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A spontaneous tRNA suppressor of a mutation in the *Chlamydomonas reinhardtii* nuclear *MCD1* gene required for stability of the chloroplast *petD* mRNA

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ABSTRACT

Numerous nuclear gene products are required for the correct expression of organellar genes. One such gene in the green alga *Chlamydomonas reinhardtii* is *MCD1*, whose product is required for stability of the chloroplast-encoded *petD* mRNA. In *mcd1* mutants, which are non-photosynthetic, *petD* mRNA is degraded by a 5′–3′ exonuclease activity, resulting in a failure to synthesize its product, subunit IV of the cytochrome b_6/f complex. Here, we report the sequence of the wild-type *MCD1* gene, which encodes a large and novel putative protein. Analysis of three mutant alleles showed that two harbored large deletions, but that one allele, *mcd1-2*, had a single base change resulting in a nonsense codon near the N-terminus. This same mutant allele can be suppressed by a second-site mutation in the nuclear *MCD2* gene, whereas *mcd2-1* cannot suppress the deletion in *mcd1-1* (Esposito,D. Higgs,D.C. Drager,R.G. Stern, D.B. and Girard-Bascou,J. (2001) *Curr. Genet.*, 39, 40–48). We report the cloning of *mcd2-1*, and show that the mutation lies in a $tRNA^{Ser}$(CGA), which has been modified to translate the nonsense codon in *mcd1-2*. We discuss how the existence of a large $tRNA^{Ser}$ gene family may permit this suppression without pleiotropic consequences.

INTRODUCTION

In photosynthetic eukaryotes, a significant portion of the nuclear genome encodes proteins required for correct functioning of the plastid. A subset of these genes encodes factors that participate in gene expression, either at the transcriptional or post-transcriptional level. Both forward and reverse genetic approaches have been used to identify and study these nuclear gene products, primarily in maize, *Arabidopsis*, and the unicellular green alga *Chlamydomonas reinhardtii* [reviewed in (1–3)].

One class of mutations that is largely unique to *Chlamydomonas* affects the stabilities of individual chloroplast mRNAs. This has led to the identification and in some cases cloning of the corresponding nuclear genes, including *NAC2*, required for the stability of *psbD* mRNA (4), *MCA1*, which stabilizes *petA* mRNA (5), *MBB1*, which stabilizes *psbB* mRNA (6) and *MDB1*, which stabilizes *atpB* mRNA (7). We have previously reported that mutations in the *MCD1* gene lead to instability of the *petD* transcript, and that because degradation in this background can be blocked by a 5′-untranslated region (5′-UTR) polyguanosine sequence, a 5′–3′ exonucleolytic mechanism is involved (8).

The mechanism by which the nucleus-encoded proteins prevent this degradation is still unresolved, but available data suggest that multiprotein complexes containing the gene-specific factors bind to sequence elements in the 5′-UTR to prevent degradation and perhaps, stimulate translation. These data include the demonstration that Mbb1 and Nac2 are members of RNA-containing complexes (4,6), and the finding that *cis* element mutations in the 5′-UTRs of *psbD* (9) and *petD* (10), for example, can phenocopy the cognizant nuclear mutants.

One way to identify additional proteins that participate in RNA stability regulation is to screen for second-site suppressors of the above-mentioned mutants, and such a screen was carried out previously by plating non-photosynthetic *mcd1-2* cells on minimal medium requiring photosynthesis. A spontaneous semi-dominant suppressor was isolated, and the mutation in this unlinked nuclear suppressor was termed *mcd2-1* (11). Curiously, *mcd2-1* suppressed *mcd1-2* but not *mcd1-1*, defining an allele-specific interaction. Since both *mcd1-1* and *mcd1-2* were expected to be point mutants as they resulted from chemical and UV mutagenesis, respectively, *mcd2-1*

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was hypothesized to encode a change-of-function mutation, perhaps in a protein which interacted with Mcd1.

The study cited above also found that *mcd2-1* was tightly linked to the *ARG7* gene, which encodes argininosuccinolyase, and had been previously cloned. This linkage, along with the newly available *Chlamydomonas* nuclear genome sequence (12), offered an opportunity to isolate the *MCD2* gene, and learn how the *mcd2-1* mutation might specifically suppress *mcd1-2*. Here, we report the cloning of *MCD1* from the insertional allele *mcd1-3*, and the cloning of *mcd2-1* based on the analysis of *mcd1* mutations and its linkage to *ARG7*. We show that *mcd2-1* is an amber suppressor tRNA, which represents the first mutation of this type to be found in plants using an undirected forward genetic screen.

MATERIALS AND METHODS

Culture conditions and quantification of chlorophyll

C.reinhardtii strains used in this study are listed in Table 1. Unless otherwise noted, cells were grown in Tris–acetate–phosphate (TAP) medium (13) in the light. Chlorophyll was quantified as described previously (13).

Nuclear transformation and molecular genetic analysis

Chlamydomonas cells were transformed either by the glass bead procedure (14) or by electroporation (15), with several modifications. For the glass bead procedure, *mcd1-1*, *cw15* cells were incubated <2 h in either minimal medium, or in N-free TAP medium (13) prior to transformation with 2–5 μg cosmid or plasmid DNA. Cosmids were not digested prior to transformation. For electroporation, CC-125 or *mcd1-2* cells were treated with autolysin, centrifuged and concentrated 100-fold in TAP medium containing 60 mM sucrose. DraI-linearized plasmid DNA (1 μg) and salmon sperm DNA (20 μg) were added to the cell suspension in a total volume of 100 μl, and electroporated in a 4 mm cuvette using a Gene Pulser (Bio-Rad, Hercules, CA). Settings were 0.8 kV (2.0 kV/cm), with a capacitance with 25 μF and no shunt resistor. Following electroporation, cells were resuspended in 3 ml TAP medium containing 60 mM sucrose, and incubated at room temperature in the light for at least 24 h before plating on TAP medium containing 5 mg/l zeocin (Invitrogen, Carlsbad, CA).

