The Plastid-encoded ccsA Gene Is Required for Heme Attachment to Chloroplast c-type Cytochromes*

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A chloroplast gene, ycf5, which displays limited sequence identity to bacterial genes (cc1/cycK) required for the biogenesis of c-type cytochromes, was tested for its function in chloroplast cytochrome biogenesis in Chlamydomonas reinhardtii. Targeted inactivation of the ycf5 gene results in a non-photosynthetic phenotype attributable to the absence of c-type cytochromes. The cloned ycf5 gene also complements the phototrophic growth deficiency in strain B6 of C. reinhardtii. B6 is unable to synthesize functional forms of cytochromes f and c6 owing to a chloroplast genome mutation that prevents heme attachment. The selected (phototrophic growth) as well as the unselected (holocytochrome c6 accumulation) phenotypes were restored in complemented strains. The complementing gene, renamed ccsA (for c-type cytochrome synthesis), is expressed in wild-type and B6 cells but is non-functional in B6 owing to an early frameshift mutation. Antibodies raised against the ccsA gene product recognize a 29-kDa protein in C. reinhardtii. The 29-kDa protein is absent in strain B6 but is restored in a spontaneous revertant (B6R) isolated from a culture of B6. Sequence analysis of the ccsA gene in strain B6R indicates that it is a true revertant. We conclude that the ccsA gene is expressed and that it encodes a protein required for heme attachment to c-type cytochromes.

The c-type cytochromes are distinguished from other cytochromes and heme proteins by virtue of the mode of attachment of the heme group to the polypeptide. Nearly all the c-type cytochromes contain a heme-binding motif, Cys-X-Y-Cys-His, which contributes thiol groups for the formation of thioether linkages between the polypeptide and the vinyl groups on the porphyrin ring, and an imidazole group, which participates as one of the two axial ligands to the heme iron. Chloroplasts contain up to two c-type cytochromes. Cytochrome f, found in all chloroplasts, is a membrane-associated subunit of the cytochrome b6f complex, and is anchored to the membrane via a hydrophobic sequence near its C-terminal end (1). A large, soluble N-terminal domain contains the heme binding site and extends into the lumen where it can interact with plastocyanin, or in some green algae, with cytochrome c6. Cytochrome c6 is a soluble, luminal-localized protein, which substitutes for plastocyanin function in copper-deficient cultures of various green algae and cyanobacteria (2).

Cytochrome f is encoded by the psaA gene in the plastome and is translated on thylakoid membrane-bound ribosomes to yield a precursor form. Post-translational maturation of pre- apocytochrome f includes proteolytic removal of the presequence after the N terminus is translocated to the lumen side and ligation of heme to the cysteinyl thiols (3–6). In Chlamydomonas reinhardtii, cytochrome c6 is encoded in the genome (Cyc6 gene) and post-translationally targeted to the lumen of the thylakoid membrane (7). Maturation of pre-apocytochrome c6 involves two sequential proteolytic cleavages after translocation across the envelope and thylakoid membranes followed by ligation of heme to the cysteinyl thiols (8). The heme attachment step appears to be common to both cytochrome f and C6 biosynthetic pathways. Characterization of a number of non-photosynthetic C. reinhardtii mutants indicated that they were pleiotropically defective in the formation of both holocytochromes c6 and f from their respective apoproteins (9). Since the two proteins are encoded in separate genomes, the defect had to occur at a common post-translational step. Indeed, the mutant strains synthesized and processed the precursor cytochromes, but were unable to form holoproteins from the newly synthesized apoproteins. The mutants were therefore determined to be blocked at the step of heme attachment. The apoproteins did not accumulate in such mutant strains but were degraded.

