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A nucleus-encoded suppressor defines a new factor which can promote *petD* mRNA stability in the chloroplast of *Chlamydomonas reinhardtii*

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Abstract Mutations in the *Chlamydomonas reinhardtii* nuclear gene *MCD1* specifically destabilize the chloroplast *petD* mRNA, which encodes subunit IV of the cytochrome b_6/f complex. The *MCD1* gene product is thought to interact with the mRNA 5′ end to protect it from degradation by a 5′ → 3′ exoribonuclease and may also have a role in translation initiation. Here we report the isolation and characterization of a semidominant, allele-specific, nucleus-encoded suppressor of the *mcd1-2* mutation. The suppressor mutation, which defines a new locus *MCD2*, allows accumulation of 10% of the wild-type level of *petD* mRNA and as much as 50% of the wild-type subunit IV level. Taken together, these results suggest the suppressor mutation restores photosynthetic growth by stabilizing *petD* mRNA. In addition, it may promote increased translational efficiency, an inference supported by direct measurements of the subunit IV synthesis rate. Thus, both *MCD1* and *MCD2* may participate in both chloroplast RNA stability and translation initiation.

Key words Chloroplast · *Chlamydomonas* · RNA stability · Translation

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Introduction

The mechanisms and machinery of chloroplast gene expression resemble those of prokaryotes, although regulation occurs primarily at the post-transcriptional level in chloroplasts (reviewed in Stern et al. 1997). Despite similarities to prokaryotic mechanisms, one major distinguishing characteristic is the involvement of nucleus-encoded factors in chloroplast gene expression (reviewed in Rochaix 1996). These factors have mainly been defined genetically by the isolation of high chlorophyll fluorescence or non-photosynthetic mutants in *Chlamydomonas reinhardtii* and vascular plants, which have subsequently been found to have defects in RNA processing (Barkan et al. 1994; Levy et al. 1997; Meurer et al. 1998) and splicing (Goldschmidt-Clermont et al. 1990; Jenkins et al. 1997), RNA stability (e.g. Drager et al. 1998; Drapier et al. 1992; Gumpel et al. 1995; Kuchka et al. 1989; Monod et al. 1992), translation initiation (Drapier et al. 1992; Girard-Bascou et al. 1992; McCormac and Barkan 1999; Yohn et al. 1996; Zerges et al. 1997) and elongation (Wu and Kuchka 1995). Interestingly, the *Chlamydomonas* mutants are generally affected in the expression of only a single chloroplast gene, whereas *Arabidopsis* and maize mutants often have pleiotropic effects.

We have previously used *Chlamydomonas* as a model system to dissect these nucleus-chloroplast interactions, in part focusing on the chloroplast *petD* gene, which encodes subunit IV (SUIV) of the cytochrome b_6/f complex. Because SUIV is essential for photosynthesis, cells defective in *petD* expression depend on a fixed carbon source (Chen et al. 1993; Drager et al. 1998; Lemaire et al. 1986). Due to pleiotropic effects on protein accumulation, such cells also have greatly diminished amounts of the other cytochrome b_6/f complex subunits (Kuras and Wollman 1994).

One nuclear gene required for *petD* expression is *MCD1* (mRNA stability of the third photosynthetic complex, c, specifically affecting *petD*; Drager et al.

1998). Recessive mutations in *MCD1* result in the complete destabilization of *petD* mRNA (although it is synthesized normally) and a consequent failure to accumulate SUIV. The use of reporter genes fused to the *petD* 5′ untranslated region (UTR) revealed that it is the 5′ UTR which confers dependence on the *MCD1* gene product for RNA stability (Drager et al. 1998), as is the case for the analogous *nac2-26* mutation, which destabilizes *psbD* mRNA (Nickelsen et al. 1994). Insertion of a poly$(G)_{18}$ sequence, which forms a ribonuclease-resistant structure (Sundquist 1993), into the *petD* 5′ UTR, stabilizes the transcript in a *mcd1-1* mutant background. This suggests that the *MCD1* gene product protects the 5′ end of the *petD* message from degradation by a 5′ → 3′ exoribonuclease, perhaps by physically interacting with the end of the message (Drager et al. 1998). Because the poly(G)-containing messages were invariably untranslatable in a *mcd1* mutant background, it was inferred that *MCD1* might also have a translational role (Drager et al. 1999).

We have undertaken traditional genetic suppressor analysis to identify other factors involved in *petD* expression, targeting those which interact functionally or structurally with *MCD1*. Suppressor analysis has been a useful tool for identifying nuclear genes involved in chloroplast RNA processing (Levy et al. 1997), RNA stability (Nickelsen 2000), and translation (Chen et al. 1993; Wu and Kuchka 1995; Zerges et al. 1997) in *Chlamydomonas*. Here we report the identification and characterization of a nucleus-encoded suppressor of the *mcd1-2* mutation. The suppressor defines a new genetic locus, *MCD2*, whose function in wild-type (WT) cells remains unknown. While the *mcd2-1* suppressor overcomes the *mcd1-2* mutation by permitting *petD* transcript accumulation, it appears to have a disproportionate effect on SUIV synthesis and accumulation, raising the possibility that the suppressor may also have a role in translation. Together with our results for *MCD1*, it can now be speculated that a dual role for nucleus-encoded chloroplast gene regulators may not be exceptional and that they may in fact meld different phases of the gene expression pathway.