Table 1. Strains used in this study

Strain	Genotype	Source
CC-124	WT	*Chlamydomonas* Stock Center
CC-406	*cw15*	
F16.6	*mcd 1-1*	(8)
F16.A20	*mcd 1-1*	This study
F16cw	mcd 1-1, *cw15*	This study
670.1	*mcd 1-2*	(8)
715.90[a]	*mcd 1-3*	This study
R1.670	*mcd 1-2*, *mcd 2-1*	(11)
R1.670.5	*mcd 1-2*, *mcd 2-1*	This study
R1.670.cw15.12	*mcd 1-2*, *mcd 2-1*, *cw15*	This study
R1.670.cw15.22	*mcd 1-2*, *mcd 2-1*, *cw15*	This study

[a]This original mutant and all derivatives (e.g. 7-90.33) have identical genotypes and phenotypic characteristics, except some derivatives exhibited better survival of tetrad progeny.

For genetic analysis of *mcd1-3*, a better-crossing derivative of strain 7-90 was created by crossing to CC-2986 (*arg2*, *nit1*, mt^+); this strain was named 7-90.33. When 7-90.33 (mt^-) was crossed to strain F16.6 (*mcd1-1*, mt^+), no photoautotrophic (low chlorophyll fluorescence) recombinants were recovered, indicating tight linkage of the mutant loci. When CC-2986 was crossed to an intermediate version of *mcd1-3*, strain 7-90.1, co-segregation of arginine prototrophy and high chlorophyll fluorescence indicated tight linkage of the inserted *ARG7* gene and the *mcd1* mutant phenotype. For molecular analysis, total *Chlamydomonas* DNA was extracted as described previously (16). One microgram of total DNA digested by BamHI was transferred to nylon membranes using standard techniques (17).

RT–PCR

Total *Chlamydomonas* RNA was extracted with TRI-Reagent according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). Poly(A)$^+$ RNA was isolated using the PolyATract mRNA Isolation System (Promega, Madison, WI) and was treated with RQ1 RNase-Free DNase (Promega). Poly(A)$^+$ RNA was used as a template to synthesize cDNA with 10 U/μl of SuperScript III RNase H$^-$ reverse transcriptase (Invitrogen) and 0.25 μM of primer Q_T (5′-GACTGCGTACGCATGGCGAGGACTCGAGCTCAA-GCTTTTTTTTTTTTTTTT-3′) with the buffer containing 1 U/μl recombinant RNasin ribonuclease inhibitor (RNasin; Promega) and 1 M betaine. The cDNA was used as a template for PCR with Ex *Taq* DNA polymerase (Takara, Ohtsu, Japan) with 1 M betaine. Primer Q_0 (5′-GACTGCGTACGCATGG-CG-3′) and gene-specific primers were used for amplification of the 3′ end, and pairs of gene-specific primers were used for internal regions. PCR conditions were 95°C for 30 s, 60°C for 1 min and 72°C for 1.5 min/kb, for 35 cycles.

Primer extension with dideoxyribonucleoside triphosphates

The primer tRNA-Ser PPE (5′-ATGCGGGAGATCCCATC-TGA-3′) was labeled at its 5′ end with 100 μCi of γ-[^{32}P]ATP and 10 U of T4 polynucleotide kinase and gel-purified. RNA was isolated from *Chlamydomonas* cells cultured in TAP medium for 7 days and then transferred into minimal medium for 7 days. Five micrograms of total RNA were incubated with 4 μl of 5× first-strand buffer (Invitrogen), 0.5 μl of RNasin (40 U/μl), 2 μl labeled primer (>10^5 c.p.m.) at 85°C for 15 min and then transferred to 50°C for 30 min. The reaction mixture was supplemented with 1 μl of 0.1 M DTT, 40 U of SuperScript III, 1 μl each of 2 mM dNTPs and 1 μl of 4 mM ddCTP or 2 mM ddTTP. The reaction mixtures with total volume of 20 μl were incubated at 50°C for 20 min, and stopped by addition of 18 μl of formamide and 4 μl of 10× TBE containing loading dyes. Reaction products were resolved in 17.5% denaturing polyacrylamide gels and visualized and quantified using a Storm scanner (Molecular Dynamics, Sunnyvale, CA).

Plasmid construction and PCR

Random Mu transposon insertions into the genomic *MCD1* fragment were made using the Finnzymes Template Generation System (MJ Research, Inc., Waltham, MA). To clone the *mcd1-2* locus, a 6.47 kb fragment was amplified using Ex *Taq*

DNA polymerase, 0.25 μM primers (mcd1 5′-2, 5′-ATGTACCCGTCTTGCTATGTAGCCGT-3′; mcd1 3′-2, 5′-CCTGCCCGTTTACATTTCCATTCCCA-3′) and total DNA as the template in manufacturer-supplied buffer containing 1 M betaine. PCR conditions were 95°C for 3 min, followed by 35 cycles of 95°C for 50 s, 60°C for 50 s, and 72°C for 9 min, with a final extension step of 72°C for 5 min. We subsequently discovered that 30–35 cycles of 98°C for 5 s, 60°C for 30 s and 72°C for 9 min (~1.5 min per kb) gave more consistent results. Genomic fragments of 1319 bp containing WT or mutant $tRNA^{Ser}$ (CGA)-1 were amplified by PCR from total DNA of CC-124 (WT) or R1.670 (*mcd1-2*, *mcd2-1*) using the primers tRNA-Ser genome2 forward (5′-CGTGCAGGATGGCGCTCTGTGACTT-3′) and tRNA-Ser genome2 reverse (5′-GCAGCTGTCACCTGTCAAGGTCGC-3′), and inserted into pGEM-T Easy (Promega). The fragments were excised with NotI and subcloned into the NotI site preceding the *ble* cassette of pMS171 (originally named Δ-196[−4]) (18), which was a gift of Michel Schroda.

