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A dominant nuclear mutation in *Chlamydomonas* identifies a factor controlling chloroplast mRNA stability by acting on the coding region of the *atpA* transcript

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Summary

We have characterized a nuclear mutation, *mda1*-ncc1, that affects mRNA stability for the *atpA* gene cluster in the chloroplast of *Chlamydomona*s. Unlike all nuclear mutations altering chloroplast gene expression described to date, *mda1*-ncc1 is a dominant mutation that still allows accumulation of detectable amounts of *atpA* mRNAs. At variance with the subset of these mutations that affect mRNA stability through the 5′ UTR of a single chloroplast transcript, the mutated version of MDA1 acts on the coding region of the *atpA* message. We discuss the action of MDA1 in relation to the unusual pattern of expression of *atpA* that associates particularly short lived-transcripts with a very high translational efficiency.

Keywords: mRNA stability, *atpA* gene, chloroplast gene expression, nuclear control, post-transcriptional regulation, *Chlamydomonas*

Introduction

The expression of chloroplast genes coding for photosynthetic proteins is controlled by nuclear factors, the vast majority of which act post-transcriptionally. This nucleo-chloroplastic interaction has been studied extensively in the unicellular green alga *Chlamydomonas reinhardtii*, as well as in higher plants (for recent reviews, see Barkan and Goldschmidt-Clermont, 2000; Monde *et al.*, 2000; Zerges, 2000). *Chlamydomonas* combines several advantages for such studies. It can grow heterotrophically, thereby allowing the isolation of photosynthetic mutants; it is particularly suitable for classical genetic approaches (Harris, 1989) and its chloroplast genome is easily transformable (Boynton *et al.*, 1988).

In the past decade, the importance of the 5′ end of chloroplast mRNAs as targets for this nuclear control has been recognized: the targets are *cis*-acting elements essential for the maturation, nucleolytic resistance and translation of chloroplast mRNAs. In several instances, it was demonstrated that a 5′ end maturation process is a pre-requisite for the stable accumulation and translation of chloroplast mRNAs, and that specific 5′ UTR-directed nuclear factors play an essential role in this post-transcriptional regulation (Bruick and Mayfield, 1998; Drager *et al.*, 1998; Nickelsen *et al.*, 1994; Vaistij *et al.*, 2000a). The characterization of the nuclear genes involved in these processes is still in its infancy. A few genes have been cloned, among which one is involved in the stability of *psbB* (Vaistij *et al.*, 2000b) and one in the stability of *psbD* (Boudreau *et al.*, 2000) The corresponding protein sequences and other data show an involvement not only in mRNA-protein interactions, but also in protein-protein interactions.

The 3′ UTR of chloroplast transcripts also contributes to their stabilization through the presence of stem-loop structures. This has been shown for instance for *atpB* (Stern *et al.*, 1991) and *psaB* (Lee *et al.*, 1996). The stem-loop structure blocks further trimming by a 3′-5′ exonuclease that acts after an endonucleolytic cut has primed the 3′ end maturation process (Stern and Kindle, 1993). A similar mechanism operates in spinach chloroplasts, where processing of the 3′ end of the *petD* mRNA has been studied in great detail (Hayes *et al.*, 1996; Stern and Gruissem, 1987; Stern *et al.*, 1989). However, with the exception of the nuclear mutation *crp3* that affects 3′ end

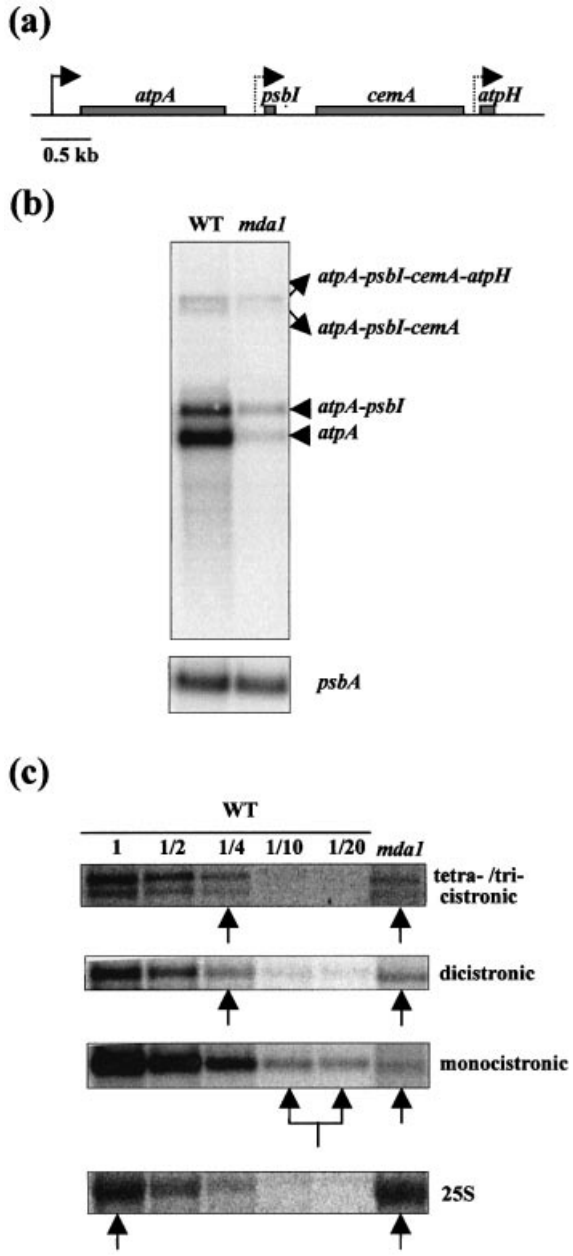

Figure 1. Accumulation of *atpA* transcripts in wild-type and *mda1*-ncc1 mutant cells.
(a) Map of the *atpA* transcription unit. The three promoters of the unit are indicated by bent arrows, with dashed lines used for promoters that are not implicated in the transcription of *atpA*. The coding regions of the genes are represented as grey boxes.
(b) RNA filter hybridization analysis of wild-type and *mda1*-ncc1. Filters were hybridized first with an intragenic *atpA* probe then with a *psbA* probe as a loading control.
(c) Dilution series of total RNA from wild-type allowing a quantitative determination of the accumulation of each *atpA* transcript species for the *mda1*-ncc1 mutant. After hybridization with an intragenic *atpA* probe, the filter was re-hybridized with 25S rRNA as a loading control.

formation for several mRNAs (Levy *et al.*, 1999), no genetic evidence for the existence of specific nuclear factors acting on a 3′ UTR has been reported. Biochemical studies have identified proteins that bind to the 3′ end in the case of the unprocessed *petD* transcript in spinach (Chen *et al.*, 1995; Hayes *et al.*, 1996; Yang *et al.*, 1996), of *psbA* in barley (Memon *et al.*, 1996) and of several unrelated mRNAs in mustard (Liere and Link, 1997; Nickelsen and Link, 1991, 1993). Several of these proteins also show endoribonuclease activity (Hayes *et al.*, 1996; Nickelsen and Link, 1993; Yang *et al.*, 1996), and thus may form part of a multiprotein RNA processing complex.