Materials and methods

Chlamydomonas strains, growth conditions, and genetic analysis

Strains used in this study are listed in Table 1. Strains F16 and 670, carrying the *mcd1-1* and *mcd1-2* mutant alleles, respectively, have been previously described (Drager et al. 1998). Strain 670R1, carrying the *mcd2-1* mutation, was isolated as a spontaneous phenotypic revertant of strain 670 by plating large numbers of cells on minimal medium (lacking acetate). For RNA and protein isolation, cells were grown in TAP medium (Harris 1989) under constant light (70 μE m^{-2} sec^{-1}). The photosynthetic growth phenotypes of strains were determined by measuring chlorophyll fluorescence transients (Bennoun and Beal 1997) and plating on minimal medium (Harris 1989).

Crosses performed in this study are listed in Table 2. Crosses and dissection of tetrads were performed according to Harris (1989). Diploids were created by generating strains which contained the *mcd1-2* mutation combined with the arginine-requiring *arg2* mutation, and the *mcd1-2, mcd2-1* mutations combined with the thiamine-requiring mutation *thia1*. After gametogenesis and mating, diploids were selected by plating in the light on medium lacking arginine and thiamine.

RNA isolation, filter hybridization, and primer extension

Total RNA was isolated as previously described (Drager et al. 1998). RNA was electrophoresed in 1.2% agarose, 2.2 M formaldehyde denaturing gels, blotted onto Gene Screen membrane (DuPont), and crosslinked by UV irradiation. Hybridization with linear double-stranded DNA probes was carried out as previously described (Drager et al. 1998). RNA accumulation was visualized and quantified using a Storm Scanner PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

5′ end mapping by primer extension was carried out with 10 μg total RNA as previously described (Higgs et al. 1998) using the primer WS5 (Sakamoto et al. 1993). Products were analyzed in a 7% denaturing polyacrylamide gel.

Protein preparation, immunoblotting, and pulse-labeling

Total protein was isolated and analyzed by immunoblotting as previously described (Drager et al. 1998; Higgs et al. 1998). Blots were reacted with an antibody raised against SUIV (Chen et al. 1993) and either an antibody raised against *Chlamydomonas* oxygen-evolving enhancer protein 2 (OEE2; 1:10,000 dilution) or *Chlamydomonas* chloroplast ATPase β-subunit (1:2,000 dilution). Proteins were visualized using enhanced chemiluminescent detec-

Table 1 Strains used in this study. Nuclear genotypes are followed by the chloroplast genotype in brackets. *PS+* Able to grow photosynthetically, *PS–* unable to grow photosynthetically, requires acetate in growth medium

Strain	Genotype[a]	Phenotype	Source
P17	wt, mt+ (wt)	PS+	Stern et al. 1991
F16	*mcd1-1*, mt+ (wt)	PS–	Drager et al. 1998
670	*mcd1-2*, mt+ (wt)	PS–	Drager et al. 1998
670R1	*mcd1-2*, *mcd2-1*, mt+ (wt)	PS+	This study
Sup670	*mcd2-1*, mt+ (wt)	PS+	This study
DG2	wt, mt+ (DG2)	PS+	Sakamoto et al. 1993
670(DG2)	*mcd1-2*, mt+ (DG2)	PS–	This study
ΔpetD	wt, mt+ (ΔpetD)	PS–	Chen et al. 1993
670*arg2*[b]	*mcd1-2*, arg2, mt+ (wt)	PS–	This study
670R1*thia1*[b]	*mcd1-2*, *mcd2-1*, thia1, mt– (wt)	PS+	This study
diploid	$\frac{mcd1\text{-}2}{mcd1\text{-}2}$ $\frac{mcd2\text{-}1}{MCD2}$ $\frac{thia1}{THIA1}$ $\frac{ARG}{arg2}$ (wt)	PS+	This study

[a] DG2, *petD-uidA-rbcL* fusion in chloroplast genome; ΔpetD, *petD* gene deleted; *arg2*, requires arginine in growth medium; *thia1*, requires thiamine in growth medium
[b] Parents for cross to produce diploid

Table 2 Crosses performed in this study. Fluorescence phenotypes: *WT* wild-type, *cyt* cytochrome *b6/f* deficient, *Arg* requiring arginine, *Thia* requiring thiamine. *PD* Parental ditype, *NPD* non-parental ditype, *T* tetratype