RESULTS

Isolation of the *MCD1* gene

C.reinhardtii mcd1 mutants are non-photosynthetic because of the instability of *petD* mRNA, which encodes subunit IV of the cytochrome b_6/f complex (SUIV). Several mutant alleles of *mcd1* were available, including the presumed point mutations *mcd1-1* (strain F16) and *mcd1-2* (strain CF670), which were described previously (11). A third allele, *mcd1-3*, is defined by the strain 715.90, which was generated from the arginine auxotroph 715 (*arg7*, *cw15*, mt^-) by random integration of the plasmid pARG7.8ϕ3, which encodes a copy of the *ARG7* gene containing a 392 bp HpaI fragment of ϕX174 DNA (19). Genetic analysis of *mcd1-3* showed that the non-photosynthetic phenotype co-segregated with the arginine prototrophy conferred by the transformed vector, and that this mutation was closely linked to the *MCD1* locus (see Materials and Methods). We therefore assumed that this strain contained the pARG7.8ϕ3 insertion at *MCD1* locus and named it *mcd1-3*.

To clone the *MCD1* gene, we began by performing DNA gel blot analysis of KpnI-digested *mcd1-3* DNA, probed with the 392 bp HpaI fragment of ϕX174 (Hpa-392; data not shown). Fragments of 9.5, 8.5 and 6 kb were detected and this, together with other restriction digests, suggested that *mcd1-3* contained tandem insertions of pARG7.8ϕ3, and that a 6 kb fragment represented one of the borders of the insertion. A plasmid library with 5.5–6 kb KpnI fragments was therefore generated from *mcd1-3* DNA, and screened using the Hpa-392 probe. Plasmids containing the desired insert were purified and sequenced. Interestingly, these clones contained sequences from the *PF28*/*ODA2* gene, which encodes the dynein gamma chain of flagellar outer arms (20), and is located on linkage group XI (21). This indicated that the *MCD1* locus was close to *PF28*, and genomic clones containing *PF28* might include *MCD1* as well.

We obtained *PF28*-containing cosmids from George Witman (Worcester Foundation for Experimental Biology) and transformed them into F16cw (*mcd1-1*, *cw15*) cells to see whether they could complement the *mcd1* phenotype. Transformations of two overlapping cosmids, *PF28* cosmids 3 and 4, yielded significant and reproducible numbers of photoautotrophic colonies. These two cosmids contained a common 12 kb BglII/HindIII fragment, which was the smallest fragment capable of complementation (data not shown). The sequence of 9.4 kb of this fragment starting at the BglII site was subsequently determined using the Mu transposon to generate randomly located primer binding sites. Once an unambiguous sequence was generated, we used GreenGenie, a gene finder program for *Chlamydomonas* (http://www.cse.ucsc.edu/~dkulp/cgi-bin/greenGenie), to predict any genes. This program indeed generated a gene model, centered within the sequenced region.

To define the borders of *MCD1* within the 12 kb fragment using functional criteria, we took advantage of the Mu insertion clones used for DNA sequencing. A series of clones with insertions spaced throughout the 12 kb region were transformed into F16cw, and photoautotrophic colonies were selected. Because the transposon has two HindIII sites, it could be predicted that if the transposon had inserted outside of *MCD1*, the plasmid could complement the mutation following HindIII digestion, whereas if Mu had inserted with *MCD1*, whether an intron or exon, it could not. Twenty-eight clones were tested in this way, and the results defined the maximum and minimum extent of *MCD1* as 6.2 and 5.6 kb, respectively. This region was mostly consistent with the GreenGenie prediction: if the 'A' of the first ATG in gene model is defined as +1, the GreenGenie model covered 1–5365 bp, whereas the maximum and minimum regions deduced from transformations were from −577 to +5701, and from −75 to +5577.

Identification of the *MCD1* mRNA

RT–PCR was performed to define the *MCD1* coding region, since no expressed sequence tag (EST) sequences were available, and no cDNA clones were found in publicly available cDNA libraries (data not shown). Using this strategy, a series of overlapping fragments were amplified. To ensure that these represented bona fide cDNA and not DNA contamination, PCR was repeated in parallel without the addition of reverse transcriptase, and where possible coding segments were amplified at least once using primers that spanned an intron/exon boundary. The *MCD1* 3′ end was identified using an oligo(dT)-based RACE procedure described in Materials and Methods. To locate the approximate 5′ end, RT–PCR was performed using the reverse primer 3+ (+624 to +606) and two upstream forward primers; −2− (−345 to −321) and −3− (−556 to −532). Because a cDNA fragment could be amplified with the primer combination (3+ and −2−), but not with (3+ and −3−), the mRNA 5′ end probably lies between positions −556 and −320. Furthermore, because there is no in-frame ATG between positions −1 and −556, the ATG at position +1 (see previous paragraph) probably corresponds to the true *MCD1* translation initiation codon.

