

Polyadenylation in Arabidopsis and *Chlamydomonas* organelles: the input of nucleotidyltransferases, poly(A) polymerases and polynucleotide phosphorylase

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SUMMARY

The polyadenylation-stimulated RNA degradation pathway takes place in plant and algal organelles, yet the identities of the enzymes that catalyze the addition of the tails remain to be clarified. In a search for the enzymes responsible for adding poly(A) tails in *Chlamydomonas* and Arabidopsis organelles, reverse genetic and biochemical approaches were employed. The involvement of candidate enzymes including members of the nucleotidyltransferase (Ntr) family and polynucleotide phosphorylase (PNPase) was examined. For several of the analyzed nuclear-encoded proteins, mitochondrial localization was established and possible dual targeting to mitochondria and chloroplasts could be predicted. We found that certain members of the Ntr family, when expressed in bacteria, displayed poly(A) polymerase (PAP) activity and partially complemented an *Escherichia coli* strain lacking the endogenous PAP1 enzyme. Other Ntr proteins appeared to be specific for tRNA maturation. When the expression of PNPase was down-regulated by RNAi in *Chlamydomonas*, very few poly(A) tails were detected in chloroplasts for the *atpB* transcript, suggesting that this enzyme may be solely responsible for chloroplast polyadenylation activity in this species. Depletion of PNPase did not affect the number or sequence of mitochondrial mRNA poly(A) tails, where unexpectedly we found, in addition to polyadenylation, poly(U)-rich tails. Together, our results identify several Ntr-PAPs and PNPase in organelle polyadenylation, and reveal novel poly(U)-rich sequences in *Chlamydomonas* mitochondria.

Keywords: polyadenylation, chloroplast, mitochondria, polyuridylation, RNA decay.

INTRODUCTION

Polyadenylation is an important post-transcriptional modification of prokaryotic, eukaryotic and organellar RNAs. In bacteria, some archaea and organelles such as plant and algal mitochondria and chloroplasts, polyadenylation is transient and occurs mainly on fragmented molecules as part of an RNA decay pathway (Dreyfus and Regnier, 2002; Kushner, 2004; Slomovic *et al.*, 2006). Therefore, the detection of non-abundant polyadenylated molecules, which are degradation intermediates, serves as a telltale sign of the presence of a poly(A)-stimulated RNA degradation mechanism.

The molecular mechanism of RNA polyadenylation and degradation in prokaryotes and organelles has been exten-

sively studied. In *Escherichia coli*, polyadenylation is mainly carried out by a nucleotidyltransferase-type poly(A)-polymerase (Ntr-PAP) that produces homopolymeric poly(A) tails. Polyadenylation is also catalyzed to a certain extent by polynucleotide phosphorylase (PNPase), which creates heteropolymeric poly(A)-rich tails containing all four nucleotides (Mohanty and Kushner, 2000). Research in *E. coli* led to the study of related mechanisms, revealing polyadenylation-stimulated RNA degradation pathways in other bacteria, in chloroplasts, in both plant and human mitochondria, in some archaea and for nucleus-encoded transcripts in yeast and human cells (Dreyfus and Regnier, 2002; Slomovic *et al.*, 2006, 2008b; Wilusz and Wilusz, 2008).

Heteropolymeric poly(A)-rich tails were first discovered when spinach chloroplast transcripts were analyzed for polyadenylation (Lisitsky *et al.*, 1996). However, the identity of the polymerizing enzyme was unknown, and likewise, the biological meaning of these extensions was not understood. Several years later, such heteropolymeric tails were detected in *E. coli* strains in which the poly(A)-polymerase gene *pcnB* was inactivated, and PNPase was identified as the enzyme responsible for this activity (Mohanty and Kushner, 2000). Following these initial observations, experiments in cyanobacteria, the Gram-positive bacteria *Streptomyces coelicolor* and *Bacillus subtilis*, as well as in spinach chloroplasts, determined that in all cases in which heteropolymeric poly(A)-rich tails were detected, PNPase was the polymerizing enzyme (Yehudai-Resheff *et al.*, 2001; Rott *et al.*, 2003; Sohlberg *et al.*, 2003; Campos-Guillen *et al.*, 2005). Therefore, the nucleotide composition of the post-transcriptionally added extensions can indicate the identity of the polymerizing enzyme.

The production of mature tRNAs for protein synthesis in plants requires a number of processing and modification steps (Hopper and Phizicky, 2003), including the addition of a 3'-terminal cytidine-cytidine-adenosine (CCA), as no plant tRNA genes encode this sequence. Thus, a tRNA nucleotidyl-transferase (Ntr) enzyme capable of adding this CCA sequence must be present in the three compartments that carry out protein synthesis. The amino acid sequence of the *E. coli* poly(A) polymerase is very similar to the CCA-Ntr, as is the case with other non-canonical PAPs (Betat *et al.*, 2004; Martin and Keller, 2007; Martin *et al.*, 2008). Therefore, it has been predicted that several of the nucleus-encoded Ntrs are organelle-targeted and function as PAPs (Martin and Keller, 2004; Zimmer *et al.*, 2008). *Arabidopsis* and *Chlamydomonas reinhardtii* encode both Ntr and PNPase proteins that may potentially be targeted to chloroplasts or mitochondria, and function in polyadenylation. Two PNPases are encoded in *Arabidopsis*, which encode, respectively, chloroplast and mitochondrial isozymes (Kudla *et al.*, 1996; Yehudai-Resheff *et al.*, 2001; Perrin *et al.*, 2004). In addition, genes encoding several members of the Ntr family were identified, and the localizations and activities of the proteins, either polyadenylation or tRNA 3' end addition/correction, were predicted (Martin and Keller, 2004). There is, however, only one PNPase-encoding gene in *Chlamydomonas*, and available evidence suggests that the protein is localized to the chloroplast (Yehudai-Resheff *et al.*, 2007). There are also several Ntr proteins encoded in *Chlamydomonas*, the functions of which remain to be determined (Zimmer *et al.*, 2008).

In this work, we aimed at the identification of the polyadenylating enzymes in the organelles of *Arabidopsis* and *C. reinhardtii*. In particular, we have focused on the contributions of PNPase and Ntrs by analyzing their subcellular localizations as well as polyadenylation and Ntr activities.

RESULTS

The *Arabidopsis* and *Chlamydomonas* Ntr proteins predicted to be localized to mitochondria and/or chloroplasts

Several *Arabidopsis* Ntr proteins were previously analyzed bioinformatically, and predicted to possess Ntr (AtNtr3) or PAP activity (AtNtr1 and AtNtr2). Based on their amino-terminal sequences, each is predicted to be targeted to mitochondria or chloroplasts (Martin and Keller, 2004). Another *Arabidopsis* Ntr protein, At2g17580, was also identified but has lower organelle localization scores when analyzed bioinformatically. Of the nine Ntr proteins identified in *Chlamydomonas*, PAP3 and PAP4 were analyzed as chloroplast localization was predicted (Zimmer *et al.*, 2008). In order to compare the selected *Chlamydomonas* and *Arabidopsis* Ntrs, the protein sequences were aligned and their domain structures examined (Figure 1a). Each has a PAP domain, and all have N-terminal extensions relative to the *E. coli* protein.

