we show that, analogous to Chlamydomonas, CCB1, CCB2, and CCB4 have a function in the c-type cytochrome maturation of heme $c_6$ in Arabidopsis. The ccb1, ccb2, and ccb4 insertion mutants show a low accumulation of cytochrome $b_{6f}$ subunits and a cytochrome $b_{6}$ in SDS-PAGE devoid of peroxidase activity with an apparent molecular mass lower than in the wild type, which corresponds to the apo-cytochrome $b_{6}$. The apo-cytochrome c ($f$ or $c_o$) shows also a low accumulation accounted by a short life span in the case of ccs mutants in Chlamydomonas (55). The phenotype of the Arabidopsis ccb2 insertion mutant (this work) is similar to that of the recently reported hef208 Arabidopsis mutant obtained by ethyl methanesulfonate mutagenesis. The mutation was identified as a glycine to arginine substitution in position 68 of the $CCB2$ gene that resulted in the introduction of a positive charge at the start of the first predicted transmembrane domain; it led to the loss of peroxidase activity on cytochrome $b_{6}$ in SDS-PAGE and interestingly still allowed detection of a small amount of assembled $b_{6f}$ complex in blue native PAGE (56). This suggests that cytochrome $b_{6}$ lacking heme $c_6$ can associate with other $b_{6f}$ subunits in a protease-sensitive form. Mutants with a limited protease sensitivity of $b_{6f}$ complex lacking heme $c_6$ would be of great interest to understand the role of heme $c_6$.

The functional complementation of the ccb mutants with the corresponding wild type cDNAs constitutively expressed under the control of the $35S$ RNA promoter of cauliflower mosaic virus yielded transformants able to grow photoautotrophically on soil. Interestingly, a $CCB4$ cDNA encoding a shorter protein missing the last 57 amino acid residues was also able to successfully restore photosynthetic growth. This is particularly surprising because the missing portion of the protein encompasses several well conserved residues including a tryptophane residue. In cytochrome c maturation systems I and II, conserved tryptophane residues have been identified as critical in heme interactions (20, 22). In the case of CCB4, it could mean that the conserved C-terminal part of the protein does not have an essential role in the cytochrome c maturation process. We also found that the expression of the $CCB1$ cDNA of Chlamydomonas in homozygous ccb1 mutant plants led to stable transformants that were able to grow photoautotrophically on soil, providing further evidence of the conserved role of the CCB1 orthologs (data not shown).

CCB Chloroplast Localization Using Fluorescent Proteins Is Consistent with Immunodetection and Proteomics Results—In Chlamydomonas we showed by immunodetection in membrane fractions that the four CCB proteins were associated with the chloroplast and absent from mitochondria (24). Arabidopsis CCB2 and CCB3 proteins were predicted to be targeted to the chloroplast by ChloroP (38), TargetP (57), and Predotar algorithms (58), CCB1 to the chloroplast by ChloroP and TargetP but possibly to the mitochondria by Predotar, and CCB4 to the mitochondria by Predotar and TargetP. The plastid proteome data base of Arabidopsis indicates the presence of

CCB3 in thylakoids (39) and that of the CCB4 in total chloroplast preparations (40). Using fluorescent tagging of the four CCB proteins, we demonstrated that these proteins are targeted to the chloroplasts in Arabidopsis (Fig. 4).

In conclusion, we show using protein fluorescent reporters that the four CCB proteins are targeted to Arabidopsis chloroplasts, and we establish using Arabidopsis insertion mutants the generality of this cytochrome maturation pathway in higher plant chloroplasts. In addition, we test the role of two YGGT proteins for which no mutants were previously characterized. The CCB proteins define an additional maturation system for c-type cytochromes and are among the few that distinguish photosynthetic cells evolving oxygen from other types of living cells. The available genomic information of Chlamydomonas and higher plants as well as mutational studies will certainly continue to provide insight into the maturation systems of the c-type cytochromes and will contribute to further elucidate the role of heme $c_6$ in the mechanisms of electron transfer in the $b_{6f}$ complex as well as the molecular nature of the signals generated by the $b_{6f}$ complex and its subsequent transduction to the cytosol/nucleus.

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