The experimentally determined *MCD1* mRNA (GenBank accession no. DQ027015DQ027015) was nearly identical to that predicted by GreenGenie, except that one intron failed to be predicted. Based on the cloned cDNA sequences, *MCD1* encodes a 1553 amino acid protein with a molecular mass of 156.1 kDa and a pI of 10.1, but bears little similarity with other proteins. A motif search identified a ribosomal protein S14 signature, which includes 7 of 24 amino acids between

positions 1081–1104 (PROSITE accession no. PS00527), however this motif is considered to arise frequently as a false positive. The apparent uniqueness of Mcd1 contrasts with the three previously cloned *Chlamydomonas* cpRNA stability factors, which possess either tetratricopeptide (TPR) or pentatricopeptide (PPR) repeats.

Analysis of *mcd1* mutant alleles

The three mutant alleles of *mcd1* mentioned above were *mcd1-1*, generated by 5-fluorouracil treatment; *mcd1-2*, which resulted from UV mutagenesis; and *mcd1-3*, which is the *ARG7::MCD1* insertional mutant. As a first step in characterizing the mutation in each allele, DNA from each strain was digested with BamHI and subjected to DNA gel blot analysis (Figure 1A) using the mixed probes indicated in Figure 1B.

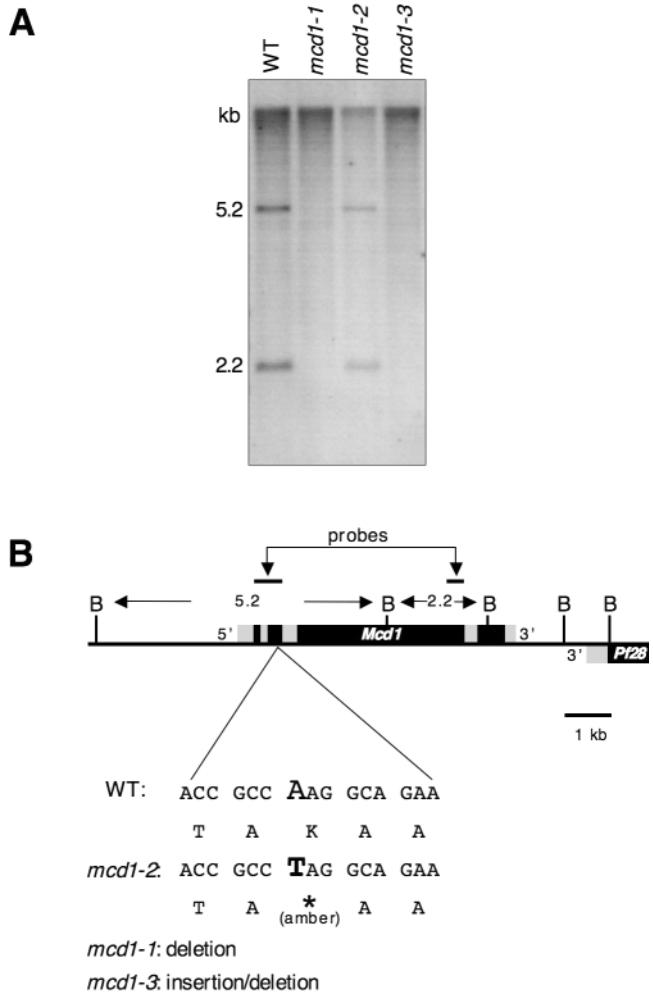

Figure 1. Gene structure of *MCD1* and its mutants. (**A**) DNA gel blot analysis of wild-type (WT) and the *mcd1* mutants indicated above each lane. Total DNA from WT (CC-124), *mcd1-1* (strain F16.A20), *mcd1-2* (strain 670.1) and *mcd1-3* (strain 7-90.33) were digested with BamHI, and the resultant blot was hybridized with the ^{32}P-labeled probes indicated at the top of panel, (**B**) which also shows a diagram of the WT *MCD1* locus. In this diagram, black rectangles represent exons and gray rectangles represent the 5′-UTR, the three introns and the 3′-UTR. BamHI restriction sites 'B' and the sizes of fragments (in kb) identified in panel A are shown. *MCD1* is adjacent to *PF28*, whose C-terminal end on the opposite strand is shown. Below the gene diagram, the nucleotide and amino acid sequences surrounding the *mcd1-2* mutation are shown.

Fragments of 5.2 and 2.2 kb were detected in the wild-type control and in *mcd1-2*, whereas *mcd1-1* and *mcd1-3* samples only gave nonspecific hybridization at the position of undigested DNA. These results suggested that *mcd1-1* had suffered a large deletion, and that in addition to the known insertion of *ARG7*, *mcd1-3* had a deletion around *MCD1*. In contrast, *mcd1-2* had a restriction pattern consistent with a point mutation(s) or small insertion/deletion.

To identify the mutation in *mcd1-2*, the entire *mcd1-2* locus was amplified by PCR using primers mcd1 5′-2 and mcd1 3′-2, which yielded a fragment extending from −625 to +5848. This 6.47 kb fragment was cloned and sequenced, revealing an A to T mutation that changed the lysine codon AAG to the amber stop codon TAG at amino acid position 113 (Figure 1B). The existence of this mutation in the genome was verified in two ways. First, multiple, independent PCRs were carried out, and the products always contained this mutation when amplified from *mcd1-2* DNA. Second, the A to T mutation creates a restriction endonuclease recognition site for the enzyme AvrII (CCTAGG). When DNA was amplified from *mcd1-2*, but not from other strains, this restriction site was present in the PCR product (data not shown). Taken together, the sequence suggests that in *mcd1-2*, translation initiation would result in production of a truncated, and possibly unstable, protein of 112 amino acids, which would be highly unlikely to be functional.