Cross no.	Cross (mt+ × mt−)	Phenotypes of tetrads	Type of tetrads	Total tetrads
1	670R1 × 670	2(cyt):2(WT)	12 (PD)	12
2	670R1 × WT	4(WT)	3 (PD)	
		2(WT):2(cyt)	3 (NPD)	
		3(WT):1(cyt)	4 (T)	
3	Sup670 × 670	2(WT):2(cyt)	6 (PD)	12
		4(WT)	2 (NPD)	
		3(WT):1(cyt)	4 (T)	
4	670 × Sup670	2(WT):2(cyt)	7 (NPD)	14
		4(WT)	3 (NPD)	
		3(WT):1(cyt)	4 (T)	
5	670(DG2) × Sup670	2(WT):2(cyt)	2 (PD)	10
		4(WT)	4 (NPD)	
		3(WT):1(cyt)	4 (T)	
6	670R1 × F16	2(WT):2(cyt)	8 (PD)	34
		4(cyt)	11 (NPD)	
		1(WT):3(cyt)	15 (T)	
7	670 × *arg2*	2(cyt):2(Arg)	7 (cyt) from	10
		2(cyt, Arg):2(WT)	10 tetrads	
		1(cyt):1(cyt, Arg):1(Arg):1(WT)		
8	*arg7* × 670	2(cyt):2(Arg)	4 (cyt) from	6
		2(cyt, Arg):2(WT)	6 tetrads	
		1(cyt):1(cyt, Arg):1(Arg):1(WT)		
9	670R1 × 670*arg7*	2(WT):2(cyt, Arg)	(PD)	2
10	670R1 × 670*arg2*	2(WT):2(cyt, Arg)	(PD)	22
11	670*thia1* × 670R1	2(cyt, Thia):2(WT)	2 (PD)	14
		2(cyt):2(Thia)	4 (NPD)	
		1(cyt, Thia):1(cyt):1(Thia):1(WT)	8 (T)	

tion (Durant 1990) and quantified by comparison to a dilution series of WT proteins.

In vivo pulse-labeling of chloroplast proteins was performed as described by Delepelaire (1983) using ^{14}C-acetate in the presence of cycloheximide, which inhibits cytosolic protein synthesis. Proteins were fractionated in a 12–18% gradient of polyacrylamide-urea gels. Labeled proteins were visualized and quantified with a PhosphorImager.

Results

mcd2-1 suppresses the *mcd1-2* mutation

The *C. reinhardtii* nuclear *mcd1-2* mutant (strain 670), characterized by Drager et al. (1998), was generated by UV mutagenesis and had a non-photosynthetic growth phenotype, due to a complete lack of chloroplast *petD* mRNA, resulting from its destabilization. We isolated a spontaneous phenotypic revertant of the non-photosynthetic mating type plus (mt+) strain 670 (*mcd1-2*) which was able to grow on minimal medium. This strain was designated 670R1. To determine whether 670R1 was an intragenic revertant or contained a second-site suppressor mutation in the nuclear or chloroplast genome, it was crossed with a 670 (*mcd1-2*) mating type minus (mt−) strain and a WT mt− strain. In the cross with 670, all tetrads (based on testing by chlorophyll fluorescence induction kinetics) contained two *mcd1-2* mutant progeny and two progeny with the phenotype of 670R1 (Table 2, cross 1). This indicated that the suppressor was in the nuclear rather than chloroplast DNA, since a chloroplast suppressor would have conferred photosynthetic growth and normal chlorophyll fluorescence to all progeny. To determine whether the suppressor mutation was in *MCD1* or in another nuclear gene, 670R1 was crossed to a WT mt− strain (Table 2, cross 2). In this cross, three types of tetrads were found with zero, one, or two *mcd1-2* mutant progeny, which can be interpreted as parental ditype (PD), tetratype (T), and non-parental ditype (NPD) tetrads, respectively. The presence of all three types of tetrads, as well as their relative frequencies (PD = NPD), indicated the segregation of two unlinked nuclear genes, *MCD1* and a new gene which we have called *MCD2*. The *MCD2* mutation in the strain 670R1 is the *mcd2-1* allele. From a NPD tetrad isolated in this last cross, we identified two photosynthetic progeny which were presumed to have the genotype *mcd2-1, MCD1*; i.e. they contained the suppressor gene in an otherwise WT background. To confirm this, we crossed them to strain 670 (*mcd1-2*) (Table 2, crosses 3 and 4). Three types of tetrads were observed with the phenotypes and frequencies predicted from the presumed genotypes (see Table 2). The *mcd2-1, MCD1* strains were named Sup670 and were used in subsequent analyses.

mcd2-1 increases the accumulation of the *petD* transcript in a *mcd1-2* background

To determine the mode of action of the suppressor, we analyzed *petD* mRNA accumulation by filter hybridization. A filter containing total RNA from WT, 670, 670R1, and Sup670 cells was hybridized with probes for *petD* and

psbA (encoding the D1 protein of photosystem II) as loading control. As shown in Fig. 1A, the 0.9-kb *petD* transcript was not detected in 670 (lane 2). In 670R1, the amount of *petD* mRNA was increased to 10% of WT (lane 3). The suppressor mutation in an otherwise WT background, however, had no effect on the accumulation of the *petD* message (lane 4; compare *petD* to *psbA*).