In the case of one of the mutant strains, B6, the pleiotropic cytochrome-minus phenotype displayed uniparental inheritance, which suggested that the affected gene was encoded in the plastome. Further, the mutant could be complemented with purified chloroplast DNA.1 Spontaneous suppressor strains, selected for their ability to grow phototrophically, were noted to have restored holocytochrome c6 function (the unselected phenotype) as well. For at least one strain, B6R, the suppressed phenotype was also uniparentally inherited, which suggested that it might be a true revertant (6). To understand the molecular basis underlying the defect in strain B6, and to identify a gene required for chloroplast cytochrome synthesis, we sought to clone the wild-type allele of the mutated gene.

The chloroplast gene, ycf5, encoding an approximately 320-residue protein of unknown function, was a likely candidate (10, 11). The deduced primary sequence of the ycf5 gene product was found to have sequence similarity, albeit quite limited, with the products of the cd1 gene of Rhodobacter capsulatus and the related cycK gene of Rhizobium meliloti and Bradyrhizobium japonicum (10, 12, 13). In a multiple alignment of Cd1 with several ycf5 gene products, the identity ranges between 23–26% over an 80–90-residue stretch of sequence at the C terminus of the ycf5 product (although there is no conservation elsewhere in the protein). The Cd1/cycK gene is one of a large

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1 B. Dreyfuss, unpublished data.
number of genes (hel, clc, or cyc loci) required for c-type cytochrome synthesis in bacteria (reviewed in Ref. 14). Mutations at these loci result in a pleiotropic c-type cytochrome-minus phenotype in the respective bacterial strains, which is reminiscent of the B6 phenotype. If ycf5 were a clc/cycd homologue, it seemed possible that the B6 strain might be affected in ycf5 expression or function. The ycf5 gene is found in the plastid genomes of vascular plants, liverwort, red algae, and cryptomonads (see, e.g., Refs. 15–21). It encodes an open reading frame, which ranges in length from 301 amino acids in the cryptomonad gene to 321 in the rice gene. The open reading frame is reasonably well conserved among the various algal and plant species (48–72% identity, 69–82% similarity), particularly in the C-terminal portion. We therefore elected to identify the Chlamydomonas ycf5 analogue on the basis of its relationship to the ycf5 family, use reverse genetics to deduce its function, and determine whether the wild-type cloned gene would indeed complement the defect in strain B6. The ability to manipulate the Chlamydomonas chloroplast genome (reviewed in Refs. 22 and 23) would also permit us to undertake a molecular genetic analysis of ycf5 expression and function.

EXPERIMENTAL PROCEDURES

Strain and Culture Conditions—C. reinhardtii strains CC503 (cw92 mt1), CC425 (cw15 arg2 mt1), and CC125 (wt) were obtained from the Chlamydomonas Genetics Center at Duke University, Durham, NC. All three strains are wild type with respect to photosynthesis and c-type cytochrome biogenesis and are accordingly labeled wt in the figures. The B6 mutant was obtained from Prof. Laurens Mets, University of Chicago, Chicago, IL. Cells were grown at 22°C in TAP medium (24), with or without copper supplementation, in dim light (25 \mu mol/m^2/s) for non-photosynthetic strains or standard illumination (125 \mu mol/m^2/s) for wild-type strains, as described previously (9), and with arginine supplementation (to 500 \mu M) where necessary.

Isolation of Chloroplast DNA—Chloroplast DNA was purified from 1-liter cultures (1 \times 10^7 cells/ml) of strain CC503 according to a procedure modified from the one described in Ref. 25. Cells were collected by centrifugation, washed once in TAP medium, and resuspended in 8 ml of a solution containing 20 mM Tris-Cl (pH 8.0), 0.1 M NaCl, 50 mM EDTA. Self-digested Pronase E (10 mg/ml) and PstI (1 unit), and the resulting fragments were separated by electrophoresis (60 V, 20 h) through 0.8% agarose in TBE solution (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10 mM EDTA), and 23 ml of a saturated solution of NaI in TEN were added. Ethidium bromide was added to a final concentration of 30 \mu g/ml, and the refractive index was adjusted to 1.4350 by the addition of a saturated solution of NaI. A gradient was established by centrifugation (VTi50 rotor, 20°C) at 150,000 \times g for 44 h. The band corresponding to chloroplast DNA, identified under UV light as the uppermost in the gradient, was drawn. The sample was extracted with saturated NaI-equilibrated isoamyl alcohol to remove the ethidium bromide, dialyzed against 4 liters of a solution containing 50 mM Tris-Cl (pH 8.0), 30 mM EDTA with two changes, and de-proteinized by phenol/chloroform extraction. The DNA was precipitated from the aqueous phase and resuspended in water. The concentration of isolated chloroplast DNA was estimated from its absorbance at 260 nm.