Identification of the suppressor mutation in *mcd2-1*

As described in the Introduction, *mcd2-1* is a semi-dominant mutation closely linked to the *ARG7* locus; no recombinants between *mcd2* and *ARG7* were found in our previous work (11). Because *mcd2-1* could suppress *mcd1-2* but not *mcd1-1*, we hypothesized that Mcd2 was most likely a protein factor that interacted with Mcd1, and presumed that *mcd1-2* carried a suppressible lesion, whereas *mcd1-1* did not. However, the fact that *mcd1-2* contains a nonsense mutation very early in the coding region argued against this hypothesis, and raised the possibility that *mcd2-1* might encode a nonsense suppressor.

Because *MCD2* is linked to *ARG7*, we examined version 2 of the *Chlamydomonas* nuclear genome sequence (http://genome.jgi-psf.org/chlre2/chlre2.home.html) around *ARG7*, and found a predicted tRNA^{Ser}(CGA) at a 320 kb distance from *ARG7* on scaffold 35 (Figure 2A). This tRNA has been previously found to become an amber (UAG) suppressor in other organisms, originally in *Escherichia coli* [reviewed in (22)]. To determine whether *mcd2-1* carried a mutation in this tRNA, we amplified and sequenced this region of the mutant *mcd1-2*, *mcd2-1*. This revealed a point mutation that changed the anticodon CGA to CUA, which would allow the mutated tRNA to recognize the amber stop codon. To confirm that this mutation truly represented the *mcd2-1* mutation, we sequenced this region in 11 *mcd2-1* strains derived from independent backcrosses, and found that all of them had the same mutation. The wild-type gene was designated as tRNA^{Ser}(CGA)-1, and the mutant allele as tRNA^{Ser}(CGA)-1^{mcd2}.

Expression of the mutant tRNA

Because the same tRNA point mutation was found in each *mcd2* strain, tRNA^{Ser}(CGA)-1^{mcd2} was an excellent candidate

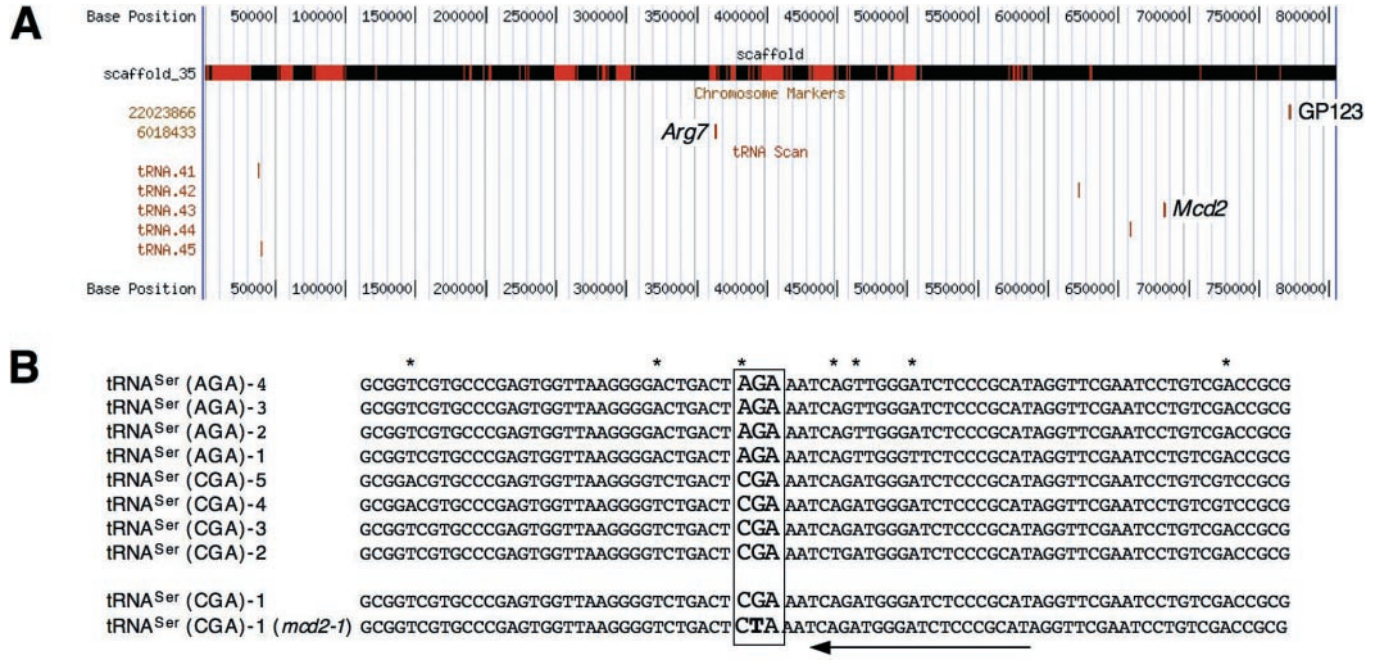

Figure 2. *MCD2* and other similar tRNA^{Ser} genes. (**A**) Scaffold 35, which includes *ARG7*, derived from the *Chlamydomonas* genome portal v2.0. The positions of *ARG7* and the chromosomal marker *GP123* (vertical brown lines) are labeled. The five tRNA genes present in the region are shown as vertical brown lines in the lower part of the diagram, and *MCD2* is labeled. Red areas are unsequenced regions, whose sizes are estimated. (**B**) Alignment of selected *Chlamydomonas* tRNA^{Ser} genes. The genes shown are highly similar to *MCD2*, and needed to be taken into consideration in designing a primer extension expression experiment. Anticodons are boxed, and asterisks indicate positions where a base difference exists in at least one gene. The lower two lines compare the WT and *mcd2-1* mutant sequences, with the point mutation shown in bold. The horizontal arrow represents the primer used for the assay shown in Figure 3 and described in the text.