While the 5′ and 3′ untranslated regions have been frequently documented as controlling RNA stability, there have been no reports of *cis*-acting elements located in the coding regions of chloroplast genes that play such a role. On the other hand, such elements do exist in prokaryotes (Kulkarni and Golden, 1997), animal and fungal cells. For example, β-tubulin mRNA stability is controlled by co-translational recognition of an amino-terminal peptide (Bachurski *et al.*, 1994), and a surveillance complex recognizes and degrades mRNAs which contain nonsense codons (reviewed in Czaplinski *et al.*, 1999). However, these studies have not defined RNA-based stability or instability determinants within coding regions, but have rather shown that changes in the RNA lifetime are a secondary effect of other cellular regulatory phenomena.

In a previous study, we described three different nuclear mutants of *C. reinhardtii* affected in the expression of the *atpA* and *atpB* genes (Drapier *et al.*, 1992). The two genes code for the α and β subunits of the chloroplast ATP synthase. One of these mutants, then named ncc1, showed unaltered rates of transcription of the *atpA* gene but displayed a decrease in the accumulation of the monocistronic *atpA* transcript; *atpA* is the first gene in the cluster *atpA-psbI-cemA-atpH* which is transcribed into four *atpA*-containing mRNA species (Drapier *et al.*, 1998). In ncc1, *atpA* mRNA was still detectable by RNA filter hybridization analysis and its translation remained efficient enough for the ATP synthase complex to accumulate in the thylakoid membranes. These characteristics contrast with other *Chlamydomonas* nuclear mutants in which the affected chloroplast mRNA was undetectable, whether the target was *atpB* (Drapier *et al.*, 1992), *psbD* (Kuchka *et al.*, 1989), *petD* (Drager *et al.*, 1998), *psbB* (Jensen *et al.*, 1986; Monod *et al.*, 1992) or *psbC* (Sieburth *et al.*, 1991).

That the ncc1 mutation might have distinct characteristics from those identified previously could be predicted from the fact that it arose as a spontaneous mutation that did not alter photosynthesis. Therefore it was not identified through regular screening procedures that allow detection of mutants showing functional alterations (reviewed in Simpson and Stern, 2001). Indeed, S1 nuclease protection experiments strongly suggested that the destabilization of the *atpA* mRNA would not occur through its 5′ end (Drapier *et al.*, 1992), in contrast to the

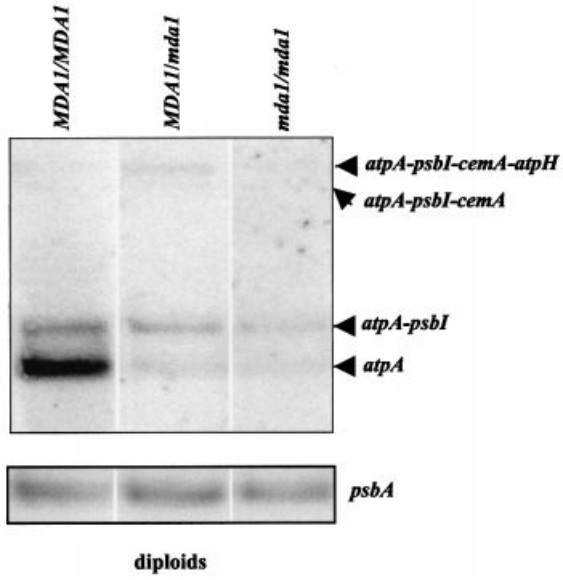

Figure 2. Accumulation of *atpA* transcripts in heterozygous vegetative diploids *MDA*1/*mda*1, relative to that in homozygous vegetative diploids *MDA*1/*MDA1* or *mda*1/*mda*1. Experimental conditions are as described for Figure 1b.

mode of action of most other nuclear-encoded stabilizing factors (Nickelsen, 1998). Here we further characterize mutant *mda1*-ncc1 (*m* for maturation of the mRNA, *d* for ATP synthase complex, *a* for *atpA*) and show that it harbours a dominant mutation that destabilizes the *atpA* transcript through a target located in its coding region.

Results

The accumulation of the four atpA*-containing transcripts is reduced in* mda1*-ncc1*

The *atpA* gene is the first gene of a transcription unit that comprises four cistrons, *atpA-psbI-cemA-atpH*, in the chloroplast genome of *Chlamydomonas* (Figure 1a). Among the eight transcripts generated from this gene cluster, four contain the *atpA* sequence (Drapier *et al.*, 1998). They are shown in Figure 1b by RNA filter hybridization with an intragenic *atpA* DNA fragment. It should be noted that the low levels of the tri- and tetracistronic *atpA* transcripts, each representing < 1% of the total *atpA* transcripts, precluded their detection in some experiments. The major phenotypic feature of the *mda1*-ncc1 nuclear mutant is the reduced accumulation of the monocistronic *atpA* transcript (Drapier *et al.*, 1992), which can be readily seen in Figure 1b. In order to characterize more precisely the respective levels of *atpA* mRNAs in *mda1*-ncc1, we performed a dilution series of total RNA from a wild-type strain. This experiment provided evidence for a decreased accumulation of all forms of *atpA* transcripts in *mda1*-ncc1, albeit to very different extents (Figure 1c): it was by far more drastic for the monocistronic transcript, a 10- to 20-fold decrease, than for the polycistronic transcripts that displayed only a 4-fold reduction. The amounts of the other transcripts from the *atpA-psbI-cemA-atpH* transcription unit were not modified (data not shown). Since the *atpA* transcription rate is not altered in *mda1*-ncc1 (Drapier *et al.*, 1992), we conclude that overall, the effects seen here are due to reduced RNA stability. However, altered processing efficiency of the longer transcripts into shorter ones may also play a role.