The *Arabidopsis* proteins described above belong to the Class II Ntrs, which in addition to the eubacterial-like PAPs, include CCA-Ntrs (Cho *et al.*, 2007). It should be noted that CCA transferase (CCATr) activity in several organisms such as *Synechocystis* sp., *Deinococcus radiodurans* and *Aquifex aeolicus* is split between two enzymes, one that adds -CC and the other that adds a terminal -A (Tomita and Weiner, 2002). Although it is difficult to assign an activity to a Class II NTR based on primary sequence, several informatic and experimental approaches (Betat *et al.*, 2004; Cho *et al.*, 2007; Martin and Keller, 2007; Martin *et al.*, 2008) identified Glu193 in *Bacillus stearothermophilus* as a residue in the PAP domain that allows discrimination between CCATrs and PAPs. In CCATrs this residue is Glu, but is variable in other family members. Only one each of the *Arabidopsis* (AtNtr3, position 302) and *Chlamydomonas* (CrNtr3, position 287; this protein was termed PAP3 in Zimmer *et al.*, 2008) proteins possess this Glu (marked with asterisks in Figure 1a). The remainder contain other amino acids (aa): Ser546 (CrPAP4), Ser230 (AtNtr1) and Gly277 (AtNtr2).

The sequence-based relationships between the *Chlamydomonas* and *Arabidopsis* Ntrs of interest are shown in Figure 1(b). The two likely CCA transferases, AtNtr3 and CrNtr3, cluster with a high bootstrap value and, excluding the putative transit peptides, are 38% identical and 54% similar. The two remaining *Arabidopsis* proteins cluster together and may derive from a gene duplication event. They are 54% identical at the amino acid level, excluding the putative N-terminal transit peptides. AtNtr1, predicted by Martin and Keller (2004) to be the chloroplast PAP, has two predicted isoforms due to alternative splicing. AtNtr1.1 lacks the first exon, and initiates at a start codon 15 bp upstream of the second exon.

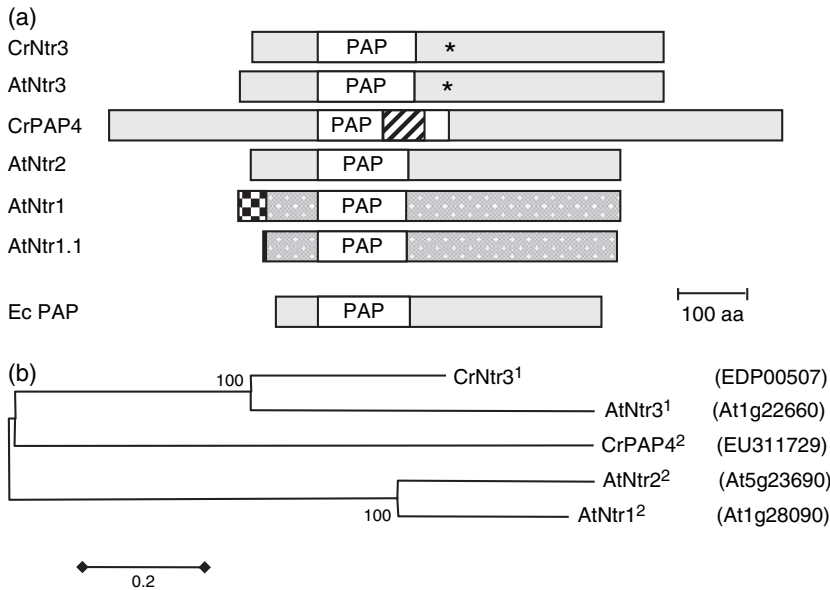


Figure 1. Comparison of putative organellar *Chlamydomonas* and Arabidopsis NTRs.

(a) Alignment of *Chlamydomonas* (Cr) and Arabidopsis (At) putative organellar Class II nucleotidyltransferase (Ntrs) with *Escherichia coli* poly(A)-polymerase (PAP). The proteins are aligned from the beginning of the catalytic and conserved PAP head domain, PFAM PF01703. The PF01703 domain in PAP4 is interrupted (hatched box). Asterisks designate the Glu residue that assigns a likely cytidine-cytidine-adenosine (CCA)-Ntr. Isoforms of Arabidopsis AtNtr1 are presented, with the identical regions in grey with white speckles, and the dissimilar N-termini in checked or black fill.

(b) Phylogenetic analysis of *Chlamydomonas* and Arabidopsis Class II Ntrs that are predicted to be organellar. The sequences were aligned and assembled using MEGA 3/4 (<http://www.megasoftware.net/>). ¹Sequences predicted to encode CCA transferases. ²Sequence is probably a PAP.

At 960 aa, CrPAP4 is larger than all other known Ntrs (Figure 1a). All of the plant and algal proteins are extended at their N-termini relative to *E. coli* PAP, and on this basis could encode transit peptides for mitochondrial or chloroplast localization. In addition, CrPAP4 has a long C-terminal extension. This region is highly repetitive, encoding a glutamine-rich domain. Another unusual feature of CrPAP4 is a region of unique sequence that interrupts its PAP domain.

Localization of Ntrs

The nucleus-encoded Ntrs analyzed here are hypothesized to be localized to the organelles, based on the targeting predictors TargetP (Emanuelsson *et al.*, 2000), Predotar (Small *et al.*, 2004) and iPSORT (Bannai *et al.*, 2002). As an experimental approach, we fused the N-terminal regions of these proteins to GFP, specifically the first 264 aa of CrPAP4, 100 aa of AtNtr3, 100 aa of AtNtr2 and 100 aa of AtNtr1, and transiently expressed the chimeric proteins in tomato protoplasts. Figure 2 shows representative results. As described previously, AtNtr3 localized GFP to both chloroplasts and mitochondria (von Braun *et al.*, 2007), with a stronger signal for mitochondrial targeting. The CrPAP4 sequence, in contrast, appeared to localize GFP exclusively to mitochondria. In the cases of AtNtr1 and AtNtr2, mitochondrial targeting is observed but some of the GFP appeared to be possibly targeted to the chloroplast. AtNtr1.1, which is shorter than AtNtr1 at the N-terminus, was not targeted, consistent with the AtNtr1 protein possessing an N-terminal transit peptide. Taken together, these results support mitochondrial localization of all the proteins examined, with likely dual targeting for AtNtr3 and possible dual targeting for AtNtr1 and AtNtr2.

It should be noted that while transient expression of GFP fusion proteins in heterologous systems often gives accurate indications of subcellular localization, these should ultimately be verified by other methods, especially in the cases of dual targeting.