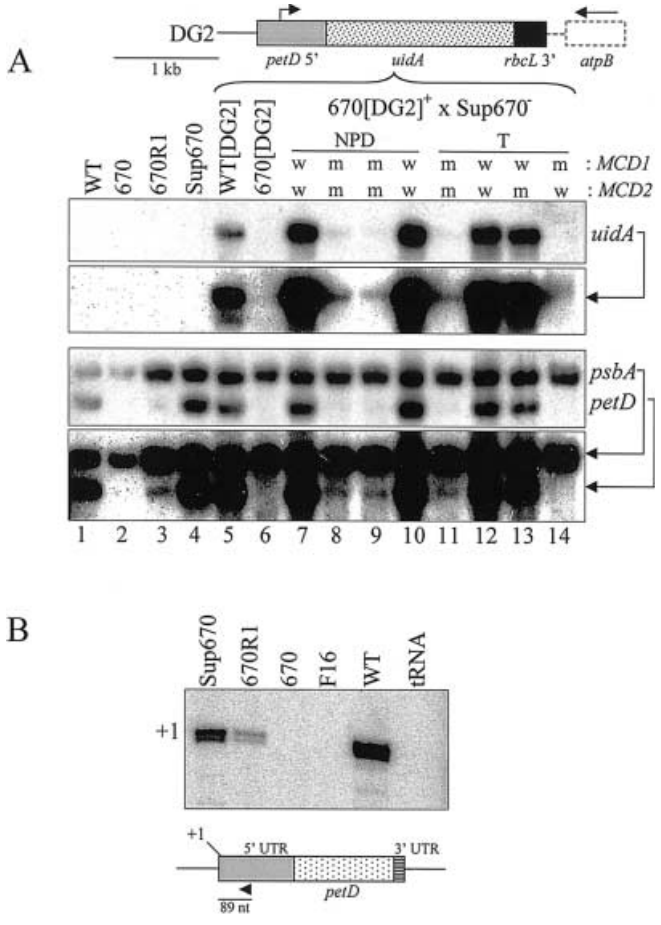

Fig. 1 A, B RNA accumulation and 5′ end mapping of *petD* mRNA in *mcd2-1* backgrounds. **A** RNA filter hybridization analysis of *petD* (0.9 kb) and *uidA* (1.9 kb) transcript accumulation in a non-parental ditype (NPD) and tetratype (T) tetrad of the indicated cross (Table 2, cross 5; see Table 1 for strain descriptions). *psbA* (1.2 kb) was used as a loading control. Short and long exposures of each image are shown. + and − indicate mating type. Wild-type and mutant strains without a chloroplast reporter construct are shown in the *first four lanes*. The inferred *MCD1* and *MCD2* genotypes are designated *w* and *m* for wild type and mutant, respectively, although the genotypes of the progeny in *lanes 12 and 13* cannot be distinguished. All progeny contain the DG2 reporter gene, diagrammed above the gel with the nearby *atpB* gene shown to indicate its site of insertion in the chloroplast genome. *Shaded box petD* 5′ untranslated region (UTR), *stippled box uidA* coding region, *filled box rbcL* 3′ UTR. The *dashed lines* indicate that the intergenic region and *atpB* are not drawn to scale. **B** 5′ end mapping of *petD* mRNA in the indicated strains, using primer extension. Strain *F16* carries the *mcd1-1* mutation. Primer extension products were sized by comparison to a sequence ladder (data not shown). The *arrowhead* below the gel shows the location of the primer used; and the size and extent of the product are indicated by *a horizontal line*. Yeast tRNA was used as a negative control. *Shaded box petD* 5′ UTR, *stippled box* coding region, *horizontally striped box* 3′ UTR

The *petD* 5′ UTR is sufficient to confer RNA instability on chloroplast reporter gene mRNAs in a *mcd1* background (Drager et al. 1998). In order to determine whether the suppressor was acting through the 5′ UTR, a Sup670 strain (*mcd2-1*, mt−) was crossed to a 670 (*mcd1-2*, mt+) strain with the DG2 reporter construct in the chloroplast genome (Table 2, cross 5). The DG2 construct contains the *petD* promoter and 5′ UTR fused to a reporter gene, *uidA*, encoding *β*-glucuronidase (Sakamoto et al. 1993). In the progeny from this cross, the DG2 construct was predicted to be in the four possible nuclear contexts obtained by the segregation of the two unlinked nuclear genes *MCD1* and *MCD2*. The mRNA accumulation in two tetrad types resulting from this cross is shown in Fig. 1A (lanes 7–14), after hybridization with probes for *uidA*, *petD*, and *psbA*. The first tetrad, a recombinant NPD tetrad, had two WT progeny and two double mutant progeny. In the double mutants (lanes 8 and 9), about 10% of the 1.9-kb *uidA* transcript accumulated, mirroring the accumulation of *petD* mRNA. In the second tetrad (a T with two parental and two recombinant progeny) the first progeny is a double mutant (lane 11), like those from the NPD. The second and third progeny (lanes 12 and 13) have WT levels of *uidA* and *petD* mRNAs. One of these progeny is WT, while the other has *mcd2-1* alone (like strain Sup670). The last progeny (lane 14) is *mcd1-2* alone (like strain 670) and has no detectable *petD* or *uidA* mRNAs. From these results, we conclude that the *mcd2-1* suppressor, acting through the *petD* 5′ UTR, increases the accumulation of the *petD* transcript in a *mcd1-2* background from an undetectable level to about 10% of the WT level.