Mda1-*ncc1 is a dominant mutation*

Vegetative diploids (3 species: *MDA1/MDA1, MDA1*/*mda1* and *mda1/mda1*) were prepared according to the usual complementation procedure between *arg2* and *arg7* mutations (for details, see Experimental procedures). Total RNA was extracted from the three types of cells and the *atpA* transcript profiles were determined by RNA filter hybridization with an intragenic *atpA* DNA fragment. The accumulation of *atpA* transcripts in the heterozygous *MDA1*/*mda1* diploid, where both the wild-type *MDA1* and mutant *mda1* loci were present, was reduced to the same extent as in the homozygous *mda1*/*mda1* diploid (Figure 2). The presence of a wild-type copy of the *MDA1* gene, i.e. the presence of the wild-type nuclear gene product MDA1 in the heterozygous diploid cells, did not allow restoration, even partially, of the wild-type amount of *atpA* transcripts, except possibly for the low abundance tetracistronic transcript. After quantification we estimated a 10- to 20-fold decrease of the monocistronic transcript, similar to what is found in haploid *mda1* mutant cells. Thus we conclude that *mda1*-ncc1 harbours a dominant mutation that affects the half-life of *atpA* mRNA. Consequently, the *mda1*-ncc1 allele is unlikely to be a null (loss-of-function) allele, and instead probably expresses a mutated version of the MDA1 protein product that acts on the *atpA* mRNAs (see Discussion).

The 5′ UTR of the atpA *gene is not the target for the mutated nuclear factor* MDA1

As a number of nuclear mutants of *C. reinhardtii* display a specific destabilization of chloroplast transcripts through their 5′ UTRs, we wondered if this was also the case with *mda1*-ncc1. We first used a strain whose chloroplast carried, besides the original *atpA* gene, a chimeric *petA* gene where the 5′ UTR and the first 75 bp of the coding sequence are derived from *atpA* (Figure 3a). The resulting strain, which was named transformant AFFF (Choquet *et al.*, 1998), expresses the *petA* product, cytochrome *f*, from a chimeric mRNA of larger size than the original *petA* transcript (compare lanes AFFF and *mda1* in Figure 3b).

In *Chlamydomonas* crosses, the chloroplast genome is uniparentally transmitted by the mt^+ parent to tetrad progeny, whereas a nuclear mutation like *mda1*-ncc1 exhibits Mendelian segregation and is transmitted only to half of the progeny. Therefore, we crossed transformant AFFF, mt^+ with *mda1*-ncc1, mt^-, in order to determine whether the tetrad products showed a 2 : 2 segregation in the amount of the AFFF mRNA, as was expected for the resident *atpA* transcripts. A filter blot containing RNA from a representative tetrad was hybridized with a mixture of an intragenic *petA* probe and an intragenic *atpA* probe to detect *petA*, *AFFF* and *atpA* mRNAs (Figure 3b). Members 3 and 4 of the tetrad bore the *mda1*-ncc1 nuclear mutation,

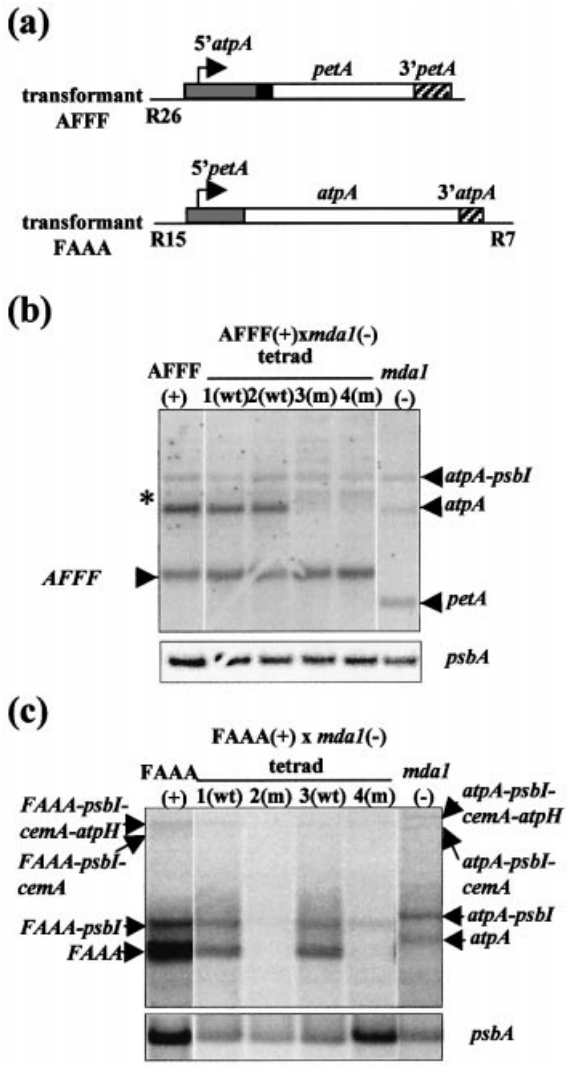

Figure 3. Accumulation of *atpA* transcripts in transformant AFFF or FAAA, in a wild-type or *mda1*-ncc1 nuclear context.
(a) Map of two constructs aimed at investigating the participation of the *atpA* 5′ UTR in the *mda1*-ncc1 phenotype. Transformant AFFF: the 5′ UTR of *petA* was replaced by that of the *atpA* gene (grey box with a bent arrow on the promoter site); note that 25 codons of the *atpA* coding sequence remain in the construct (black box). Transformant FAAA: the 5′ UTR of *atpA* was replaced by that of *petA*. R26 and R15 are the *Eco*RI restriction fragments, named after Rochaix (1978), where are located the chimeric genes.
(b) RNA filter hybridization analysis of a representative tetrad from an AFFF × *mda1*-ncc1 cross. Combined probes for *atpA* and *petA* intragenic fragments. Polycistronic *atpA* transcripts are poorly visible because of the numerous minor *petA* transcripts, the most abundant migrating between the mono- and dicistronic *atpA* transcripts (marked by an asterisk). A *psbA* probe was used as a loading control. The inferred genotype at the *mda1* locus is given for the tetrad progeny (wt = *MDA*1; m = *mda1* mutant).
(c) RNA filter hybridization analysis for a representative tetrad from an FAAA × *mda1*-ncc1 cross. Same experimental conditions as in Figure 1b and same tetrad labelling as in panel b.