Southern Hybridization for Localization of ycf5—Purified chloroplast DNA (1.5 \mu g) was digested (3 h) with BamHI (4 units), BglII (4 units), EcoRI (4 units), and PstI (1 unit), and the resulting fragments were separated by electrophoresis (60 V, 20 h) through 0.8% agarose in TBE (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA (pH 8.3)). After alkaline denaturation, the DNA was transferred to GeneScreen membranes (DuPont NEN) by capillary blotting in 10 \times SSC, fixed by UV irradiation, and hybridized (7% SDS, 0.5 Na_2EDTA (pH 7.2), 1% bovine serum albumin) to the following 32P-labeled oligonucleotides: ycf5-1 (5' -ATGCAYGTNWM-3'), ycf5-2 (5' -ATGCAYGTNWM-3'), ycf5-3 (5' -ATGCAYGTNWM-3'). The abbreviations used are: kb, kilobase pair(s); PVDF, polyvinylidene difluoride; ORF, open reading frame.

The relevant BamHI fragments of the chloroplast genome are indicated on the top of the figure. The positions of a few well-characterized genes in this region are indicated in boldface type. The coding region of ycf5 is shaded, with the arrow indicating the position of the first ATG in the open reading frame. The boxes numbered 1–3 represent the positions of complementarity with oligonucleotide probes ycf5-1, -2, and -3, respectively. The filled circles indicate the positions of various oligonucleotide primers referred to in succeeding portions of the manuscript. Oligonucleotides ending in odd numbers have a 5' to 3' directionality corresponding to left to right. The lower part of the figure shows the portion that was sequenced. Each horizontal arrow represents an independent determination of the nucleotide sequence. Thus, the entire sequence shown in Fig. 2 was determined on both strands. The indicated restriction sites are as follows: X, XhoI; P, PstI; B, BamHI; H, HindIII; S, SphI; N, NdeI; E, EcoRI.
The fragment was amplified in 50-restriction sites of interest (see Fig. 1). The reactions (50) and 15-6 (5 CA CT GGA A-3) of the spectinomycin-resistant transformants, according to the procedure described in (29). Amplification of Chloroplast DNA—RNA was isolated as described previously (9). About 15 μg of total RNA was digested with 25 units of RNaseI (Promega) for 30 min at 37°C. The reaction was deproteinized by a standard extraction with phenol/CHCl3, and the RNA precipitated with ethanol. The precipitated RNA was washed, dried, and resuspended in 10 μl of H2O. Five μl of RNA was used as a template for Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s suggested procedure (Life Technologies, Inc.). The oligonucleotide (200 pmol) 15-15 (5′-CCATAACTGTAAAACC-3′) and 15-18 (5′-AGACATCCCTGTAAAGAGA-3′). The conditions were identical to those described above except that the annealing temperature in the first three cycles was 44°C. The amplified product was digested with NdeI and HindIII and cloned into the appropriate restriction sites in vector pTZZM (+) (Novagen, Madison, WI). The insert was sequenced completely on both strands as described above. Any differences between the cloned wild-type ccsA sequence and that of the ccsA gene in B6 was confirmed by sequencing of an independent amplification product.