Based on RNA gel blot analysis, the *petD* transcript in the suppressed strains appeared to be the same size as the WT transcript, suggesting it had the same 5′ ends (*petD* mRNA has two 5′ ends that differ by one nucleotide). To verify this, primer extension was used to map the 5′ end of the *petD* message in *mcd2-1*, as shown in Fig 1B. There was no detectable RNA in 670 or in F16, both *mcd1* mutants. Both 670R1 and Sup670 had *petD* mRNA with WT 5′ ends, with the signal being diminished for 670R1, as expected.

mcd2-1 increases the synthesis and accumulation of SUIV in a *mcd1-2* background

To determine the level of SUIV accumulation, we performed immunoblot analysis of total protein, using the nucleus-encoded OEE2 of photosystem II as a loading control. Figure 2A shows that there was no detectable SUIV in 670, nor in a *petD* deletion strain (ΔpetD). In 670R1 however, SUIV increased to about 50% of the WT level (based on the average of several protein preparations and immunoblots). This is a higher level of protein than might be expected from the 10% level of *petD* mRNA (Fig. 1A), suggesting that *mcd2-1* might boost translation as well as RNA stability (see Discus-

sion). The *mcd2-1* mutation alone (Sup670) had no effect on the accumulation of subunit IV.

To determine whether the suppressor increased SUIV synthesis, in vivo chloroplast protein pulse-labeling was performed in the presence of cycloheximide to inhibit cytosolic protein synthesis. Figure 2B shows that SUIV synthesis increased from undetectable in 670 and ΔpetD to 30–50% of the WT level in 670R1. In Sup670, SUIV synthesis was equivalent or slightly higher than in WT cells. In general, synthetic rates and accumulation for SUIV are in agreement, suggesting that any effects of *mcd2-1* were at the translational rather than post-translational level.

mcd2-1 is an allele- and gene-specific suppressor

To determine if *mcd2-1* could suppress another mutation in the *MCD1* gene, we crossed the double mutant 670R1 (*mcd1-2*, *mcd2-1*) to the *mcd1-1* mutant F16 (Table 2, cross 6). Because this cross involved two unlinked nuclear loci, three types of tetrads (PD, NPD, and T) were expected in which the *mcd2-1* mutations were in combination either with *mcd1-1* or *mcd1-2*. These three types of tetrad gave different phenotypes, indicating that *mcd2-1* could suppress only the *mcd1-2* mutation and not *mcd1-1*. Since a weak phenotypic suppression of *mcd1-1* by *mcd2-1* might not be detected by fluorescence tests, we elected to analyze RNA accumulation. While PD tetrads would possess two progeny with a reduced level of *petD* mRNA and two progeny without *petD* mRNA, NPD tetrads would show either a 2:2 segregation of progeny with a reduced level of *petD* and progeny without *petD* mRNA if *mcd2-1* could suppress *mcd1-1*, or a 4:0 segregation of progeny without *petD* mRNA and progeny with a reduced level of *petD* if it could not. By similar reasoning, T tetrads would show a 2:2 segregation of progeny with reduced:null levels of *petD* mRNA or 1:3 reduced:null levels of *petD* mRNA, respectively.

RNA accumulation from representative NPD and T tetrads is shown in Fig. 3 and is consistent with the photosynthetic phenotypes of these progeny. The NPD progeny all lack the *petD* transcript, indicating that *mcd1-1* is not suppressed by *mcd2-1*. One progeny of the T tetrad contains *petD* mRNA, suggesting it has the parental *mcd1-2*, *mcd2-1* genotype, whereas the other three completely lack the *petD* message. We conclude that *mcd2-1* can suppress *mcd1-2*, but not *mcd1-1*; and it is therefore allele-specific. This, taken with the fact that no suppressors or revertants have been isolated for *mcd1-1*, suggests that it may be a null allele, whereas *mcd1-2* may produce a non-functional gene product.