Polyadenylation activity of recombinant AtNtr1 and CrPAP4

To test the predictions based on sequence analysis, recombinant proteins were analyzed for polyadenylation activity. To this end, the proteins were expressed in an *E. coli* strain that lacks polynucleotide phosphorylase (PNPase), an efficient poly(A) polymerase whose co-purification with the recombinant protein would be problematic. In addition, the absence of detectable *E. coli* PAP in the purified recombinant protein preparation was verified by immunoblot analysis (Figure 3a). The assay revealed that AtNtr1 and CrPAP4, but not AtNtr3, polyadenylated the A₂₀ substrate (Figure 3b–d). This substrate was elongated in some assays to a high molecular weight form of several hundred nucleotides. The activity of AtNtr2 was not analyzed here because only a small amount was obtained as a soluble recombinant protein, and we were unable to purify it to homogeneity.

CCA addition activity of recombinant AtNtr3

To determine whether the proteins of interest could catalyze the addition of CCA to tRNA, the assays shown in Figure 4 were conducted. First we analyzed AtNtr3, since it lacked polyadenylation activity, possesses the Glu residue characteristic of CCAtrs and had been shown to confer the addition of CCA to tRNA in yeast strains lacking endogenous CCAtr (von Braun *et al.*, 2007). Indeed, the recombinant protein, similar to its *E. coli* counterpart, added CCA to an artificial tRNA substrate (panel A).

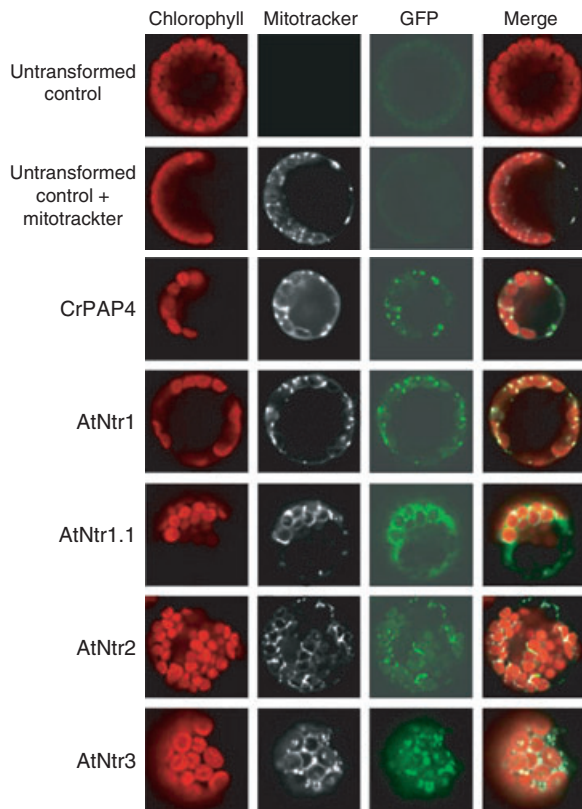


Figure 2. Targeting of GFP fusions in tomato protoplasts. Each image is a single protoplast within which chloroplasts can be visualized by their chlorophyll autofluorescence, and mitochondria by Mitotracker dye. In the last column, the chlorophyll and Mitotracker channels were overlaid with the GFP channel to show the location of the GFP.

As mentioned above, some organisms employ two separate enzymes to add either CC or A to tRNAs. To test whether the putative *Arabidopsis* PAPs had such activity, CC- and A-adding activities were tested separately, as shown in Figure 4(b). In the absence of ATP, CCAtr can only add two cytidines to the tRNA 3' end. The resulting product is thus 2 nucleotides (nt) longer than the original substrate, i.e. 75 nt (Figure 4b, lane 7). Addition of a single adenosine to the 3' end would bring the RNA size to 76 nt. Such an RNA was produced when the input 75 nt tRNA + CC was incubated with AtNtr3 (Figure 4b, lane 2), but no change in size was obtained following incubation with either AtNtr2 or AtNtr1 (Figure 4b, lanes 3 and 4, respectively). As shown earlier, AtNtr1 displayed polyadenylation activity when incubated with A₂₀ RNA and ATP (Figure 3b,d). However, this activity was not observed here since tRNA is a very poor substrate for polyadenylation activity, probably due to its secondary structure (Lisitsky *et al.*, 1996).

CrPAP4 and AtNtr1 polyadenylate *Escherichia coli* transcripts *in vivo*

To see whether CrPAP4 and AtNtr1 could act as PAPs *in vivo*, we attempted to complement an *E. coli* PAP1 deletion mutant ($\Delta pcnB$) with genes encoding these proteins. As a negative control, an inactive version of CrPAP4, in which two amino acids located in the active site were mutated (see Experimental procedures) was used. In the case of AtNtr1, cells uninduced for AtNtr1 expression were compared with those in which its expression had been induced. Accumulation of the recombinant proteins was verified by immunoblotting, as shown in Figure 5.

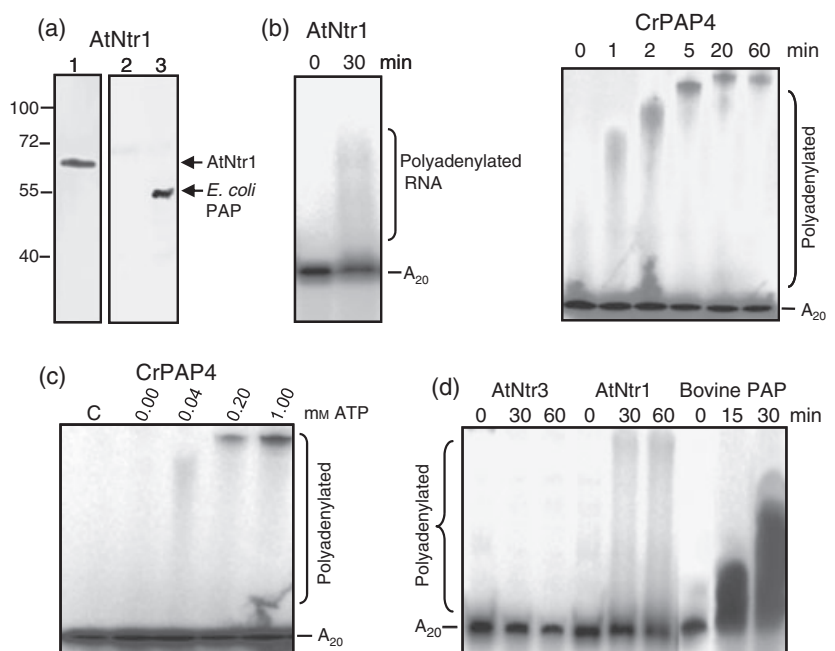
Figure 3. Polyadenylation activity of recombinant proteins.

(a) Coomassie Blue-stained gel of purified recombinant AtNtr1 (lane 1). To verify the lack of *Escherichia coli* poly(A)-polymerase (PAP) contamination, the same protein preparation was analyzed by immunoblot with specific antibodies to *E. coli* PAP (lane 2). The *E. coli* proteins were analyzed in lane 3 as a positive control.

(b) 5'-[³²P]A₂₀ RNA was incubated with *Arabidopsis* nucleotidyltransferase 1 (AtNtr1) in a buffer supplemented with 1 mM ATP. The polyadenylation kinetics of CrPAP4 were analyzed using the same substrate and 1 mM ATP (right panel).