Reconstruction of a Complete ccsA Gene and Generation of the Disruption Plasmid—The BamHI fragment was cloned into the BamHI site of plasmid p207 to generate plasmid p207Bam17 with the orientation shown in Fig. 1. The resulting plasmid was digested to completion with PstI, followed by a partial digestion with BamHI to obtain the desired 2.2-kb PstI-BamHI fragment containing the 3′ end of the ccsA gene. The 2.2-kb fragment was ligated to plasmid pEB digested with PstI and BamHI to yield plasmid pEBP. The adaA expression cassette (obtained from Dr. M. Goldschmidt-Clermont, University of Geneva, Switzerland) (27) was cloned into the BamHI site within the ccsA gene to generate the plasmid pEBP-ADA (see Fig. 4).

Manipulation of the Chloroplast Genome—Cells in liquid culture (0.5–1.0×106/ml) were collected by centrifugation, resuspended in TAP medium (at 4×103/ml), and spread on 82-mm Nytran membranes (Schleicher & Schuell) layered on agar plates (0.5 ml cells/plate) (28). Gold particles (1.0 μm) were coated with DNA (10 mg/ml of gold 30% in Hg vacuum) with the PDS-1000/He particle delivery system (Bio-Rad). Following bombardment, cells were incubated in the dark for 16 h. For the complementation experiments, the bombarded cells on the nylon membranes were transferred to solid minimal medium and incubated at a higher light intensity. For the ccsA gene disruption experiments, the nylon membranes were transferred to fresh solid medium containing spectinomycin (100 μg/ml) and incubated in dim light until colonies appeared.

Purification of Strains with a Disrupted ccsA Gene—Spectinomycin-resistant colonies were screened for the presence of the adaA expression cassette in the ccsA coding region by amplification of the targeted sequence from total DNA preparations with primers, method 15-9 (5′-ACCCATACGATGTAACCC-3′) (see Fig. 1). Wild-type DNA was expected to yield a 440-base pair product, while the DNA containing the adaA insertion was expected to yield a 2.3-kb product. Colonies that contained the insert were resuspended in 0.5 ml of TAP medium and replated on spectinomycin (~1000 cells/plate). Colonies were re-isolated and retested and the procedure repeated for a total of five rounds until the wild-type amplification product was not detected or only marginally detected.

Isolation of Total DNA—Total DNA was prepared for amplification reactions exactly as described in Ref. 30, and for Southern blot analysis of the spectinomycin-resistant transformants, according to the procedure described in Ref. 31.

Amplification of Chloroplast DNA—RNA was isolated as described previously (9). About 15 μg of total RNA was digested with 25 units of RNaseI (Promega) for 30 min at 37°C. The reaction was deproteinized by a standard extraction with phenol/CHCl3, and the RNA precipitated with ethanol. The precipitated RNA was washed, dried, and resuspended in 10 μl of H2O. Five μl of RNA was used as a template for Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s suggested procedure (Life Technologies, Inc.). The oligonucleotide (200 pmol) 15-15 (5′-CCATAACTGTAAAACC-3′) and 15-18 (5′-AGACATCCCTGTAAAGAGA-3′). The conditions were as follows: 95°C for 5 min prior to addition of the enzyme, 30 cycles of 94°C for 1 min, 50°C for 30 s, 72°C for 1 min; and a final 5-min extension at 72°C.

Quantitative real-time PCR (QPCR) was performed as described previously (9) and analyzed by melting-curve analysis after separation of products on SDS-containing polyacrylamide (10–12%) gels. Separated products were transferred either to PVDF membranes (Millipore, Billerica, MA) or nitrocellulose membranes (Pharmacia Biotech, Piscataway, NJ) and subjected to Western blotting as described in Ref. 12. Protein Preparation and Analysis—Cell extracts were prepared as described previously (9) and analyzed by SDS-PAGE as described.
cross-linked to keyhole limpet hemocyanin and used to generate antibodies against the ccsA gene product by Research Genetics, Huntsville, AL. The serum was used at a 1:1000 dilution. Bound antibodies were detected with alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibody according to instructions provided by the manufacturer (Bio-Rad). For detection of heme proteins, the membranes were washed in Tris-buffered (20 mM (pH 7.5)) saline and incubated with chemiluminescence reagents (DuPont NEN) according to the manufacturer's instructions.