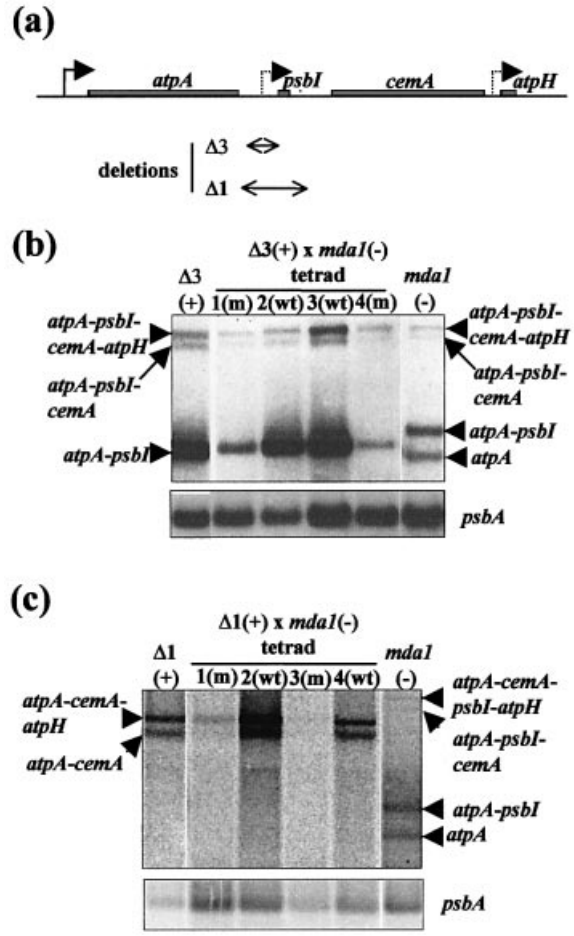

Figure 4. Accumulation of *atpA* transcripts in transformants lacking the *atpA*-3′ UTR in wild type and *mda1*-ncc1 nuclear contexts.
(a) Position of Δ1 and Δ3 deletions are indicated on a map of the *atpA* gene cluster (same representation of the transcription unit than for Figure 1a); Δ3 eliminates the *atpA-psbI* intergenic region; Δ1 eliminates the intergenic *atpA-psbI* region as well as the whole *psbI* gene and part of the *psbI-cemA* intergenic region.
(b) RNA filter hybridization analysis for a representative tetrad from a Δ3 × *mda1*-ncc1 cross. Similar experimental conditions as in Figure 1b; tetrad labelling is the same as for Figure 3b.
(c) Transformant Δ1 was crossed with *mda1*-ncc1; RNA filter hybridization analysis for a representative tetrad is shown. Experimental conditions were as those described for Figure 1b; tetrad labelling is described in the legend to Figure 3b.

since they showed a substantial decrease of monocistronic *atpA* mRNA. However, their content in chimeric *AFFF* mRNA was similar to that of the wild-type members 1 and 2 of the tetrad. We conclude that the chimeric *AFFF* mRNA does not contain the *cis*-elements involved in the *mda1*-ncc1-mediated destabilization of *atpA* transcripts.

Although the 5′ UTR of *atpA* was not sufficient to confer sensitivity of a chimeric transcript to the *mda1*-ncc1 mutation, it could still harbour some of the motifs involved in the destabilization process. If such was the case, a chimeric *atpA* transcript with a 5′ UTR distinct from the original *atpA* 5′ UTR should no longer be sensitive to the *mda1*-ncc1 mutation. To assess this hypothesis, we used transformant FAAA, in which the resident *atpA* gene was replaced by a chimeric version of the gene corresponding to a fusion of the 5′ UTR of *petA* with the coding region and 3′ UTR of *atpA (*see Figure 3a). After crossing FAAA, mt^+ with *mda1*-ncc1, mt^-, we examined the abundance of *atpA* transcripts in the resulting tetrads. As shown in Figure 3c, we observed a 2 : 2 segregation in abundance of the *FAAA* transcript (in this representative tetrad, members 2 and 4 are mutant and members 1 and 3 are wild-type). Since the chimeric transcript was still sensitive to the *mda1*-ncc1 mutation, we concluded that the 5′ UTR of *atpA* was not required for the destabilization of *atpA* mRNA in a *mda1*-ncc1 context.

The 3′ UTR of the atpA *gene is not the target of the mutated nuclear factor MDA1*

In an earlier study we had suggested that *atpA* transcripts in the *mda1*-ncc1 mutant are principally degraded from their 3′ ends, based on comparative S1 nuclease protection experiments (Drapier *et al.*, 1992). To test this hypothesis, we crossed *mda1*-ncc1, mt^- with the transformant Δ3, mt^+ Δ3 carries a deletion of most of the *atpA* 3′ UTR, from 62 bp to 375 bp downstream of the translation stop codon (see Figure 4a). If the target of the mutated nuclear factor MDA1 was in this deleted region, the four members of the tetrad should have the same *atpA* transcript profiles as the Δ3 parent, by virtue of their insensitivity to the presence or absence of the *mda1*-ncc1 mutation. Results for a representative tetrad are shown in Figure 4b. As previously shown (Drapier *et al.*, 1998), only polycistronic *atpA* transcripts accumulate in transformant Δ3, since the processing site for monocistronic *atpA* mRNA is located within the deleted region. We observed a 2 : 2 segregation in the abundance of *atpA* transcripts, with the mutant members 1 and 4 showing a markedly reduced accumulation of *atpA* mRNA as compared to the wild-type members 2 and 3. Thus, the target for the mutated MDA1 factor is not located in the 3′ UTR of the *atpA* monocistronic transcript. We note that the dicistronic *atpA* transcript in Δ3 (i.e. the smallest *atp*A-containing mRNA in this strain) showed a much larger accumulation than the regular dicistronic transcript expressed in the presence of monocistronic *atpA (*strain *mda1*), but also exhibited a much larger relative decrease in the *mda1*-ncc1 context.

We then performed a similar analysis with transformant Δ1, mt^+ which harbours a longer 3′ end deletion, beginning 7 bp downstream of the translation stop codon and extending 766 bp to a *Hin*dIII site in the *psbI-cemA* intergenic region (Figure 4a). In this strain, the 3′ end processing sites of both *atpA* and *psbI* are deleted and thus, the only *atpA* transcripts present are the tri- and tetracistronic forms (see lane Δ1(+) in Figure 4c. Although their accumulation is much greater in Δ1 than in wild-type cells, the total content of *atpA* mRNA, taking into account all *atp*A-containing transcripts, showed a 10-fold reduction. In Δ1, $mt^+ \times$ *mda1*-ncc1, mt^- crosses, we observed a 2 : 2 segregation in the abundance of the tri- and tetracistronic transcripts (Figure 4c). They were hardly detectable in members 1 and 3 of the tetrad shown, but accumulated as in the Δ1 parent as well as in tetrad members 2 and 4. After quantification, we deduced that the sum of the tri- and tetracistronic transcripts was 15-fold lower in Δ1-*mda1* progeny. This reduction was comparable to that of the monocistronic *atpA* transcript in *mda1*-ncc1 cells carrying a wild-type chloroplast (Figure 1c). Taken together, these deletion studies led us to the conclusion that the 3′ UTR of *atpA* does not contain the *cis*-acting targets for the mutated factor MDA1.