(c) *Chlamydomonas* PAP4 polyadenylation was analyzed with different ATP concentrations, as indicated above the figure. Incubation was for 20 min.

(d) Recombinant AtNtr3 and AtNtr1 were assayed for polyadenylation as described for panel (b). Recombinant bovine PAP served as a positive control.



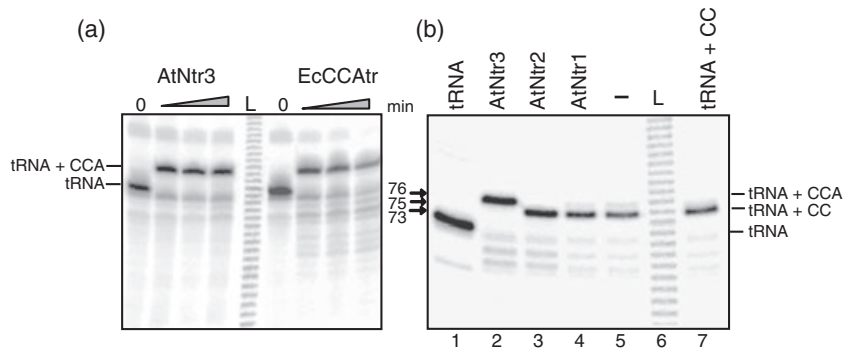


Figure 4. Analysis of recombinant proteins for cytidine-cytidine-adenosine (CCA) transferase activity.

(a) *Escherichia coli* [³²P]-tRNA^{Phe} was incubated with recombinant Arabidopsis nucleotidyltransferase 3 (AtNtr3), or the *E. coli* CCA transferase (EcCCATr) as a positive control. Following incubation for 5, 15 or 45 min, the RNA was isolated and analyzed by high-resolution denaturing PAGE alongside a RNA ladder (L). (b) [³²P]UTP-labeled tRNA^{Phe} (lane 1) was incubated with EcCCATr and CTP in order to add the two cytosine residues at the 3' end (lane 7). Next, this RNA was purified and incubated for 30 min separately with either AtNtr3 (lane 2), AtNtr2 (lane 3), AtNtr1 (lane 4) or without protein (lane 5), and analyzed by high-resolution PAGE. An alkaline ladder of a longer transcript is shown in lane 6.

One phenotype of $\Delta pcnB$ is that transcripts including *lpp3*, which encodes outer membrane murein lipoprotein, lack the homogeneous poly(A) tails which are the predominant species in wild-type cells (Mohanty *et al.*, 2004). Instead, heteropolymeric tails are observed, and their presence has been attributed to the activity of PNPase, unmasked by the absence of PAP1. Therefore, *lpp3* tails were amplified using oligo(dT) RT-PCR, and sequenced tails were classified as being either homopolymeric or heteropolymeric. While 20/29 or 14/22 homopolymeric tails were found in two cDNA preparations from wild-type *E. coli*, no homopolymeric tails were found out of 39 analyzed in $\Delta pcnB$ in the two complementation experiments (Figure 5a,b and Table S1 in Supporting Information). However, 10 homopolymeric tails out of the 43 examined were found when $\Delta pcnB$ expressed active CrPAP4, indicating that this protein possessed PAP activity in *E. coli*. Similar results were obtained for AtNtr1, where 11/40 tails were homopolymeric (Figure 5b and Table S1). In contrast, 0/30 tails were homopolymeric when $\Delta pcnB$ expressed the presumably catalytically inactive form of CrPAP4. In the case of the Arabidopsis AtNtr1, homopolymeric tails were obtained only in the bacterial cells in which its expression was induced by isopropyl β -D-1-thiogalactopyranoside (IPTG). Taken together, the *in vitro* analysis of the recombinant proteins and the complementation of $\Delta pcnB$ strongly suggest that CrPAP4 and AtNtr1 are PAPs.

Polynucleotide phosphorylase may be largely responsible for polyadenylation in *Chlamydomonas* chloroplasts

Polynucleotide phosphorylase was shown to be entirely responsible for the post-transcriptionally added RNA tails in the cyanobacterium *Synechocystis*, believed to be closely related to the evolutionary ancestor of the chloroplast (Rott *et al.*, 2003). In addition, PNPase contributes a polymerization activity producing a certain number of the tails in plant

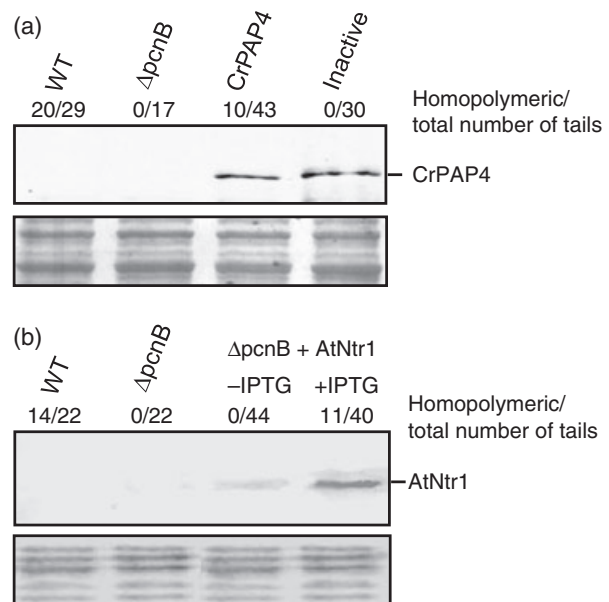


Figure 5. *Chlamydomonas* poly(A)-polymerase (CrPAP4) and Arabidopsis nucleotidyltransferase 1 (AtNtr1) complementation of *Escherichia coli* $\Delta pcnB$. (a) The $\Delta pcnB$ strain was transformed with expression plasmids for CrPAP4 or a catalytically inactive version. Proteins from each strain were analyzed by immunoblot with CrPAP4 antibodies. Some of a Coomassie Blue-stained gel is shown to reflect loading. The composition of tails added to the *lpp3* transcripts is shown above the blot as the fraction of homopolymeric A tails, over the total number of tails sequenced.

(b) An assay similar to that described for part (a) was performed using the AtNtr1 expression construct. Immunodetection was carried out with an antibody against the His₆ tag.

chloroplasts (Yehudai-Resheff *et al.*, 2001; Schuster and Stern, 2008). Therefore, while in cyanobacterial PNPase is uniquely responsible for polyadenylation activity, in chloroplasts and some bacteria, PNPase and a Ntr-PAP are thought to (or have been proposed to) share this function

(Slomovic *et al.*, 2008b). As described above for *E. coli*, while the tails produced by PNPase are heteropolymeric, those synthesized by Ntr-PAP are homo-adenosine. Initially, only very short and homopolymeric tails were described for *Chlamydomonas* chloroplasts (Komine *et al.*, 2000). However, subsequent isolation and analysis of longer tails revealed that although rich in adenosines, most of the tails contained other nucleotides, suggesting that the tails in *Chlamydomonas* chloroplasts are often heteropolymeric (see below and Figure S3).