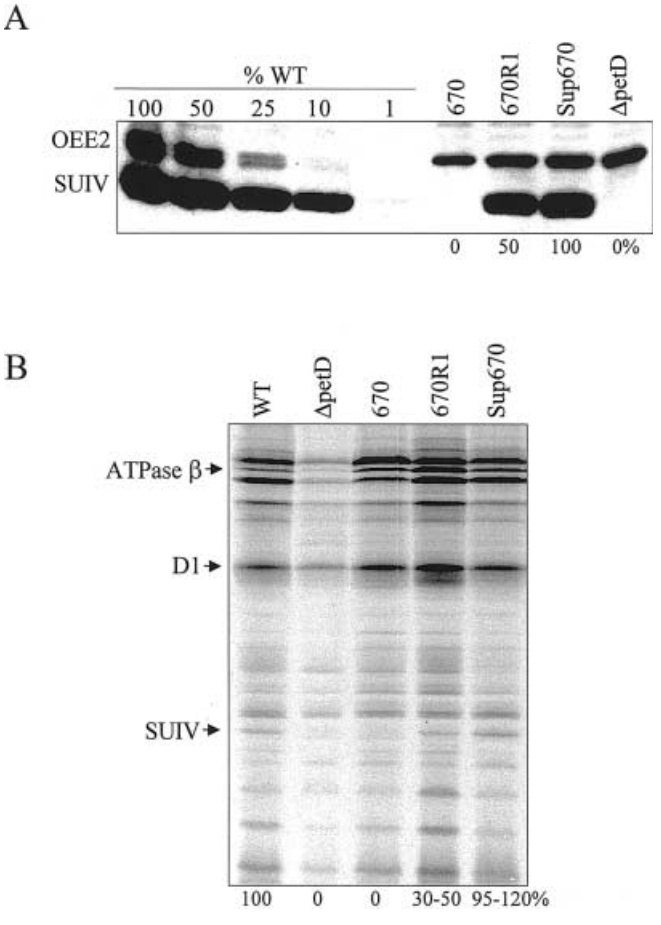

Fig. 2 A, B Protein accumulation and synthesis in a *mcd2-1* background. **A** Immunoblot analysis was used to measure the accumulation of subunit IV (*SUIV*; 17 kDa) in the indicated strains, with the oxygen-evolving enhancer 2 (*OEE2*) protein (23 kDa) as a loading control. Quantification was based on the average of several immunoblots. **B** Pulse-labeling (5 min) of chloroplast proteins with ^{14}C-acetate. The positions of *SUIV*, *ATPase β*, and *D1* are indicated. Quantification of SUIV synthesis, *shown as a percentage at the bottom*, was based on the average of several gels, using ATPase *β* and D1 as normalizing bands

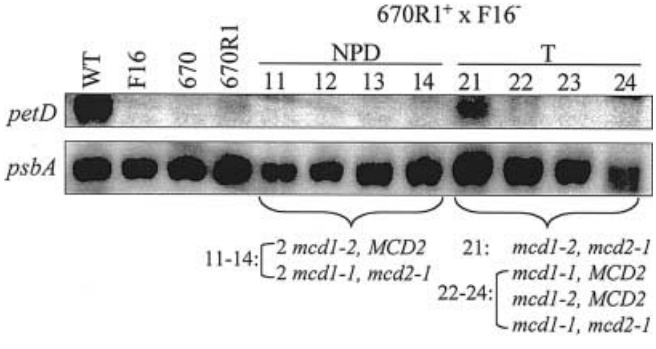

Fig. 3 RNA accumulation in the progeny of a cross to determine whether *mcd2–1* suppresses another allele of *MCD1*. Filter hybridization analysis was performed on RNA from two tetrads of the indicated cross (Table 2, cross 6), with *psbA* used as a loading control. + and − indicate mating type. In the first tetrad, a NPD, all four progeny lack the *petD* message. The genotypes of individual progeny were not determined, but are listed below the gel. In the second tetrad, a T, *lane 21* contains the level of *petD* mRNA expected for a *mcd1-2*, mcd2-1 strain. The other three progeny do not contain the *petD* transcript. Their individual genotypes were not determined but are listed below the gel. The *shadows* present at the top of the *petD* box (0.9 kb) in *lanes 11–14* and *lanes 22–24* are from an overexposed *psbA* band (1.2 kb)

It was evident from pulse labeling (Fig. 2B) that *mcd2-1* greatly increased SUIV translation, but it could not be unequivocally ascertained from these data that the increase was specific to *petD*, rather than a general or pleiotropic increase in chloroplast translation. To address this issue, Sup670 (*mcd2-1*) was crossed as a mt− parent, both to several chloroplast mutants affected at the level of translation initiation or RNA stability and to a nuclear translation mutant. Among the chloroplast mutants were iniD2, in which the *petD* initiation codon was changed from AUG to AUU, reducing SUIV translation to 20% of the WT rate (Chen et al. 1993), an *atpB* AUG to AUU initiation codon mutant (Rimbault et al. 2000), FUD6, which contains a deletion of the *petD* promoter and part of the 5′ UTR (Sturm et al. 1994), and LS2 and LS6, which contain linker-scanning mutations in element I of the *petD* 5′ UTR. Element I is the presumed binding site for *MCD1*: and these linker-scanning mutations both decrease RNA stability and abolish translation (Higgs et al. 1999). Finally, *mcd2-1* was crossed to strain FuD34, which has a *psbC* 5′ UTR mutation which prevents translation (Rochaix et al. 1989). When tetrad progeny from each of these crosses were analyzed, no increase in protein accumulation for the mutated chloroplast gene was observed (data not shown). This showed that *mcd2-1* cannot overcome weak initiation codons, nor can it compensate for the lack of a *MCD1* binding site.

Sup670 (*mcd2-1*) was also crossed to strain F34, which contained a mutation in the nuclear *TBC1* gene required for the translation of the photosystem II protein P6, encoded by the chloroplast *psbC* gene. No effect on translation attributable to *mcd2* was observed in any tetrads of Sup670 × F34 (data not shown), although another nuclear suppressor, *tbc3-rb1*, has been characterized that can suppress F34 (Zerges et al. 1997). In summary, the *mcd2-1* mutation does not simply increase translation of *petD* or other chloroplast messages.