The target of the mutated nuclear factor MDA1 is located in the last 1360 bp of the atpA *coding sequence*

The experiments presented above strongly suggested the surprising conclusion that the *atpA* coding region is the target for the destabilizing action of the mutated MDA1 factor. To test this directly, we assembled another chimeric *atpA* gene in which its 5′ and 3′ UTRs were both replaced by their counterparts from the *petA* gene (Figure 5a); this yielded transformant FAFA. As shown in Figure 5b, only three *atpA*-containing transcripts are present in the transformant, since the 3′ end processing site for *atpA* had been deleted and replaced by the single site derived from *petA*. We crossed transformant FAFA, mt^+ with the *mda1*-ncc1, mt^- and analysed the resulting tetrads for the presence of *atpA*-containing transcripts, as shown in Figure 5b. A 2 : 2 segregation was observed in their accumulation, with *mda1*-ncc1 mutant members 3 and 4 having nearly undetectable amounts of the *atpA*-containing transcripts. This experiment provided direct evidence that the target for mutated factor MDA1 was present within the coding region of *atpA*.

Because 76 bases of the coding region of *atpA* remained in the AFFF construct, which was insensitive to the presence of the *mda1*-ncc1 mutation (Figure 3a), we

could further restrict the target for the mutated MDA1 factor to sequences downstream of position 77. We took advantage of another cross between strain ΔatpA, mt^+ (Drapier *et al.*, 1998) and *mda1*-ncc1, mt^- to investigate the contribution of the next 85 bases, since the deletion in ΔatpA begins at an *Eco*RI site 160 bp downstream of the ATG initiation codon (see Figure 5a). Figure 5(c) shows the abundance of the two *atpA* 5′ UTR-driven transcripts within a representative tetrad: they were present in comparable amounts in each of the four members. Therefore, critical elements of the target for the mutated factor MDA1 are located in the last 1360 bases of the *atpA* coding sequence.

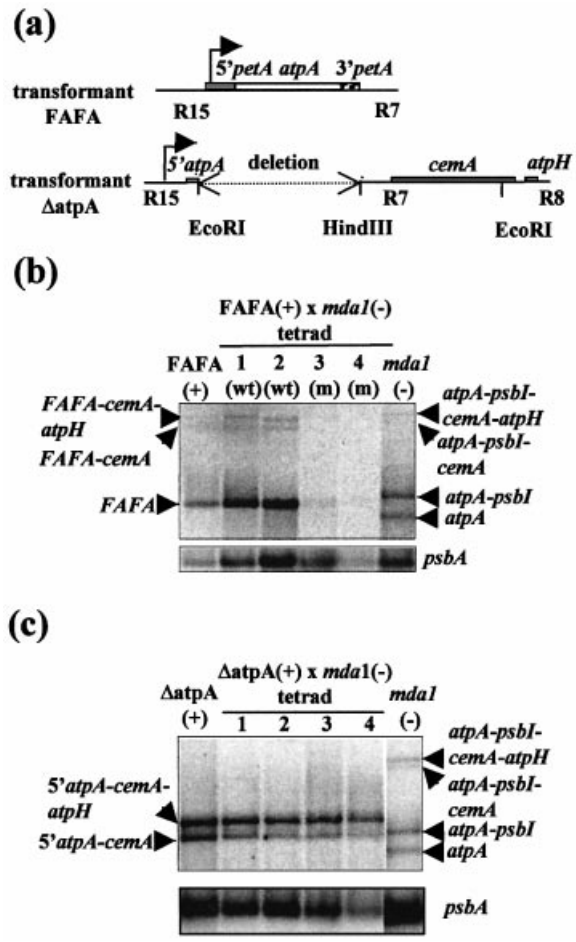

Figure 5. Accumulation of *atpA* transcripts in transformant FAFA or ΔatpA in wild-type and *mda1*-ncc1 nuclear contexts.
(a) Schematic representation of the reporter gene FAFA in which the 5′ and the 3′ UTRs of *atpA* were replaced by those of *petA* (the promoter of *petA* is indicated by a bent arrow), and map of ΔatpA showing deletion of the 2 kb *Eco*RI-*Hin*dIII subfragment. R7, R8 and R15, *Eco*RI restriction fragments named after Rochaix (1978) where are located the chimeric genes.
(b) RNA filter hybridization analysis for a representative tetrad from a FAFA × *mda1*-ncc1 cross. Same experimental conditions as in Figure 1b; tetrad labelling similar to that in Figure 3b.
(c) RNA filter hybridization analysis for a representative tetrad from a.atpA × *mda1*-ncc1 cross. Same experimental conditions as in Figure 1b. No effect of the *MDA1/mda1* locus was observed on the members of the tetrad progeny.

The level of the expression of the α -subunit varies between strains expressing modified versions of the atpA *gene*

Since translation rates in *Chlamydomonas* chloroplasts cannot be predicted from the extent of chloroplast mRNA accumulation (Eberhard *et al.*, 2002), we measured directly the rate of synthesis of the α-subunit in strains that showed an altered pattern of accumulation of atpA transcripts. To this end, we pulse-labelled chloroplast translates from whole cells with ^{14}C acetate for 5 min in the presence of cycloheximide, which inhibits cytosolic translation. The patterns of chloroplast protein synthesis were similar in transformants Δ1 and Δ3, and wild-type cells, whereas those of the Δ1-*mda1* and the Δ3-*mda1* mutant strains were similar to that in the *mda1*-ncc1 single mutant. This is illustrated in Figure 6a for transformant Δ1 and the members of a tetrad resulting from a Δ1,mt^+ × *mda1*-ncc1,mt^- cross (same observations for transformant Δ3 and members of a tetrad resulting from a Δ3,mt^+ × *mda1*-ncc1,mt^- cross, data not shown). The rate of synthesis of the α-subunit decreased but that of the β subunit was stimulated in mutant Δ1-*mda1* as it is in *mda1*-ncc1, thus inverting the ratio of labelling between the two CF1 subunits with respect to that in the strains possessing a wild-type nuclear genome.