As mentioned earlier, *Chlamydomonas* appears to encode a single, chloroplast PNPase (Yehudai-Resheff *et al.*, 2007), although a dual targeting to mitochondria has not been excluded. In order to analyze the possible involvement of PNPase and CrPAP4 in the polyadenylation of chloroplast or mitochondrial transcripts, we used RNAi to reduce expression of these enzymes and analyzed the tail composition in the resultant strains. The generation of a stable PNPase-depleted strain PNPI3 (also called ip3; with a reduction in the protein amount of approximately 90%) has been previously described (Yehudai-Resheff *et al.*, 2007), and an analogous strain, PAP4i, was obtained for CrPAP4 (Figures S1 and S2).

We elected to sequence *atpB* tails, since we had prior data from wild-type *Chlamydomonas* for comparison (Komine *et al.*, 2000). In agreement with the apparent mitochondrial localization of CrPAP4 (Figure 2), the *atpB* tails were similar whether obtained from the vector-transformed control or the PAP4i strain (Figure 6 and Figure S3). Most of the polyadenylation sites were located in the vicinity of the endonucleolytic cleavage site located in the 3' untranslated region following the stem-loop structure, as previously reported (Komine *et al.*, 2000). However, in spite of extensive screening of amplified RT-PCR products to find *atpB* tails in the PNPI3 strain (Table S1), only three short tails were found, two of which were the length of the RT primer and thus may have been only a few nt long. Since the purified

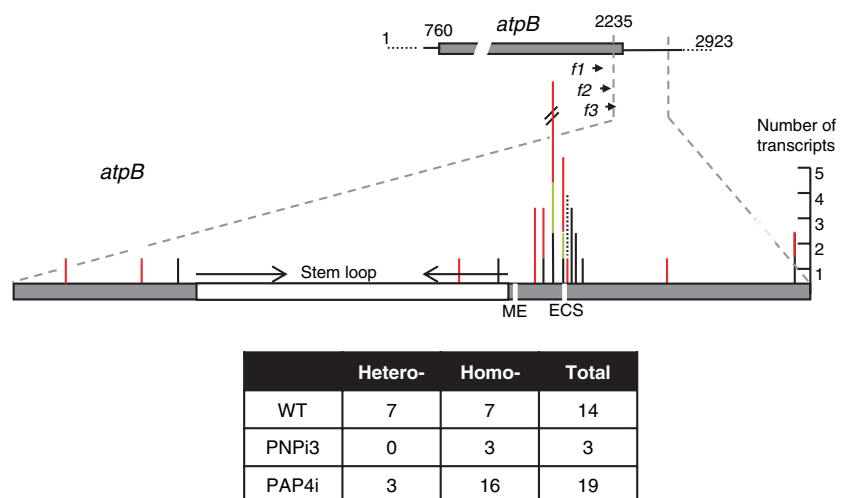
PNPI3 RNA appeared intact when examined for the ribosomal RNAs by agarose gel electrophoresis and ethidium bromide staining, and since mitochondrial cDNAs could be readily obtained from this strain (Figures S4 and S5), the very low yield of *atpB* tails could not be accounted for by the quality of the RNA preparation. Therefore, we tentatively conclude that down-regulation of PNPase significantly reduced the number of poly(A) tails for the *atpB* transcript, and perhaps also for all other chloroplast transcripts, and therefore that this enzyme is mainly or even solely responsible for chloroplast polyadenylation in *Chlamydomonas*. This result is in agreement with the observations that CrPAP3 is predicted to be a Ntr (Figure 1), and that CrPAP4 is localized to mitochondria (Figure 2).

Both poly(A)- and (U)-rich tails are present in *Chlamydomonas* mitochondria

Chlamydomonas possesses a short, linear mitochondrial genome, with divergent transcription units emanating from a dual promoter/replication origin region analogous to what is found in animal mitochondrial DNAs (Cardol *et al.*, 2008). The *cox1* transcript, encoding cytochrome oxidase subunit 1, is an easily detectable mRNA, and was chosen for analysis of mitochondrial polyadenylation. In this case we chose primers in the 5' rather than 3' end of the transcript, since these proved more reliable for *cox1*.

When oligo(dT)₁₀ was used to prime first-strand *cox1* cDNA synthesis, the resultant tails were homo- and heteropolymeric poly(A), similar to those observed in the chloroplast (Figure 7, top, and Figure S4). However, unlike the results obtained for *atpB*, where few tails were obtained from the PNPase-deficient strain, no significant differences were obtained for *cox1* when the vector control, PNPI3, and PAP4i were compared (Figures S4 and S5). This suggested that neither PNPase nor PAP4 is responsible for producing mitochondrial tails, that they are redundant (or another

Figure 6. Depletion of polynucleotide phosphorylase (PNPase) results in significant reduction of chloroplast poly(A) tails in *Chlamydomonas*. The coding region of *atpB* is indicated, as are the features downstream of the stop codon, where a majority of the tails are added. ME, mature 3' end of transcript; ECS, endonucleolytic cleavage site involved in transcript maturation (Stern and Kindle, 1993). Primers f1 and f2 were used to amplify the tails (f2 is nested); primer f3 was used to screen *Escherichia coli* colonies for those that contained *atpB* cDNA. Vertical lines indicate sites of polyadenylation, and their heights indicate the number of tails obtained. Tails colored black are from the vector control, red tails are from PAP4i and green tails are from PNPI3. The number of hetero- and homopolymeric tails identified is given in Table S1 and the sequences are presented in Figure S3.



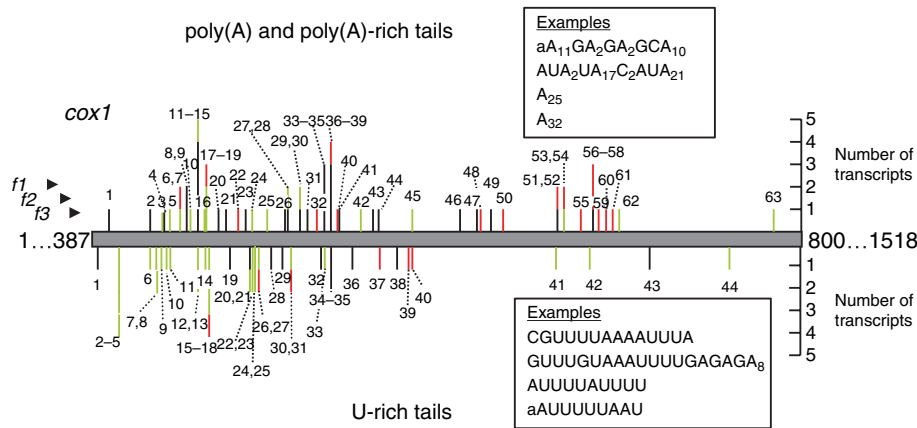


Figure 7. *cox1* mRNA tails in *Chlamydomonas* mitochondria.