Table 2. Positions of selected tRNA^{Ser} genes in the nuclear genome[a]

Gene name	JGI *Chlamydomonas* genome v2.0
tRNA^{Ser} (CGA)-1	scaffold_35: 681829–681909
tRNA^{Ser} (CGA)-2	scaffold_10: 21195–21275
tRNA^{Ser} (CGA)-3	scaffold_10: 14902–14822
tRNA^{Ser} (CGA)-4	scaffold_48: 598155–598235
tRNA^{Ser} (CGA)-5	scaffold_48: 598768–598848
tRNA^{Ser} (AGA)-1	scaffold_10: 20500–20580
tRNA^{Ser} (AGA)-2	scaffold_10: 15591–15511
tRNA^{Ser} (AGA)-3	scaffold_13: 711455–711535
tRNA^{Ser} (AGA)-4	scaffold_13: 715098–715178

[a]tRNAs were identified using *MCD2* in a Blast search of available nuclear genome sequence.

for the suppressor. We therefore examined its expression using a primer extension assay. Experimental design was complicated by the fact that the available nuclear genome sequence includes eight other tRNA genes which are nearly identical to tRNA^{Ser}(CGA)-1 (Figure 2B; Table 2). This was overcome by using appropriate ddNTPs during primer extension, which would terminate elongation by reverse transcriptase at a position in the mutant tRNA calculated to yield a differently sized product than in the wild-type. The primer chosen for this experiment is drawn at the bottom of Figure 2B.

Predicted and actual results from primer extension are shown in Figure 3. Panel A shows that when no ddNTPs are included, the major product from the collective WT tRNA^{Ser} would be 26 nt. This premature termination results from methylation at the cytidine marked by an asterisk in

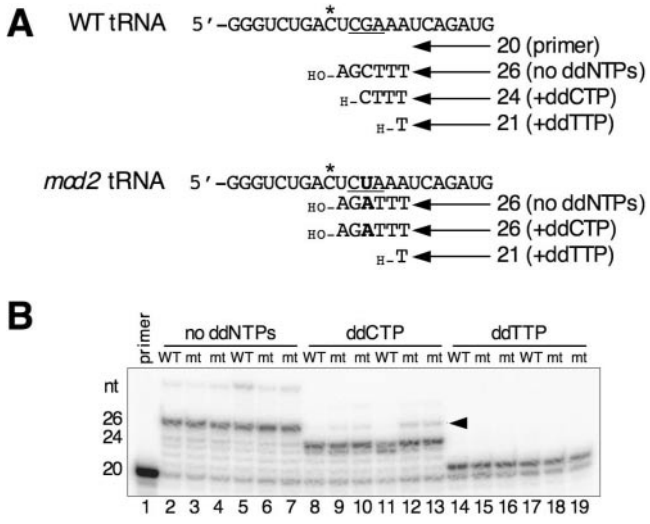

Figure 3. Expression of a tRNA^{Ser} encoded by *mcd2-1*. (**A**) Design of primer extension experiment using ddNTPs, showing the WT and mutant versions of tRNA^{Ser} (CGA)-1 (*MCD2*). The anticodon is underlined and the *mcd2-1* point mutation is shown in bold. The horizontal arrows represent the 3′ part of the primer, and numbers to the right predicted product sizes in nucleotides, which are terminated by hydroxy (–OH) or dideoxy (–H) nucleosides. The asterisk indicates a putative methylation site discussed in the text. (**B**). Primer extension assay without or with ddNTPs, as indicated across the top. The arrowhead indicates the *mcd2-1*-specific band. Total RNA from cells with WT tRNA^{Ser} (CGA)-1 was derived from CC-124 (WT; lanes 2, 8 and 14) and CC-406 (*cw15*; lanes 5, 11 and 17). RNAs carrying the *mcd1-2* mutation were isolated from the following strains: R1.670 (lanes 3, 9 and 15), R1.670.5 (lanes 4, 10 and 16), R1.670.cw15.12 (lanes 6, 12 and 18) and R1.670.cw15.22 (lanes 7, 13 and 19).

Figure 3A, which has been previously reported for this tRNA in tobacco (23). With the inclusion of ddTTP, all reverse transcription reactions should terminate at the 3′-A of the anticodon, generating 21 nt products. However, the presence of ddCTP should differentiate between tRNA^{Ser}(CGA)-1 and tRNA^{Ser}(CGA)-1^{mcd2}, with the mutant anticodon allowing readthrough to the 26 nt size, rather than ddCTP-mediated termination at 24 nt. Thus, a 26 nt product derived uniquely from *mcd2* RNA preparations, in the presence of ddCTP, would be diagnostic of expression of the amber suppressor.

Figure 3B shows the results of primer extension experiments, which bore out the predictions stated above. Without ddNTPs, most of the products were 26 nt, although some longer (>30 nt) products were also observed (lanes 2–7). However, when ddCTP was used, strong signals at 24 nt were detected for each sample (lanes 8–13), whereas a 26 nt band could be detected uniquely in the four independent samples derived from *mcd2* cells (lanes 9, 10, 12 and 13). RNAs from *mcd2* cells also generated a 24 nt product, derived from non-mutated tRNA^{Ser} transcribed from other loci. The fact that only 21 nt products could be detected when ddTTP was used (lanes 14–19) suggested that ddNTP incorporation can fully impede extension under the conditions used. We therefore concluded that the 26 nt signals represented the expression of tRNA^{Ser}(CGA)-1^{mcd2} in *mcd2*.