RESULTS

Analysis of the Sequence of the C. reinhardtii ycf5 Homologue—The longest open reading frame contained in the N1-B2 fragment (see Fig. 1) encodes a protein of 353 amino acids and an apparent molecular mass of 40 kDa. There are two in-frame ATG codons, either one of which may serve as initiation codons. Since neither one is associated with a consensus Shine-Dalgarno sequence, it is not obvious which functions as the initiator. Alternative non-ATG initiation codons or an ACG triplet that could be edited to generate an ATG are not found upstream of the first ATG nor between the first and second ATG. It is unlikely that initiation of translation occurs downstream of the second ATG (see analysis of strain B6 under "Experimental Procedures"). Thus, we propose that the C. reinhardtii ycf5 gene encodes a protein between 334 and 353 amino acids in length. Although this makes the putative C. reinhardtii protein slightly longer than its counterparts in other species, it should be noted that the N-terminal region of the ORF is not well conserved and displays considerable length variation (Fig. 3).

Comparison of the deduced amino acid sequences of the ycf5 gene product from seven species indicates that there are three blocks that display a high degree of sequence identity. The most highly conserved block is at the C-terminal end (53 residues out of the last 100–105 at the C terminus are identical in all seven species), which is also the region that contains the WGXXW sequence motif and displays some sequence identity with bacterial genes involved in cytochrome biogenesis (10, 12, 13). Comparison of a total of 14 chloroplast, bacterial, and mitochondrial sequences identified at least 10 residues in the conserved region that are identical in all 14 sequences. These might form the active site of the protein.

The ycf5 Gene Is Required for Cytochrome Biogenesis—To test the function of the ycf5 gene in wild-type cells, the gene was inactivated by replacement of the endogenous gene with a disrupted copy (Fig. 4). Phototrophic cells were bombarded with the disruption construct containing a selectable spectinomycin resistance marker, and transformants were selected for spectinomycin resistance. Spectinomycin-resistant transformants containing the interrupted ycf5 gene were tested for
would yield a 2.3-kb product. Likewise, Southern analysis of genomes containing an interrupted copy of ccsA revealed a fragment that is 1.9 kb larger than the corresponding fragment from a wild-type genome. The probe used for Southern analysis is indicated as a patterned box.

Fig. 4. Targeted inactivation of the ccsA gene. Structure of the plasmid pEBP-AAD containing the disrupted ccsA gene (filled portion of the insert). The aadA gene (27) is indicated as a hatched rectangle inserted into the BamHI site within the ccsA coding region. Restriction sites are indicated as follows: P, PstI; B, BamHI; E, EcoRI. The filled circles indicate the position of sequences corresponding to oligonucleotide primers 15-6 and 15-9. Amplification of wild-type template would yield a 0.44-kb product, while amplification of templates containing the interrupted gene would yield a 2.3-kb product. Likewise, Southern analysis of genomes containing an interrupted copy of ccsA reveals a fragment that is 1.9 kb larger than the corresponding fragment from a wild-type genome. The probe used for Southern analysis is indicated as a patterned box.

Fig. 5. Phenotype of a strain (ΔccsA) containing a disrupted ccsA gene. Soluble proteins were prepared from copper-supplemented (+ Cu) or copper-deficient (− Cu) cultures of the indicated strains and analyzed for the accumulation of holocytochrome c6 (heme stain and anti-cytochrome c6) and plastocyanin (anti-pc). Nitrocellulose membranes were used for the transfer, and an alkaline phosphatase-conjugated secondary antibody was used for detection.

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their ability to grow phototrophically and were found to be non-photosynthetic. The defect in photosynthesis was ascribed to the electron transfer apparatus, since the non-photosynthetic cells were also determined to be methronidazole-resistant (data not shown). Western blot analysis of soluble and membrane fractions indicated that the strain carrying the disrupted ycf5 gene (ΔccsA) was severely depleted in both holocytochromes c6 and f although other lumenal proteins accumulated to normal levels (Fig. 5). The ycf5 gene was therefore concluded to be essential for the biosynthesis of c-type cytochromes and was accordingly renamed ccsA (for c-type cytochrome synthesis).