mcd2-1 is semidominant

To test the dominance of *mcd2-1*, diploids homozygous for *mcd1-2* but heterozygous for *mcd2-1* were generated. To do this, 670 (*mcd1-2*) was crossed to an *arg2* strain, defective in arginosuccinate lyase, to obtain progeny which contained both mutations and thus required both acetate and arginine for growth (Table 2, cross 7). To generate the other parent, 670 was first crossed to an *arg7* strain (Table 2, cross 8) to create a 670*arg7* strain which was then crossed to 670R1 (*mcd1-2*, mcd2-1) to obtain an arginine-requiring triple mutant strain for complementation (Table 2, cross 9; *arg2* and *arg7* are two complementing mutant alleles of the *ARG7* locus; Matagne 1978). Interestingly, of the *mcd2-1* mutant progeny examined, none were recombinant *mcd2-1*, arg7 progeny This was also true for a cross to 670*arg2* (Table 2, cross 10), indicating that *MCD2* is actually linked to the *ARG* locus. Therefore, 670R1 was instead crossed to the *thia1* thiamine-requiring mutant (Table 2, cross 11), generating the triple mutant 670R1*thia1* (*mcd1-2*, *mcd2-1*, *thia1*). Gametes from this strain were then mated to gametes from the 670*arg2* strain and diploids were selected by their ability to grow without added arginine or thiamine. These cells also had the minus mating-type and enlarged cell size, characteristic of vegetative diploids (data not shown).

Photosynthetic electron transport in diploids was tested by fluorescence transients (data not shown). Based on the fluorescence data, the phenotype of the heterozygous diploid appeared to be intermediate between 670R1 and 670. Moreover, the rate of colony formation on minimal medium also suggested that the diploids do not grow as well as the haploid 670R1 parent, although they are photosynthetic (data not shown).

To test the molecular phenotypes of the heterozygous diploid, RNA and protein accumulation were determined. Figure 4A shows filter hybridization of total RNA from WT, haploid parents, and a representative diploid with gene-specific probes for *petD* and *atpB* (encoding the β subunit of chloroplast ATPase) as a loading control. No *petD* mRNA was detected in the original 670 strain or the 670*arg2* parent. The amounts of *petD* mRNA in 670R1, the 670R1*thia1* parent, and the heterozygous diploid were equivalent at approximately 10% of the WT level, which cannot account for

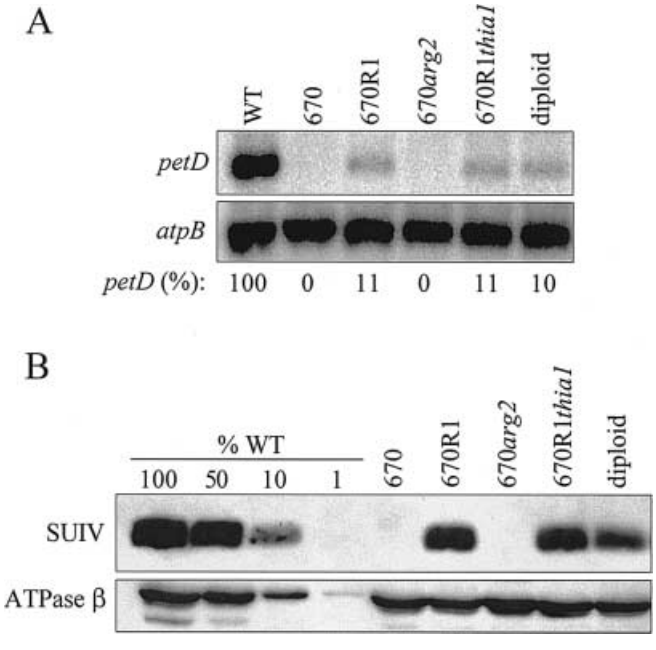

Fig. 4 A, B RNA and protein accumulation in *mcd2-1* heterozygous diploids. **A** RNA filter hybridization analysis was used to determine the accumulation of *petD* mRNA in the strains shown, including one representative diploid. *atpB* mRNA (1.9 kb) was used as a loading control. The accumulation of *petD* mRNA, relative to wild type, was determined using a PhosphorImager. **B** Immunoblot analysis was used to measure the accumulation of *SUIV* (17 kDa) in the indicated strains, with the *ATPase β* subunit (55 kDa) as a loading control. A dilution series of wild-type proteins was used to estimate the amounts of SUIV

the difference in their photosynthetic growth rates. Figure 4B shows an immunoblot reacted with antibodies against SUIV and the chloroplast ATPase β subunit as a loading control. Based on a dilution series of WT proteins, the heterozygous diploid accumulated about 20% of the WT level of SUIV, down from 50% in the original suppressor. This two-fold decrease in SUIV accumulation could account for the decreased photosynthetic electron transport and growth phenotype. Thus *mcd2-1* appears to be semidominant in terms of SUIV accumulation, but completely dominant in terms of *petD* mRNA accumulation.

Discussion

Suppressor analysis has been a useful tool for identifying genes involved in a particular biological process. We have used suppressor analysis to identify a nucleus-encoded factor, Mcd2, involved in the expression of the chloroplast *petD* gene in *C. reinhardtii*. In the current model of *petD* gene expression, the *MCD1* gene product physically interacts with the 5′ end of the *petD* 5′ UTR to prevent degradation by a $5' \rightarrow 3'$ exoribonuclease (Drager et al. 1998) and possibly promotes translation. In the absence of Mcd1, the *petD* transcript is rapidly degraded. The *mcd2-1* mutation allows at least one allele of *MCD1*, *mcd1-2*, to partially function by increasing *petD* mRNA accumulation to about 10% of the WT level. This amount of mRNA leads to restoration of SUIV synthesis, permitting photosynthesis. Through the use of a reporter construct containing the *petD* 5′ UTR, we have shown that, like *MCD1*, *MCD2* acts through the 5′ UTR.