The rate of synthesis of the α-subunit in FAAA was lower than in wild-type cells, and similar to that in *mda1*-ncc1 (Figure 6b). This is easily explained by the presence of 5′-*cis*-acting signals derived from the *petA* gene, upstream the atpA coding sequence. In 5 min pulses with ^{14}C-acetate, these *petA cis*-acting signals drive the synthesis of cytochrome *f*, that incorporates far less ^{14}C than the α-subunit in a wild-type strain, which is indicative of lower translation rates. The comparison of the rates of synthesis of the α and β subunits among the tetrad progeny of Figure 6b, is hampered by the lower protein labelling in members 2 and 4 as compared to members 1 and 3 of the tetrad. However, after quantification of the labelling of the α and β subunits relative to that in bands P5 and P6 (compare these bands along the tracks of Figure 6b), we observed that all members of a tetrad progeny from the FAAA × *mda1*-ncc1 cross showed (i) similar rates in α-subunit synthesis — close to that in the two parental strains but lower than that in the wild-type strain. They also displayed the same enhanced synthesis of the β-subunit as compared to that in the wild-type strain. We observed similar changes in the pattern of synthesis for the α and β subunits in FAFA × *mda1*-ncc1 crosses

(experiment not shown). The *mda1*-ncc1 nuclear background in the daughter cells from crosses with FAAA and FAFA became apparent only when probing the steady-state protein levels with antibodies against the α and β subunits (Figure 6c): the α-subunit remained below detection in the *mda1*-ncc1 daughter cells, whereas the β-subunit was present in amounts comparable to that in mutant *tda1*-F54, which is impaired in the synthesis of the α-subunit (Drapier *et al.*, 1992). Overall, these results establish that in the FAAA-*mda1* and FAFA-*mda1* background, the combined effects of a drop in *atpA* mRNA accumulation and a change is *cis*-acting signals for translation prevents functional assembly of the chloroplast ATP synthase. Accordingly these double mutant strains did not grow phototrophically (data not shown), allowing the *mda1*-ncc1 to be used as a dominant mutation preventing photosynthesis.

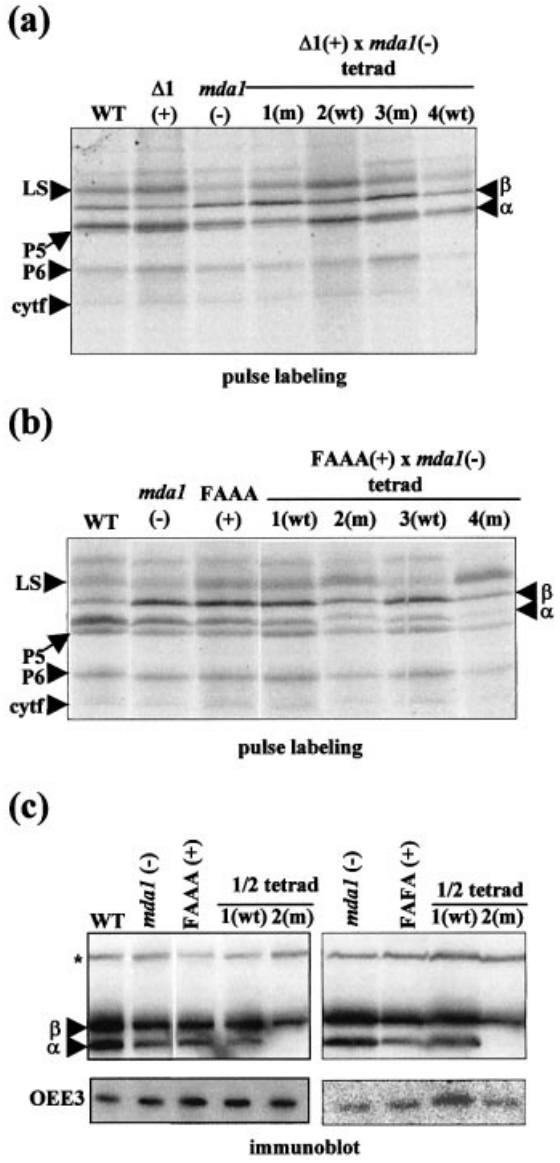

Figure 6. Expression levels of the α-subunit in transformants Δ1, FAAA and FAFA, in a wild-type or *mda*1-ncc1 nuclear context.
(a) The same tetrad as in Figure 4c (1 × *mda1*-ncc1) and the parental strains, together with the wild-type used as a control, were pulse-labelled for 5 min with ^{14}C-acetate in the presence of an inhibitor of cytoplasmic translation, then solubilized in SDS and separated by polyacrylamide gel electrophoresis. Indicated on the left side are reference electrophoretic bands for LS (Rubisco large subunit, *rbcL* gene product), P5 (*psbB* gene product), P6 (*psbC* gene product) and cytf (*petA* gene product).
(b) Same experimental conditions and labelling as in panel a, the tetrad used is the one shown in Figure 3c (from the cross FAAA × *mda1*-ncc1).
(c)Immunoblot of whole cell protein extracts of daughter cells from FAAA × *mda1* and FAFA × *mda1* crosses. Asterisk indicates a band resulting from a cross reaction of the anti-β antibody with the β-subunit of the mitochondrial ATP synthase. The oxygen evolving enhancer protein 3 (OEE3) was used as a loading control.

Mda1-*ncc1 exhibits a dominance in the synthesis of the ATP synthase* α *and* β *subunits*

To establish the dominance of *mda1* at the translational level, we measured the rates of synthesis of the α and β subunits in the three types of vegetative diploids that we had isolated (Figure 2). The patterns of synthesis were similar in the homozygous diploids *MDA1*/*MDA1* or *mda1*/*mda1* (Figure 7) and in the corresponding haploids, showing a large decrease of the α/β ratio in *mda1* context (Figure 6a,b). On the other hand, the labelling pattern of α and β subunits was similar in the homozygous *mda1/mda1* diploid cells and the heterozygous *MDA1/mda1* diploid cells, confirming the dominance of the *mda1* mutation at the translational level.

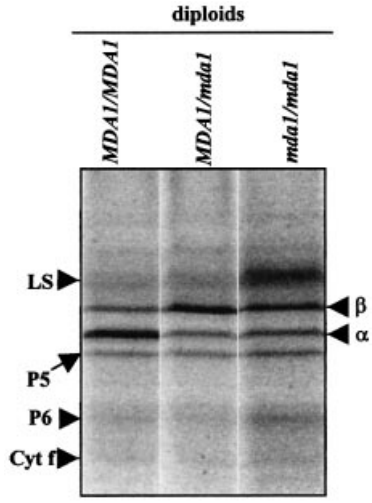

Figure 7. Rate of synthesis of the α-subunit in homozygous (*MDA1/MDA1* or *mda1/mda1*) and heterozygous (*MDA1/mda1)* diploids. Same experimental conditions as in Figure 6a.