Tails added to *cox1* mRNA fragments were analyzed using oligo(dT) to detect polyadenylation (top part). In the lower part, tails are shown when the RNAs were first elongated by poly(G) addition and then reverse transcribed using oligo(dCdT). The number of sequenced tails is shown by the heights of bars, and the boxed sequences are representative examples. Tails obtained from PNPI3 are colored in green, those obtained from the vector control in black, and those from PAP4i in red.

enzyme is redundant with PAP4) and/or that residual PAP4 was sufficient to synthesize a relatively normal number of tails.

Since the oligo(dT) primer can anneal at any position in a long poly(A) or A-rich tail, we used the method of initially adding poly(G) to the RNA 3' end, followed by RT with a chimeric dC₅dT₈ primer. This method is designed to amplify complete poly(A) tails (Komine *et al.*, 2000). Unexpectedly, using this method for *cox1* revealed in addition to poly(A) and poly(A)-rich tails, a significant portion of (U)-rich tails (Figure 7, bottom, and Figure S4). These tails had been added apparently stochastically throughout the part of the coding region analyzed, presumably to RNA degradation intermediates or truncated transcription products. They comprised approximately 70% U and 30% A, with minor contributions of G and C. Typically, runs of three to five Us were punctuated by one to four As. This experiment was repeated on a smaller scale for a second mitochondrial transcript, *nad4*, and similar results were obtained (Figure S5).

These results imply that apart from the well-characterized post-transcriptional addition of poly(A) and poly(A)-rich sequences as part of the degradation pathway, (U)-rich tails are added to truncated transcripts in *Chlamydomonas* mitochondria, although the same method failed to reveal (U)-rich transcripts in *Chlamydomonas* chloroplasts. While poly(U) polymerases and poly(U) or (U)-rich tails were recently observed in fission yeast, human cells and also for miRNAs in *Chlamydomonas* (Ibrahim *et al.*, 2006; Rissland *et al.*, 2007; Heo *et al.*, 2008; Mullen and Marzluff, 2008; Wilusz and Wilusz, 2008), only poly(A) tails have been observed to date in animal and plant mitochondria. Therefore, the finding of both heteropolymeric poly(A) and (U)-rich tails in *Chlamydomonas* mitochondria is a novel feature.

DISCUSSION

In this work, we have analyzed polyadenylation enzymes and poly(A) tails in the organelles of *Arabidopsis* and *C. reinhardtii*. Two Ntr proteins, one in each organism, were found to display PAP activity as recombinant proteins and when expressed in an *E. coli* mutant lacking the endogenous enzyme. One *Arabidopsis* protein displayed CCA-Ntr activity and localization to both organelles. In addition, these experiments yielded two unexpected observations. The first was that depletion of PNPase virtually eliminated our ability to detect poly(A) tails for *atpB* in *Chlamydomonas* chloroplasts, suggesting PNPase to be the sole polyadenylation enzyme in this organelle. The second was the presence of (U)-rich tails in the mitochondria of this organism.

The organellar Ntr- and CCA-PAPs

Two template-independent polyadenylation types of enzyme have been described for prokaryotes, organelles and eukaryotic cells. The first is the Ntr-PAP group, which are very similar in amino sequence to the CCA-Ntr proteins, making it very difficult to predict informatically their biochemical activities (Betat *et al.*, 2004; Martin and Keller, 2004). Therefore, enzyme activity assays are required to determine whether a given protein carries out polyadenylation or CCA addition. In this work we applied two methods – characterizing recombinant proteins and bacterial complementation. Although sequence-based predictions had been performed, none of the predicted organellar Ntrs in plants and algae had been analyzed biochemically. In prokaryotes, the only analyzed Ntr protein displaying polyadenylation activity is the *E. coli* Ntr-PAP called PAP1, encoded by *pcnB* (Raynal and Carpousis, 1999; Yehudai-Resheff and Schuster, 2000). Other Ntrs analyzed in *B. sub-*

tilis and *Bacillus synechocystis* were found to be CCA-Ntrs (Raynal *et al.*, 1998; Rott *et al.*, 2003).

Our work revealed that *Arabidopsis* AtNtr3, which was shown to be targeted to both chloroplasts and mitochondria, is active as a CCA-Ntr. We therefore suggest that this protein is responsible for this activity in both organelles and perhaps also the nucleus/cytoplasm (von Braun *et al.*, 2007). Indeed, AtNtr3 contains the Glu residue which is a characteristic of CCA-Ntrs (Martin and Keller, 2004). *Chlamydomonas* Ntr3 also displayed CCA-Ntr activity when incubated with a tRNA substrate to which CC had not already been added (data not shown). Therefore, we suggest AtNtr3 and CrNtr3 to be CCA-Ntrs, functioning in tRNA maturation in mitochondria and chloroplasts. Interestingly, the next step in tRNA metabolism, namely charging with an amino acid, is also generally carried out by dually targeted enzymes, at least in *Arabidopsis* (Duchene *et al.*, 2005).

In contrast to AtNtr3, AtNtr1 and CrPAP4 displayed polyadenylation activity as recombinant proteins. In addition, these proteins produced homopolymeric poly(A) tails in *E. coli* $\Delta pcnB$, which lacks endogenous PAP. Furthermore, neither protein was able to complete CCA addition to tRNA in its recombinant form. Therefore, in accordance with sequence-based analysis (Martin and Keller, 2004), we suggest that these proteins function as Ntr-PAPs. While mitochondrial localization was observed for both proteins based on transient expression of GFP fusions, AtNtr3 could also be located in the chloroplast. Indeed, multiple targeting locations for Ntr proteins to organelles, the nucleus and cytosol of yeast and plant cells have been described (von Braun *et al.*, 2007). Accordingly, the CCA-Ntrs were suggested to be 'sorting isozymes', i.e. multiple isoforms of proteins are synthesized from a single gene and distributed to different subcellular compartments (Chen *et al.*, 1992; Reichert *et al.*, 2001; Keady *et al.*, 2002; von Braun *et al.*, 2007). It is possible that the use of multiple in-frame start codons allows for the production of variant forms of the enzyme containing different targeting information.

In *Chlamydomonas* chloroplasts, PNPase may be the sole polyadenylation enzyme

Two type of proteins polyadenylate prokaryotic and organellar RNAs, namely the Ntr-PAPs and PNPase. While Ntr-PAP produces homopolymeric poly(A) tails using ATP as a substrate, PNPase, as a phosphorylase, polymerizes heteropolymeric poly(A)-rich tails using nucleotide diphosphates (NDPs) as substrates (Slomovic *et al.*, 2006). In addition to these two enzymes, in archaeal species in which polyadenylation takes place, the archaeal exosome complex, which is very similar to PNPase, is responsible for polyadenylation, producing heteropolymeric tails (Portnoy *et al.*, 2005). Indeed, PNPase is present in every bacterium