In addition to stabilizing *petD* mRNA, *mcd2-1* may increase the translational efficiency of the *petD* message; the synthesis and accumulation of SUIV (50% of WT) is higher than might be expected for the level of *petD* mRNA (10% of WT) in the suppressed strain. However, it is also possible that only 20% of the *petD* mRNA is required to synthesize 100% of SUIV: some other *Chlamydomonas* chloroplast mRNAs can be reduced by 50–70% following growth in the chloroplast DNA synthesis inhibitor 5-fluorodeoxyuridine, without a discernable effect on protein synthesis rates (Hosler et al. 1989).

It would not be surprising if *MCD1* and *MCD2* were involved in both mRNA stability and translation, as the two processes are interdependent. Other factors which play a role in both RNA stability and translation have been previously identified. The Nac2 protein, which is required for *psbD* message stability, may also be involved in translation because *psbD* mRNA stabilized by a poly(G) tract is not translated in the absence of Nac2 (Nickelsen et al. 1999). The nucleus-encoded F35 mutant in *Chlamydomonas* has a primary defect in the translation of the chloroplast *psbA* mRNA, but also destabilizes the transcript due to reduced ribosome association (Yohn et al. 1996). In yeast, the nuclear *PET127* locus was originally identified as a suppressor of a C-terminal truncation of the nucleus-encoded *PET122* translational activator of the mitochondrial *COX3* gene (Haffter and Fox 1992). *PET127* was subsequently identified as a suppressor of mutations in the 5′ UTRs of both *COX3* (Wiesenberger and Fox 1997) and *COB* (Chen et al. 1999) which destabilize the transcript, suggesting a dual role for the protein. To determine whether *mcd2-1* increased translation of *petD* or chloroplast mRNAs in general, which in turn would lead to increased message stability through ribosome association, we crossed Sup670 to several chloroplast and nuclear mutants which primarily affect translation of *petD* or other chloroplast transcripts. No increase in translation was observed, suggesting the suppressor does not act simply by increasing *petD* translation or chloroplast translation in general.

Further evidence of a role for *MCD2* in translation comes from the phenotype of the heterozygous diploid: *mcd2-1* is semidominant with respect to SUIV accumulation. The photosynthetic growth phenotype and fluorescence transience of a heterozygous diploid in a homozygous *mcd1-2* background is intermediate between the unsuppressed 670 strain (*mcd1-2* mutation) and the haploid 670R1 (*mcd1-2*, *mcd2-1*) strain. While the *petD* mRNA levels in the haploid and heterozygous diploid are equivalent, the level of subunit IV in the diploid is about half of that in the haploid suppressor strain, again suggesting a potential role in translation.

Several nucleus-encoded factors required for the stability of a specific chloroplast mRNA have been identified genetically in *Chlamydomonas* (Drager et al. 1998; Drapier et al. 1992; Gumpel et al. 1995; Kuchka et al. 1989; Monod et al. 1992; Sieburth et al. 1991). Only for *psbD* have multiple factors been previously implicated in message stability (Nickelsen 2000). Conversely, multiple nucleus-encoded factors required for the splicing (Goldschmidt-Clermont et al. 1990) and translation of a specific chloroplast transcript have been characterized (Kuchka et al. 1988; Zerges et al. 1997). This disparity may reflect either the number of gene products involved in each process or simply that exhaustive mutagenesis has not been performed. Looking at the analogous system of yeast mitochondrial gene expression suggests that the latter possibility may be the case. Many nucleus-encoded factors have been identified which are involved in the stability and, in some cases, processing of the *COB* mRNA (Chen et al. 1999; Dieckmann and Mittelmeier 1987; Staples and Dieckmann 1994; Wallis et al. 1994). For example, the nucleus-encoded *CBP1* gene is required for the stability and processing of mitochondrial *COB* mRNA (Dieckmann and Mittelmeier 1987). The nuclear gene *SOC1* was identified as a suppressor of *COB* RNA instability due to mutations in either *CBP1* (Staples and Dieckmann 1994) or the *COB* 5′ UTR (Chen et al. 1999); and it is believed to encode a nuclease involved in mitochondrial mRNA turnover. *mcd2-1* is unlikely to be a mutation in a nuclease which degrades *petD* mRNA, because it is not recessive, as a loss-of-function mutation of that sort would be. Moreover,

mcd2-1 is allele-specific, suggesting it neither eliminates the need for Mcd1 nor functions in its place. *mcd2-1* may restore partial function to the *mcd1-2* gene product, but it cannot compensate for the *mcd1-1* defect.

In one model which could explain the phenotype of *mcd2-1*, Mcd1 and Mcd2 interact with each other and with the *petD* transcript. The *mcd1-2* mutation prevents the interaction with Mcd2 and the *petD* message. The *mcd2-1* mutation restores the protein–protein interaction and binding to the transcript. Mcd2 may interact with another translational activator as well as with Mcd1. This activator could be transcript-specific, or it could be part of the general translation machinery. WT Mcd2 may out-compete the suppressor form of the protein and sequester the activator, preventing it from interacting with the *petD* mRNA and lowering translation in the heterozygous diploid.

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