The ccsA Gene Rescues the Photosynthetic Deficiency in Strain B6 and Restores Holocytochrome c6 Levels—The B6 strain is unable to grow phototrophically owing to a deficiency in cytochrome b6f function (32). In previous work, we attributed the loss of cytochrome b6f function to a defect in holocytochrome f formation (6, 9). Cells of strain B6 synthesize apocytochrome f but are unable to convert the apoprotein to its holoform. The mutant is also defective in cytochrome c6 function for the same reason, but the defect is only evident in copper-deficient cells where cytochrome c6 replaces plastocyanin.

To determine whether the ccsA gene was indeed the candidate wild-type allele of the gene mutated in B6, plasmid pEBP DNA was introduced into E. coli cells by biolistic bombardment. Phototrophic transformants were identified by their ability to grow on minimal medium. Cells bombarded with pEBP consistently yielded phototrophic colonies, while unbombarded cells or those bombarded with vector sequences did not yield phototrophic colonies (Fig. 6A). To further localize the complementing sequences, plasmids pEBH, containing less 3'-flanking DNA, and pNH, containing less 5'-flanking DNA, were tested for their ability to rescue the B6 phenotype. Both plasmids were found to complement strain B6. The mutation in strain B6 was thus restricted to a 1.4-kb fragment containing the coding region of the ccsA gene. The frequency with which photosynthetic activities are recovered appears to decline progressively as the size of the complementing DNA is reduced, which is not unexpected because complementation occurs via gene replacement by homologous recombination between the introduced wild-type DNA and the recipient mutated plastome (33).

Since the selection for complemented cells requires only restoration of holocytochrome f synthesis (and hence cytochrome b6f function), restoration of holocytochrome c6 synthesis in the complemented colonies was tested directly (Fig. 6B). As expected, copper-deficient cells of (two) representative transformants accumulated holocytochrome c6 to wild-type levels. Thus, we concluded that the B6 phenotype resulted from a defect in a single gene, namely ccsA, which functions in the maturation of both c-type cytochromes.

Fig. 6B. Phenotype of a strain (ΔccsA) containing a disrupted ccsA gene. Soluble proteins were prepared from copper-supplemented (+ Cu) or copper-deficient (− Cu) cultures of the indicated strains and analyzed for the accumulation of holocytochrome c6 (heme stain and anti-cytochrome c6) and plastocyanin (anti-pc). Nitrocellulose membranes were used for the transfer, and an alkaline phosphatase-conjugated secondary antibody was used for detection.
type versus B6 cells for the presence of ccsA transcripts by amplification of cDNA synthesized from a ccsA-specific primer (Fig. 7, lanes 2 and 4). B6 cells did not appear to be defective in accumulating ccsA transcripts. To test for the presence of the protein product of the ccsA gene, antibodies were raised against a synthetic peptide (corresponding to residues 238–255 in the open reading frame; see dashed underline in Fig. 2). The antiserum recognized a protein with an apparent molecular weight of 29 × 10^3 in total extracts of wild-type cells that was absent in extracts prepared from the B6 strain or from the ΔccsA strain (Fig. 8). Membrane fractions did not contain the immunoreactive polypeptide. It appeared, therefore, that the protein product of the ccsA gene was lacking in extracts prepared from B6 cells. The absence of the ccsA gene product in the ΔccsA strain emphasizes the functional importance of its C-terminal region because the disruption construct interrupts the protein coding region only at position 282 out of 353 residues in the open reading frame. The ΔccsA strain does not appear to accumulate a shorter protein (as might be expected), but this could be attributed either to degradation of the non-functional protein or to decreased stability of the ΔccsA mRNA template owing to misplacement of its normal 3' untranslated sequence.