Ectopically expressed tRNA^{Ser}(CGA)-1^{mcd2} complements *mcd1-2*

To confirm that this mutant tRNA could suppress the amber mutation at a phenotypically significant level, we performed a complementation experiment. To do this, genomic fragments containing tRNA^{Ser}(CGA)-1 were amplified from wild-type and *mcd2-1* DNA, and inserted into pMS171, a *Chlamydomonas* vector containing a zeocin resistance marker (Figure 4A). The amplified fragment was large enough to very likely contain complete expression elements for the tRNA gene. The resulting plasmids, or the original vector, were then used to transform strain 670.1 (*mcd1-2*), and colonies were selected on zeocin-containing TAP medium, to which the *ble* gene confers resistance. As expected, numerous transformants were obtained with each plasmid, since TAP medium does not select for photosynthesis.

To see whether the ectopically expressed suppressor tRNA could restore photosynthetic activity to *mcd1-2*, transformants and suitable controls were replica-plated on TAP and minimal media, as shown in Figure 4B. All strains grew on TAP medium, although the non-photosynthetic strains showed less robust growth, a phenomenon which is commonly observed with *Chlamydomonas*. On the other hand, only five strains were viable on minimal medium. These were CC-406 (*MCD1*) cells, two independent clones of *mcd1-2* and *mcd2-1*, and two independent strains where *mcd1-2* had been transformed with pMS171 co-expressing tRNA^{Ser}(CGA)-1^{mcd2}. When *mcd1-2* was transformed with pMS171 alone, or with pMS171 co-expressing (non-suppressing) tRNA^{Ser}(CGA)-1, cells failed to grow on minimal medium. This indicates that the suppressor tRNA confers the biological function of allowing sufficient expression from the *mcd1-2* locus to permit photoautotrophic growth.

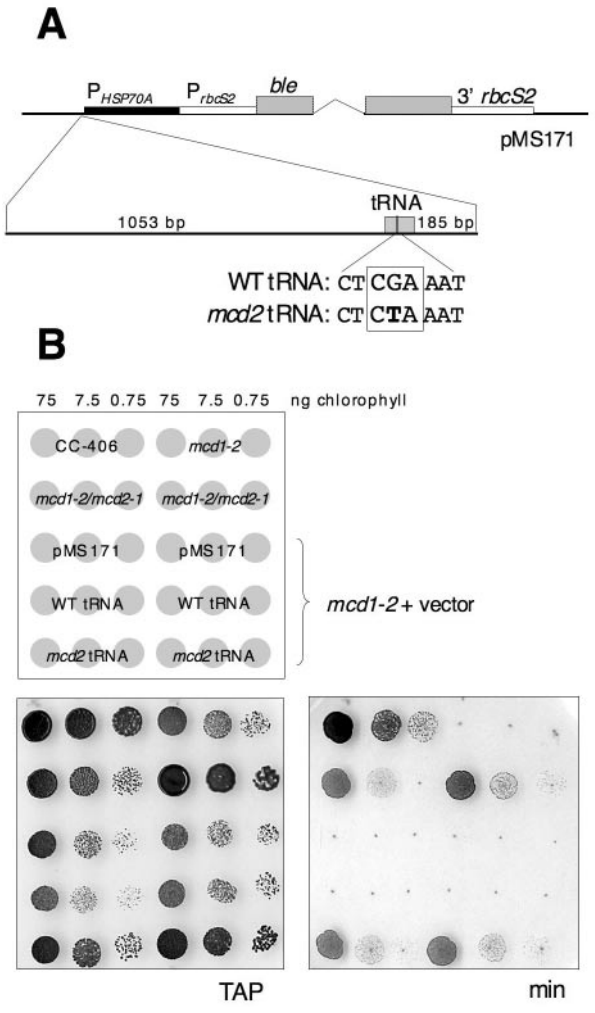

Figure 4. Complementation of non-photosynthetic phenotype of *mcd1-2* by mutant tRNA. (**A**) Schematic diagram of the constructs. Genomic DNA fragments including WT or *mcd2-1* tRNA^{Ser} (CGA)-1 were inserted into a position preceding the *ble* cassette of pMS171. (**B**) Cells with the indicated amount of chlorophyll were spotted on TAP or minimal medium and incubated for 8 days in the light.

It should be noted that occasional escapes were seen in the transformation experiments described above. Although 16/16 zeocin-resistant *mcd1-2* transformants expressing the suppressor tRNA were able to grow on minimal medium, 1/16 transformants from pMS171 alone, and 2/14 pMS171 transformants co-expressing non-suppressing tRNA^{Ser} also exhibited some growth on minimal medium. Although the reasons for these occasional unexpected growth phenotypes was unclear, the fact that all the transformants possessing the *mcd2-1* tRNA gene recovered photosynthetic activity strongly suggested that the transformed mutant tRNA could suppress the *mcd1-2* mutation.

DISCUSSION

In this report, we describe the isolation of *MCD1* and *MCD2*, two genes which were identified by mutations affecting the stability and possibly translation of *Chlamydomonas petD* mRNA. The predicted *MCD1* gene product, a novel 1553

amino acid protein, is posited to interact with of 5′-UTR of *petD* mRNA and protect this transcript from 5′–3′ exoribonucleolytic degradation. *mcd2-1*, a semi-dominant, allele-specific suppressor of *mcd1-2*, was found to encode a nonsense suppressor tRNA. In the haploid state, *mcd1-2* and *mcd2-1* show partial restoration of *MCD1* function (11), consistent with incomplete suppression of the *mcd1-2* amber mutation.