Discussion

The characterization of numerous nuclear mutants of *Chlamydomonas* impaired in the synthesis of a single chloroplast-encoded protein has defined distinct phenotypic classes, each one corresponding to a purported step in the regulation of chloroplast gene expression (Barkan and Goldschmidt-Clermont, 2000; Monde *et al.*, 2000; Zerges, 2000). Nuclear factors acting either on RNA maturation or translation were thus identified genetically. The maturation mutants have been studied in great detail, particularly in the cases of *nac2, mbb1 and mcd1*, nuclear mutations that destabilize the *psbD*, *psbB* and *petD* mRNAs, respectively. In each case, the studies provided evidence that 5′ end processing is required for mRNA stabilization and translation, and that the mutated gene encoded a protein involved in these steps *via* an interaction with the cognisant 5′ UTR (Drager *et al.*, 1998; Nickelsen *et al.*, 1994; Vaistij *et al.*, 2000a).

The nuclear mutant *mda1*-ncc1 here described does not belong to this well-described class, since the 5′ UTR of *atpA* plays no role in the *mda1*-ncc1-mediated destabilization process. *atpA* is the upstream cistron of a transcriptional unit comprising four genes, and the *mda1*-ncc1 mutation has a much stronger destabilizing effect on monocistronic *atpA* than on the polycistronic forms of the transcript. Since the four transcripts had identical 5′ ends, mutant *mda1*-ncc1 was a reasonable candidate for being altered in a nuclear factor involved in the 3′ end processing of monocistronic *atpA*, as suggested previously (Drapier *et al.*, 1992). Initially we wondered whether *mda1* might resemble *crp3*, another nuclear mutation that affects the processing of the 3′ ends of several transcripts, including *atpA*, *psbI*, *cemA*, and *atpB* (Levy *et al.*, 1997). We found, however, that *mda1* was not allelic to *crp3* (which is recessive), and furthermore that *mda1* is *atpA*-specific (Levy *et al.*, 1999). Moreover, as shown here *atpA*-containing transcripts are still sensitive to the *mda1*-ncc1 mutation in transformants bearing extensive deletions at the 3′ end of the *atpA* coding region. Finally, the analysis of tetrads resulting from a cross *mda1*-ncc1 with a transformant that retains only the central part of the *atpA* gene provided direct experimental evidence that the *cis*-acting elements conferring sensitivity of the *atpA* transcript to the *mda1*-ncc1 mutation were located in its coding region. The *mda1*-ncc1 nuclear mutation has two unprecedented characteristics in the field of the chloroplast gene expression: (i) it disclosed a target in the coding region of a chloroplast gene that controls the half-life of the transcript. Coding region elements that contribute positively or negatively to the half-lives of transcripts have been described earlier in prokaryotes (Kulkarni and Golden, 1997) as well as in mammalian (Ross, 1995) and fungal (Hennigan and Jacobson, 1996) cells (ii) it is a dominant mutation whereas all other nuclear mutations altering chloroplast mRNA stability identified to date have been recessive. There are two major ways to explain the dominance of a mutation: the 'dominant negative hypothesis' or the 'gain of function hypothesis'.

The dominant negative hypothesis. Several copies of MDA1 would be present in a multimeric protein complex that confers nucleolytic resistance to the *atpA* mRNA This protection would occur through binding to some endonucleolytic cleavage site in the coding region as is the case of c-*fos* and c-*myc*, in mammalian system. The presence of even one mutated copy of MDA1 would be sufficient to prevent the protective binding of the protein complex to *atpA* mRNAs.

The gain of function hypothesis. MDA1 possesses a regulated nuclease activity directed against the *atpA* transcripts. The mutated version of MDA1 would have modified regulatory properties, for instance because of the loss of binding of a negative regulator and would incorrectly target the *atpA* transcripts. Alternatively, it could have acquired a new recognition site for the coding region of *atpA*. In this way it might be analogous to the endoribonuclease which cleaves c-*myc* mRNA in its wild-type form (Lee *et al.*, 1998).

An extreme version of the 'gain of function' hypothesis is the recruitment by the *mda1*-ncc1 mutation of a nuclease active on *atpA* mRNA. In this scenario, the wild-type version of MDA1 would not recognize *atpA* transcripts. We think this is unlikely for two reasons. First, the de-repression of a nucleolytic activity would most likely target several chloroplast transcripts, whereas the *mda1*-ncc1 mutation is specific for *atpA* mRNA degradation, to the extent that protein pulse labelling and a limited number of filter blots allow that conclusion. Second, we have shown that *atpA* transcripts in a wild-type nuclear context are much more short-lived than all other chloroplast transcripts we have tested, an observation that suggests specific or enhanced sensitivity to a nucleolytic process (Eberhard *et al.*, 2002). It is reasonable to propose that this process has merely been enhanced by the *mda1*-ncc1 mutation.

Neither of the two hypotheses that may account for dominance of *mda1*-ncc1 can explain by themselves the discrimination among *atpA*-containing mRNAs by the mutated MDA1 factor: monocistronic *atpA* transcripts were far more affected in *mda1*-ncc1 than any of the polycistronic *atpA*-containing transcripts. However, when the monocistronic form was missing because of a 3′-end modification, the dicistronic transcript became the major *mda1*-ncc1 target. In turn the tri- and tetracistronic transcripts became major targets when neither the mono- nor the dicistronic transcripts could be formed. Taken together, these results suggest that the

