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 *Chlamydomonas*. 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 nucleochloroplastic 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
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 cotranslational 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-psbl-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
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-psbl-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., 1999). 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-psbl-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.

Figure 2. Accumulation of atpA transcripts in heterozygous vegetative diploids MDA1/mda1, relative to that in homozygous vegetative diploids MDA1/MDA1 or mda1/mda1. Experimental conditions are as described for Figure 1b.

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*. (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 = MDA1; 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 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 *atpA*-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 *HindIII* site in the *psbl-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 *atpA*-containing transcripts, showed a 10-fold reduction. In Δ1, mt+ × *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 FAAA. 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 FAAA, 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+ (Draper et al., 1998) and mda1-ncc1, mt- to investigate the contribution of the next 85 bases, since the deletion in ΔatpA begins at an EcoRI 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.

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 14C 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 14C-acetate, these petA cis-acting signals drive the synthesis of cytochrome f, that incorporates far less 14C 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 FAAA × mda1-ncc1 crosses.
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 in 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.

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.
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 *mdc1*, 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 endonuclease 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 policistronic transcripts were increasingly engaged in translation: indeed, the tri- and tetracstronic 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 monocistrionic 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 5′ UTR of the atpA gene, the ΔatpA strain bearing a deletion of the major part of the coding sequence of atpA and the entire psbl gene were constructed as described in Drapier et al. (1998); the construction of the strain AFFE 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 EcoRI restriction site, was created from plasmid p5F (Choquet et al., 1998): p5F was linearized by the unique cutter EcoRI, 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 (GGGCTGCT) and 3FHpa(3′CGGATCTGCGGAATCTTCA-GAA BamH I, Ncol) and ATPA3 (GCTCTAGATCTGGGAGAC- CATGAAGCT XbaI) as primers. In the resulting fragment, a Ncol site has been created at the level of the initiation codon. This fragment was digested with XbaI and Ncol 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 EcoRV (located upstream of the petA 5′ UTR) and EcoRI (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 Aafl and filled-in with Klenow enzyme, then with EcoRI to result in plasmid pFAAA. Plasmid pWIF comprising the complete petA sequence (Kuras and Wollman, 1994) was used as a template to amplify by PCR the 3′ part of the petA gene using oligonucleotides 3FPac(5′GTTAACTTCGCAACTGCCACTG-TTAATTAATATTTAATTTTTTGTA-CATGGCAATGCGTACTCCA-GATGAAGCT XbaI) and 3FHpa(3′CGGATCTGCGGAATCTTCA-GAA BamH I, Ncol) as primers. The amplified fragment (491 bp), after digestion with PacI and HpaI, 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′ 5′ UTR. This created plasmid pFAPA, 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 pFAPA 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 (mda1-nc1, arg2 and mda1-nc1, 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, 1987; Harris et al., 1988).

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 EcoRI-PstI fragment (947 bp) or the 5′ UTR of atpA to detect the atpA transcripts, the intragenic petA HindIII-AccI 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 14C-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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