Several nuclear genes are known whose products are required for chloroplast mRNA stability (see Introduction), but their precise mechanisms of action are poorly understood. To clarify how Mcd1 might protect the *petD* mRNA 5′ end, we sought to identify the *MCD1* gene, and ultimately obtained cDNA and genomic sequence. Surprisingly, the deduced Mcd1 protein does not contain any significant identity to proteins in the database, nor any motifs that hint at its mode of action. This contrasts with the three other *Chlamydomonas* cpRNA stability regulators, which possess TPR (4,6) or PPR (5) protein–protein interaction motifs. These domains are present in vastly expanded numbers in land plants, and are thought or known to be frequent regulators of organellar processes (24,25). Based on the currently available *Chlamydomonas* nuclear genome sequence, however, they are relatively uncommon in the alga: v2.0 has seven genes annotated as containing PPR motifs, and 80 annotated as containing TPR motifs. This suggests that Mcd1 may contain a previously unknown domain involved in protein–protein and/or protein–RNA interactions.

The lack of relationship between Mcd1 and known chloroplast regulatory proteins may reflect a small sample size, but might also be related to unique features of *petD* in *Chlamydomonas*. Located downstream of *petB* in organisms as diverse as cyanobacteria (26), land plants (27) and the *Chlamydomonas* relative *Chlorella vulgaris* (28), *petD* in *Chlamydomonas* is located downstream of *petA*, transcribed from both the *petA* and its own promoter, and matured to its monocistronic form through uncharacterized RNA processing steps (29,30). One might speculate that Mcd1 evolved to serve dual roles of RNA maturation and 5′ end protection, and indirect evidence suggests that Mcd1 may also play a role in translational activation of *petD* mRNA (10,31). Biochemical analysis of Mcd1, and any homologues discovered in the future, would be highly informative.

We originally hypothesized that the *mcd2-1* mutation was in an Mcd1-interacting protein, as it had been obtained in a classical suppressor screen designed to yield such a result. The finding that *mcd1-2* possessed a point mutation changing the lysine codon AAG (Lys-113) to the amber stop codon TAG led us to believe that *mcd2-1* might be a nonsense suppressor mutation. Although in principle a truncated protein could be reactivated by a second-site suppressor in an interacting protein, K113 is near the N-terminal of Mcd1, and any protein expressed in *mcd1-2* would be expected to consist largely of a chloroplast targeting signal. Our finding raises the possibility that suppressors isolated in analogous screens [e.g. (32)] could be of a similar nature.

To test the hypothesis that *mcd2-1* expressed a suppressor tRNA, we first examined the genomic sequence around the *ARG7* locus, to which *mcd2-1* was closely linked. This led to a mutated $tRNA^{Ser}$ (CGA), which was subsequently found in all 11 strains carrying *mcd2-1*. Although the original *mcd2* mutation was isolated only once, the 11 strains differ in their sources in that they were derived from a variety of crosses to test specificity or dominance of the mutation. Our hypothesis was supported by expression of an ectopic copy of the suppressor tRNA, which restored photosynthetic activity to the *mcd1-2* mutant.

Nonsense suppression is a well-known phenomenon in prokaryotes and eukaryotes, but naturally occurring examples in plants are rare, to our knowledge. In *Chlamydomonas*, an amber suppressor tRNA was identified in the chloroplast (33), as it restored photosynthesis to a strain bearing a nonsense mutation in the *rbcL* gene, which encodes the Rubisco large subunit. Although the $tRNA^{Trp}$ which was mutated is a single-copy gene, the polyploid nature of the chloroplast allowed heteroplasmic maintenance of the suppressor. Curiously, the frequency of the mutant allele was ~70%, suggesting that a preponderance of $tRNA^{Trp}$(CUA) was required for biological activity. This contrasts with the apparently minor contribution of *mcd2-1* to total $tRNA^{Ser}$ transcription (Figure 3B). An important class of nonsense suppressors in plants are naturally occurring tRNAs which are able to promote readthrough of stop codons, particularly in single-stranded RNA viruses [reviewed in (34)]. These contrast with *mcd2-1* because they possess a wild-type anticodon, but are able to recognize the viral stop codons through sometimes unusual wobble base-pairing mechanisms.

The principle that mutant tRNAs in plants can promote nonsense suppression has been demonstrated through transient or stable transgenic approaches. In one example, $tRNA^{Ser}$ was engineered to become an amber suppressor, and transient expression resulted in 10% of β-glucuronidase activity relative to a gene without a nonsense codon (35). Stable expression of amber suppressor $tRNA^{Leu}$, or other tRNAs, were similarly shown to confer GUS (36,37), luciferase (38) or chloramphenicol acetyltransferase (39) activity. As in the case of chloroplast tRNA suppression cited above, *mcd2-1* is viable because wild-type $tRNA^{Ser}$(CGA) genes are still expressed. As shown in Figure 2B, available *Chlamydomonas* sequence information reveals five $tRNA^{Ser}$(CGA) genes, as well as four with an AGA anticodon. This may well be an underestimate; the *Arabidopsis* genome has 65 loci annotated as encoding $tRNA^{Ser}$; four of which feature a CGA anticodon and the vast majority of the remainder AGA. Assuming that *mcd2-1* represents 20% of the $tRNA^{Ser}$ (CGA) activity, expression of suppression is reasonable based on the fact that cognizant *E.coli* amber suppressor, *supD*, exhibited a 28% efficiency, second only to the 55% conferred by the Tyr amber suppressor [reviewed in (22)].

Our results suggest that *Chlamydomonas*, like other organisms, will be amenable to site-directed protein mutagenesis through expression of engineered suppressor tRNAs (40,41). The ability to recover *MCD2* without a laborious map-based cloning approach also illustrates the utility of the newly available nuclear genome sequence, when combined with the powerful genetic approaches long utilized in *Chlamydomonas* (42). The tight linkage of *MCD2* to *ARG7* was indeed fortuitous, but the proliferation of chromosome markers (43) makes mapping of suppressors a fully practical endeavor, which should quickly enhance our understanding of how nuclear gene products (44–47) regulate chloroplast gene expression in this organism.

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