most abundant *atpA* transcript is the major target of the mutated MDA1 factor. In parallel with their increased susceptibility to degradation in an *mda1*-ncc1 context, we observed that the polycistronic transcripts were increasingly engaged in translation: indeed, the tri- and tetracistronic transcripts that remained in strain Δ3 and represented as little as 10% of the total *atpA* transcripts in wild-type cells, were sufficient to drive the synthesis of the α-subunit at a wild-type rate. Since the target for mutated MDA1 is in the coding region of *atpA*, it is tempting to speculate that it destabilizes *atpA*-containing transcripts only during, or soon after, translation. In fact, the c-*myc* ribonuclease mentioned above, along with other known ribonuclease activities, are polysome-associated. The potential of association between *mda1*-ncc1 action and translation could be assessed in two sets of experiments. In the first, abundance of *atpA* transcripts would be probed in the double mutant *mda1-tda1*, resulting from a cross between *mda1*-ncc1 and the nuclear mutant *tda1*-F54, where translation of *atpA* mRNA is blocked (Drapier *et al.*, 1992); and in the second, effects on *atpA* transcripts would be measured after treating *mda1*-ncc1 cells for up to 24 h with chloroplast translation inhibitors. In the latter case, we tested chloramphenicol, lincomycin and kasugamycin, and found no protective effect of the *atpA* mRNA in a *mda1*-ncc1 context (data not shown). In the former case, we still observed the destabilization of monocistronic *atpA* mRNA in the translation defective *tda1*-F54 mutant. However, these transcripts are still found in polysomal fractions in *tda1*-F54 (S. Eberhard, personal communication), which suggests that the mutation plays a role at the elongation step rather than at initiation. We would conclude from both sets of experiments that completion of *atpA* translation is not a prerequisite for the action of the mutated MDA1 factor. However, one should consider the possibility that the *mda1*-ncc1 mutation mediates degradation of polysome-bound *atpA* mRNA, perhaps after translation initiation has taken place and in response to the inevitable changes in RNA secondary structure that accompany ribosome passage.

Initially, cloning the *MDA1* gene appeared as a most difficult task, since its mutated version in *mda1*-ncc1 had neither a selectable nor an easily screenable phenotype. However, we have been able to produce non-phototrophic strains harbouring the *mda1*-ncc1 mutation, where the *petA* promoter/5′ UTR has replaced that of *atpA*. Although we have at present no clues as to the molecular mechanism that prevents the subunit expressed in this genetic background from accumulating in a functional ATP synthase complex, these strains open the way to a cloning strategy for the *MDA1* gene.

Experimental procedures

Strains and growth conditions

The wild-type strain of *C. reinhardtii* used here was derived from strain 137c; the ncc1 nuclear mutant was obtained from a spontaneous mutation that occurred in the wild-type strain (Drapier *et al.*, 1992); the Δ3 and Δ1 strains bearing deletions in the 3′ UTR of the *atpA* gene, the ΔatpA strain bearing a deletion of the major part of the coding sequence of *atpA* and the entire *psbI* gene were constructed as described in Drapier *et al.* (1998); the construction of the strain AFFF bearing the *petA* gene under the control of the 5′ UTR of *atpA* was described in Choquet *et al.* (1998). All strains were grown on Tris-acetate-phosphate (TAP) medium (pH 7.2) at 25°C with a light intensity of 5.9 μmol photons m^{-2} s^{-1}.

Plasmid constructions and chloroplast transformation

The pFAAA plasmid was constructed as followed: Plasmid p5FΔR, lacking any *Eco*RI restriction site, was created from plasmid p5F (Choquet *et al.*, 1998): p5F was linearized by the unique cutter *Eco*RI, filled-in with Klenow enzyme and re-ligated on itself. A 644-bp fragment containing the 5′ part of the *atpA* coding sequence was amplified by PCR, using plasmid pATPA2 as a template and oligonucleotides ATPA5 (G*CGGATC*CATGGCAATGCGTACTCCA-GAA *Bam*HI, *Nco*I) and ATPA3 (GCTCTAGATACTTGAGCTACA-GATGAAGCT *Xba*I) as primers. In the resulting fragment, a *Nco*I site has been created at the level of the initiation codon. This fragment was digested with *Xba*I and *Nco*I and cloned in the plasmid p5FΔR digested with the same enzymes, to yield plasmid p5F-atpA. In this new plasmid the 628 first nucleotides of the *atpA* coding sequence are fused in frame to the *petA* 5′ UTR. Plasmid p5FatpA was then digested by *Eco*RV (located upstream of the *petA* 5′ UTR) and *Eco*RI (located in the *atpA* coding sequence, 165 bp downstream of the initiation codon), and the 863 bp band was eluted. It was then cloned into the pATPA2 vector, first digested with *Aat*II and filled-in with Klenow enzyme, then with *Eco*RI to result in plasmid pFAAA. Plasmid piWF comprising the complete *petA* sequence (Kuras and Wollman, 1994) was used as a template to amplify by PCR the 3′ UTR of the *pet* gene, using oligonucleotides 3FPac(5′-GCGTTAATTAATATTTAATTTTTTGTA-GGGCTGCT) and 3FHpa(3′-CGCGTTAACTTCGCAACTGCCACTG-ACG); the amplified fragment (491 bp), after digestion with *Pac*I and *Hpa*I, the two restriction sites underlined in the sequences of the oligonucleotides, was inserted into the vector pFAAA that had been previously digested with the same enzymes and was consequently missing the 366 bp within the *atpA*-3′ UTR. This created plasmid pFAFA, with the 5′ and 3′ UTRs of *petA* replacing those of *atpA* on either side of the *atpA* coding sequence ΔatpA cells were bombarded with tungsten particles coated with plasmids pFAAA or pFAFA as described in Kuras and Wollman (1994) and the transformants were selected by photosynthetic growth on minimal medium under bright light. The homoplasmy of the cells was checked by DNA filter hybridization.

Genetic analysis

For gametogenesis, cells were grown for 3–4 days on TAP plates containing one-tenth the usual amount of nitrogen. Mating, germination, and tetrad analysis were performed according to Harris *et al.* (1989). Diploid progeny from crosses were selected by incorporating the complementary auxotrophic mutations *arg2*

and *arg7* into the parental strains (*mda*1-ncc1, *arg2* and *mda1*-ncc1, *arg7)* and plating on TAP medium. After 12 days in low light, large colonies appeared on each plate that were comprised of larger cells that wild type haploid cells and displayed a mating type minus phenotype (12–20 colonies tested from each cross), as expected for diploid cells (Ebersold, 1967; Harris *et al*., 1989).

RNA analysis

Isolation of total RNA and RNA filter hybridization analysis were performed as described in Drapier *et al*. (1998). The probes were as follows: the intragenic *atpA Eco*RI-*Pst*I fragment (947 bp) or the 5′ UTR of *atpA* to detect the *atpA* transcripts, the intragenic *petA Hin*dIII-*Acc*I fragment (620 kb) to detect *petA* transcripts, the chloroplast R14 fragment according to the nomenclature of Rochaix (1978) to detect the *psbA* transcript or the 25S nuclear DNA fragment as loading controls for the RNA gels.

Protein analysis

Chloroplast proteins were pulse labelled in the presence of ^{14}C-acetate and a cytosolic translation inhibitor (cycloheximide) and analysed by denaturing gel electrophoresis according to Drapier *et al*. (1992). Immunoblotting experiments were performed as in de Vitry *et al*. (1989).

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