and organelle where polyadenylation takes place, excluding the mitochondria of trypanosomes, although in mammalian mitochondria it is located in the intermembrane space, physically separated from mtRNA (Chen *et al.*, 2006, 2007). In several bacteria such as cyanobacteria and the Gram-positive *S. coelicolor*, there is no Ntr-PAP, and PNPase is solely responsible for polyadenylation activity, producing heteropolymeric tails (Rott *et al.*, 2003; Sohlberg *et al.*, 2003). In other bacteria, such as *E. coli*, both Ntr-PAP and PNPase are active, producing both homo- and heteropolymeric tails (Mohanty *et al.*, 2004). Similarly, both hetero- and homopolymeric tails were found in *Arabidopsis* chloroplasts (data not shown), and depletion of PNPase did not abolish polyadenylation (Walter *et al.*, 2002). The results shown here, where depletion of PNPase in *Chlamydomonas* virtually eliminated the chloroplast poly(A) tails of *atpB*, suggests that PNPase is solely responsible for the polyadenylation activity, apparently a different situation than in *Arabidopsis* chloroplasts. This situation is similar to that of cyanobacteria and archaea where the phosphorolytic activity, using NDPs, is solely used for polyadenylation, which perhaps reflects the ancestral version of the polyadenylation process (Slomovic *et al.*, 2008b). It should be noted that because a certain length of poly(A) tail is necessary for binding of the oligo(dT) RT primer, we cannot exclude that the PNPase-deficient *Chlamydomonas* strain has very short poly(A) tails that would not be detected by this method. Indeed, some evidence for such tails was found in wild-type *Chlamydomonas* (Komine *et al.*, 2000), and in human mitochondria short oligo(A) tails were found to be added neither by PNPase nor the canonical Ntr-PAP (Slomovic and Schuster, 2008).

As mentioned above, tails produced by PNPase contain all four nucleotides, reflecting their relative concentrations (Slomovic *et al.*, 2008b). Compared with cyanobacteria, the tails in *Chlamydomonas* chloroplasts are rich in adenosines, making them closely related to homopolymeric tails. This observation could be explained by assuming a relatively high concentration of ADP in this organelle, relative to other NDPs. Polynucleotide phosphorylase has recently been characterized to be very important for phosphorus starvation response-related processes in *Chlamydomonas*, including regulation of RNA accumulation in the chloroplast (Yehudai-Resheff *et al.*, 2007). Taken together, the observations that PNPase may be solely responsible for chloroplast polyadenylation in *Chlamydomonas* and that it has a key role in stress response raise the possibility that polyadenylation, and degradation of those tails, which also requires PNPase (Nishimura *et al.*, 2004), are integral to both RNA and phosphorus metabolism.

(U)-rich tails in *Chlamydomonas* mitochondria

When we polyguanylated total RNA of *Chlamydomonas* and then amplified mitochondrial cDNAs using a C₅T₈

primer, we expected to identify poly(A) tails as we had previously done for chloroplast transcripts (Komine *et al.*, 2000). Instead, we found along with poly(A)-rich tails, numerous sequences that could be characterized as (U)-rich. This contrasts strongly with previous findings in plants and human mitochondria of only homopolymeric poly(A) tails (Gagliardi and Leaver, 1999; Lupold *et al.*, 1999; Slomovic *et al.*, 2005; Holec *et al.*, 2006). However, (U)-rich tails were found in trypanosome mitochondria and in the mitochondria of human cells where PNPase was significantly down-expressed by RNAi (Ryan and Read, 2005; Etheridge *et al.*, 2008; Slomovic and Schuster, 2008). In addition, polyuridylated histone transcripts were recently found in human cells during the cell cycle stage in which these mRNAs undergo rapid degradation (Mullen and Marzluff, 2008; Wilusz and Wilusz, 2008). Uridine-containing poly(A) tails were found in *Schizosaccharomyces pombe* (Rissland *et al.*, 2007), and oligo(U) tails are added to siRNAs in *Chlamydomonas*, probably as part of their degradation (Ibrahim *et al.*, 2006). In addition, members of the Ntr-PAP family were found to display poly-uridylation activity when the recombinant proteins were assayed *in vitro*, or suspected to be responsible for the uridylation of histone transcripts (Rissland *et al.*, 2007; Mullen and Marzluff, 2008).

The mitochondrial (U)-rich sequences described are likely to be intermediates in the degradation process, like their poly(A) counterparts. This suggestion is based on the observations that a multistep PCR protocol is necessary to detect these tails, and that the tails are not added at the 3' end but apparently to a truncated transcript. We propose that these truncated and elongated transcripts are of low abundance in mitochondria because they undergo rapid degradation. Therefore, the (U)-rich tails may function as poly(A) tails do, by providing a platform for an exoribonuclease to bind and, while progressively degrading the molecule, successfully overcome secondary structures. Indeed, the spinach chloroplast and human mitochondrial PNPases display high affinity for poly(U) (Yehudai-Resheff *et al.*, 2003; Portnoy *et al.*, 2008). However, as mentioned above, it is as yet unknown whether the single PNPase of *Chlamydomonas* is targeted to mitochondria, as it is to the chloroplast (Zimmer *et al.*, 2008). In addition, it is unknown whether the RNase R type or oligoribonuclease type exoribonucleases encoded in the *Chlamydomonas* genome are localized and function in mitochondria (Zimmer *et al.*, 2008). An additional open question is the identity of the enzyme polymerizing the (U)-rich tails and whether it is one of the Ntr-PAPs described in this work. Taken together, further studies are required in order to explore if the mitochondrial (U)-rich tails are also present in other organisms and whether the function in the degradation process is indeed similar to the destabilizing poly(A) tails.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

For CrPAP4 expression, *E. coli* ENS134-3 cells (Mohanty and Kushner, 2000) were grown in LB medium containing 1 M sorbitol and 50 mM Na phosphate buffer (LBSP) containing ampicillin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) at 30°C. Expression was induced with 0.004% arabinose for 10 h at 22°C. For expression of AtNtr1 and AtNtr3, M15 [pRep4] *E. coli* cells were transformed with appropriate expression vectors (see below). For expression of AtNtr2, *E. coli* XL-1 Blue was used. Expression was induced by 1 mM IPTG at OD 0.6 and cultures transferred to 20°C for 8 h.

For the complementation of *E. coli* Δ *pcnB* (SK7988) (Mohanty and Kushner, 2000) with CrPAP4 or AtNtr1, cultures were initiated in LB supplemented with 50 µg ml⁻¹ thiamine. The cells were grown for 40 min at 37°C and then induced for 30 min with 0.005% arabinose (CrPAP4) or 0.1 mM IPTG (AtNtr1). The plasmids used were the same as those used for expressing recombinant proteins for *in vitro* assays (see DNA constructs, below). The *E. coli* Δ *pcnB* was a kind gift of Dr Sidney Kushner (University of Georgia, Athens, GA, USA).

Chlamydomonas transformation with RNAi vectors

Cell-wall-deficient *Chlamydomonas* (CC-849) cultures were grown in 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-acetate-phosphate (TAP) medium (Harris, 1989) plus 1% sorbitol under continuous light at 25°C. *Chlamydomonas* transformants of the recipient strain CC-849, generated by electroporation (Shimogawara *et al.*, 1998), were selected on TAP medium containing 12 µM 5-fluorouracil and 1.5 mM tryptophan (Yehudai-Resheff *et al.*, 2007).