Sequence analysis of the ccsA gene in strain B6 (1.4 kb from NdeI to HindIII corresponding to the smallest complementing fragment) revealed a single mutation. One T in a run of 7 T nucleotides is deleted in the B6 gene relative to the wild-type. This results in a frameshift at the 23rd codon and immediate termination, which accounts for the absence of the protein product of this gene in B6 cells. The frameshift mutation does not appear to affect greatly the accumulation of the ΔccsA message (Fig. 7).

Earlier, we had characterized a spontaneous phenotypic revertant of strain B6, called B6R (6). Holocytochromes c6 and f were restored in B6R as was phototrophic growth. B6R was not likely to be a wild-type contaminant of the B6 culture because it was streptomycin-resistant (like B6, but unlike the standard laboratory wild-type strains). Since the suppressed phenotype displayed uniparental inheritance, we suspected that B6R might be a true revertant. To test this hypothesis, the ccsA gene of B6R was sequenced. Indeed, strain B6R contained a wild-type ccsA gene, and extracts of B6R accordingly accumulate wild-type levels of the ccsA gene product (Fig. 8).

**DISCUSSION**

Functional Conservation of ccsA and ccl1/cycK—The biogenesis of c-type cytochromes is among the best studied of the post-translational assembly pathways, particularly in the case of mitochondrial and bacterial cytochromes (reviewed in Refs. 14 and 34–36). Genetic analysis of cytochrome c and c1 maturation in yeast and Neurospora led to the cloning of cytochrome c heme lyases, which catalyze the stereospecific addition of cysteinyll thios in the apoprotein to heme vinyl groups (37–39). Each cytochrome c heme lyase appears to be specific for its apoprotein substrate. A similar approach, applied to the study
of bacterial cytochrome synthesis, identified a number of genetic loci required for periplasmic heme attachment. In contrast to mitochondrial cytochrome synthesis, where the genes appear to be pathway-specific, these bacterial genes are required for the assembly of all c-type cytochromes. Whether or not some of the genes encode bacterial cytochrome c-heme lyases is not known, and the lack of sequence similarity between the putative protein products of these genes and the mitochondrial cytochrome c-heme lyases precludes homology-based assignment of function. If some of the bacterial genes encode a multisubunit bacterial heme lyase, their protein products would appear to be unrelated to the mitochondrial ones (12, 36).

A relationship between the cytochrome assembly pathway in chloroplasts versus those in mitochondria and bacteria has been suggested (35). In this work, we describe the isolation of a chloroplast gene, ccsA, required for the formation of membrane and soluble holocytochromes. The gene was identified on the basis of sequence similarity to the cyck/cd1 gene in bacteria, but the assignment of function was based on the well characterized biochemical phenotype of strain B6, which was shown (in this work) to carry a frameshift mutation in the ccsA gene. B6 displays normal synthesis of pre-apocytochromes \( c_6 \) and \( f \) and normal processing of the prepeptides to the respective holoproteins; however, the mutant strain is unable to attach heme to apocytochromes \( c_6 \) and \( f \) to convert them to their respective holoproteins (6, 9). Complementation of B6 with a cloned (wild-type) ccsA gene restores both cytochromes to wild-type levels (Fig. 6). We therefore concluded that the ccsA gene indeed encodes a product required for cytochrome biogenesis, possibly one that is a functional homologue of CycK/Cd1. We further concluded that ccsA function is required during cytochrome biogenesis, specifically that it is a functional homologue of CycK/Cd1. We further concluded that ccsA function is required during cytochrome biogenesis at the step of heme attachment. A mitochondrial homologue of the ccsA gene (orf 577/589 in Oenothera and wheat, respectively) has also been identified (11, 40), but the extent of identity among the respective genes is significantly less than the sequence identity between the mitochondrial cytochrome c-heme lyases (only 32% identity between Saccharomyces cerevisiae and Neurospora crassa cytochrome c-heme lyase) but is approximately equivalent to the degree of identity between Cd1 (R. capsulatus) and CycK (R. meliloti).

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