DNA constructs

To generate GFP fusions, the sequences encoding the fragment lengths described in the Results were amplified from cDNA, inserted by BP recombination into the Gateway entry vector pDONR207, and recombined into pMDC83 (Curtis and Grossniklaus, 2003).

The CrPAP4 expression construct was generated by inserting nucleotides 175–2376 into pENTR (Invitrogen, <http://www.invitrogen.com/>). Gateway LR Clonase facilitated recombination of the fragments into the pBAD-DEST49 expression vector. Site-directed mutagenesis, in which amino acids 315 and 317 were changed from aspartic acid to alanine, was carried out to generate the catalytically inactive form.

To produce the CrPAP4 RNAi vector, nucleotides 1–171 of the coding region, corresponding to the predicted transit peptide, were inserted into the pENTR vector. A double Gateway LR recombination was then used to insert this fragment in inverted orientations into pGwyRNAi (Rohr *et al.* (2004).

To produce the AtNtr1 and AtNtr3 expression vectors, cDNAs encoding these proteins were inserted into pQE30 (Qiagen, <http://www.qiagen.com/>) in-frame with an N-terminal His₆ tag. Transit peptide-encoding sequences of AtNtr1 (nucleotides 1–225) were removed by site-directed deletion, using the QuickChange site-directed mutagenesis kit (Stratagene, <http://www.stratagene.com/>). Nucleotides 1–252 of AtNtr3 were removed by *SacI* digestion and religation. For AtNtr2, a DNA fragment encoding amino acids 71–1524 was PCR-amplified and introduced into pMal-c2x (New England Biolabs, <http://www.neb.com/>), to express a maltose-binding protein-AtNtr2 fusion protein.

Generation of antibody

The CrPAP4 antibody was generated against recombinant protein at Lampire Biological Laboratories. The *E. coli* PAP antibodies were obtained from Dr Agamemnon Carpousis (University of Toulouse, Toulouse, France).

Confocal microscopy

Tomato protoplasts were transformed using the method described in Xing *et al.* (2001). Protoplasts were visualized 19–22 h post-transformation and images were collected on a Leica TCS-SP5 confocal microscope (Leica Microsystems, <http://www.leica.com/>). To visualize mitochondria, incubation in Mitotracker CMXROS (Molecular Probes, <http://www.invitrogen.com>) at 200 nm for 25 min was performed.

Purification of recombinant proteins

For CrPAP4, cells were collected and lysed in lysis buffer (50 mM Tris pH 7.5, 500 mM NaCl, 2 mM imidazole, 2% Triton X-100, 7.5% glycerol, 20 mM β -mercaptoethanol and 1 mM EDTA). Following purification on HisTrap and MonoQ columns (Amersham, <http://www.gelifesciences.com>), the protein was concentrated and frozen in aliquots. The bovine PAP expression plasmid was obtained from Dr E. Wahle (University of Halle, Germany). Expression and purification were carried out as described (Wahle, 1991).

For the purification of the recombinant proteins AtNtr1 and AtNtr3, Bacteria were collected following transformation and induction and lysed with a microfluidizer in lysis buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 2% Triton X-100, 10% glycerol and 20 mM β -mercaptoethanol). The recombinant proteins were purified by a HisTrap affinity column following by fractionation on a MonoQ column (Amersham). For obtaining the recombinant AtNtr2 protein, induced cells were resuspended in a column buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.25% Tween-20), lysed and the recombinant protein purified by a bench-top amylose resin column (New England Biolabs). The purified fusion of the maltose-binding protein and AtNtr2 was then cleaved by incubation with 10 μ g TEV protease (Invitrogen) for 4 h at 16°C. The maltose-binding protein was removed by passage through an amylose resin column, and AtNtr2 was further purified by MonoQ column chromatography (Amersham).

Activity assays

The CCAtr activity assay. The [32 P]-labeled tRNA^{Phe} substrate utilized in the CCAtr assays was generated from a plasmid described in Schurer *et al.* (2001). This plasmid and a vector encoding full-length *E. coli* CCAtr, were kind gifts of Dr Heike Betat (University of Leipzig, Germany). The CCAtr assay was performed as follows: After *in vitro* transcription and ribozyme cleavage as described (Schurer *et al.*, 2001), 73-nt-long [32 P]-labeled tRNA^{Phe} was gel-purified and incubated with CTP and the recombinant *E. coli* CCAtr to generate the tRNA^{Phe} with a CC 3' extension. Following phenol-chloroform extraction and ethanol precipitation, this modified tRNA was used as the substrate for the CCAtr reaction performed in buffer E (Portnoy *et al.*, 2008) containing 1 mM ATP and 120 ng protein for 15 min at 25°C. Alternatively, the [32 P]-labeled tRNA^{Phe} was incubated in a buffer containing 1 mM ATP and CTP.

Polyadenylation assay. Purified recombinant protein (150 ng) was incubated in buffer D (25 mM Tris-HCl, pH 8, 40 mM KCl, 0.5 mM MnCl₂, 0.005 mM EDTA and 10% glycerol) with 9000 c.p.m. (approximately 2 ng) of [32 P]-labeled A₂₀ oligoribonucleotide and 0.5 mM NTP or NDP for different times as indicated in the figures.

The RNA was purified and analyzed as described (Stern and Grissem, 1987).

Analysis of polyribonucleotide tails

SuperScript III Reverse Transcriptase (Invitrogen) was used to synthesize cDNA in a 20- μ l reaction from 2 μ g total RNA using the dT-adaptor primer GACTCGAGTCGACATCGA(T)₁₀ at 45°C for 1 h. When the dCdT-adaptor primer GACTCGAGTCGACATCGA(C)₅(T)₈ was used instead, the RNA was first G-tailed as described (Komine *et al.*, 2000). Two microliters of the cDNA was used in 20- μ l PCR amplification (PCR 1) using the adaptor primer and an f1 primer (Table S2). Reactions were separated in a 1.5% agarose gel and the resulting smear of appropriately sized products was excised and gel-purified with the Qiaex II gel purification kit (Invitrogen). Two microliters of the purified PCR 1 reaction was used as a template in a nested PCR amplification (PCR 2) using the adaptor primer and an f2 primer, and the product purified as for PCR 1. All of the precipitated products of PCR 2 were resuspended and TOPO-cloned into pCR2.1 (Invitrogen), then transformed into Top10 cells. Selected colonies were analyzed by colony PCR using the gene-specific f3 primer and the adaptor primer, and products examined in 2% agarose gels (Slomovic *et al.*, 2008a).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Depletion of PNPase in *Chlamydomonas*.

Figure S2. Depletion of PAP4 in *Chlamydomonas*.

Figure S3. The poly(A) and poly(A)-rich tails fused to the chloroplast *atpB* transcript in *Chlamydomonas*.

Figure S4. Poly(A)- and poly(U)-rich tails associated with the mitochondrial *cox1* transcript in *Chlamydomonas*.

Figure S5. Heteropolymeric polyadenylated tails identified in *Chlamydomonas* for the mitochondrial *nad4* transcript.

Table S1. Summary of nucleotide tails.

Table S2. Primers used.

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