An *Arabidopsis* Dual-Localized Pentatricopeptide Repeat Protein Interacts with Nuclear Proteins Involved in Gene Expression Regulation [W]

Kamel Hammani,[a,b,1] Anthony Gobert,[a] Kamal Hleibieh,[a] Laurence Choulier,[c] Ian Small,[b] and Philippe Giegé[a,2]

[a] Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université de Strasbourg, 67084 Strasbourg Cedex, France

[b] Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley, Western Australia 6009, Australia

[c] Institut de Recherche de l'Ecole de Biotechnologie de Strasbourg, Centre National de la Recherche Scientifique/Université de Strasbourg, 67400 Illkirch Cedex, France

Following the endosymbiotic acquisition of mitochondria by eukaryotic cells, most of the genes in this organelle were transferred to the nucleus. To maintain mitochondrial biogenesis and function, nuclear and mitochondrial genomes require regulated and coordinated expression. In plant organelles, nuclear-encoded proteins targeted to the organelles control posttranscriptional and posttranslational mechanisms. Pentatricopeptide repeat (PPR) proteins are good candidates to play such regulatory roles. Here, we identify PNM1 (for PPR protein localized to the nucleus and mitochondria 1), a novel PPR protein that is dual localized to mitochondria and nuclei in *Arabidopsis thaliana*, as observed by green fluorescent protein fusions and immunodetection on subcellular fractions and on histological sections. Genetic complementation showed that loss of *PNM1* function in mitochondria, but not in nuclei, is lethal for the embryo. In mitochondria, it is associated with polysomes and may play a role in translation. A genetic screen in yeast identified protein partners of PNM1. These partners, the nucleosome assembly protein NAP1, and the transcription factor TCP8 interact with PNM1 in the nucleus in planta. Furthermore, TCP8 can bind the promoter of *PNM1*. This suggests that PNM1 might be involved in the regulation of its own gene expression in the nucleus and could thus play a role in gene expression adjustments between mitochondria and the nucleus.

INTRODUCTION

In eukaryotic cells, mitochondria originated from free-living bacterial ancestors (Gray et al., 2001). Mitochondria have retained a genome; however, during evolution, the vast majority of the endosymbiont's genes have been transferred to the nucleus, allowing the nucleus to consolidate its genetic control over the organelle. As a result, the mitochondrial genome encodes a limited number of proteins that are mainly components of the respiratory chain and translational machinery to express their genes (Burger et al., 2003). On the other hand, proteomic and genomic analyses of protein localization sequences estimate that organelles might contain up to several thousand different proteins (Millar et al., 2005). As these numbers imply, most proteins found in organelles are encoded in the nucleus, synthesized in the cytoplasm, and then imported into the organelles. Hence, components of the respiratory chain and organellar

ribosomes are multiprotein complexes made up of nuclear- and organelle-encoded subunits. This implies the existence of mechanisms that coordinate the expression of organellar and nuclear genomes to ensure correct assembly of complexes and to maintain organelle function. However, we are still largely ignorant of the mechanisms that lead to the coordination of expression of nuclear and organellar genomes in individual cells and tissues.

Among the estimated 1000 or more proteins that constitute the functional plant mitochondrion (Millar et al., 2005), only 33 proteins, three rRNAs, and 20 tRNAs are encoded in the *Arabidopsis thaliana* mitochondrial genome (Unseld et al., 1997). The remaining proteins and tRNAs are encoded in the nuclear genome and imported into the mitochondrion from the cytosol (Braun and Schmitz, 1999; Salinas et al., 2008). Although our understanding of the signaling pathways between the nucleus and chloroplast has improved significantly in recent years (Pogson et al., 2008; Woodson and Chory, 2008), comparable studies on plant mitochondria have mainly focused on understanding the nuclear control of mitochondrial biogenesis (Mackenzie and McIntosh, 1999; Edqvist and Bergman, 2002; Giraud et al., 2009). Studies in *Arabidopsis* by Brennicke and coworkers have shown that regulation of mitochondrial gene expression involves complex transcriptional and posttranscriptional processes, including RNA processing, intron splicing, RNA editing, and RNA stability (Binder and Brennicke, 2003). A comparative study of the transcriptional

activities and steady state levels of the various mRNAs encoded in the *Arabidopsis* mitochondrial genome revealed little correlation between relative promoter activities and transcript abundance, suggesting extensive posttranscriptional regulation (Giegé et al., 2000).

Genetic analysis of mutants defective in organellar functions in yeast, *Chlamydomonas reinhardtii*, and land plants has revealed many nuclear-encoded posttranscriptional regulators of organelle gene expression. It is likely that these proteins act as adaptors; they bind specific RNA transcripts and recruit translation machinery or RNA metabolism enzymes. Many of these regulators belong to the pentatricopeptide repeat (PPR) protein family. The PPR family is a eukaryote-specific protein family and is particularly large in land plants, with >450 members in *Arabidopsis*. PPR proteins are characterized by the signature motif of a degenerate 35–amino acid repeat always arranged in tandem arrays. Most PPR proteins are predicted to be targeted to either mitochondria or chloroplasts (Lurin et al., 2004). A rapidly growing number of publications relate the function of PPR proteins in posttranscriptional processes in organelles (Schmitz-Linneweber and Small, 2008). Among these processes, PPR proteins have been linked to translation, as suggested in plants by the involvement of CRP1 as a chloroplast translation regulator (Schmitz-Linneweber et al., 2005) and by the association of PPR336 with polysomes in mitochondria (Uyttewaal et al., 2008).

Many posttranscriptional steps are shared between organelles and the nucleus, leading to the expectation to find proteins involved in DNA or RNA metabolism that are targeted to all three compartments in plant cells (Small et al., 1998). The first example of a protein dual targeted to an organelle and the nucleus within the same cell came from Whirly1 (Grabowski et al., 2008; Prikryl et al., 2008). In most cases, the dual-targeted proteins (nuclear-organellar proteins) that we know today are RNA and/or DNA binding proteins (e.g., transcription factors and telomere binding proteins). Such dual-targeted factors are good candidates for coordinating the expression of nuclear and organellar genomes (Krause and Krupinska, 2009).

Here, we identified an *Arabidopsis* embryo-lethal mutant designated *pnm1* (for PPR protein localized to the nucleus and mitochondria 1). *PNM1* encodes a novel pentatricopeptide repeat protein that is dual localized to mitochondria and nuclei. PNM1 has RNA binding capacity and has an essential function in mitochondria that may be related to translation. We showed that PNM1 interacts with the nucleosome assembly protein NAP1 and the nuclear transcription factor TCP8 in yeast and in the nucleus of plant cells. Altogether, our findings suggest that PNM1, by interaction with both nuclear transcriptional factors and mitochondrial ribosomes, is a potential coordinator of the expression of the two genomes.

RESULTS

PNM1 Is Essential for Embryo Development

To gain insights into the involvement of PPR proteins in mitochondrial translation as suggested by the interaction of PPR336 with mitochondrial polysomes (Uyttewaal et al., 2008), we initially performed a reverse genetic screen of *Arabidopsis* mutants representing PPR genes of unknown function. The collections from the Syngenta *Arabidopsis* Insertion Library and Salk Institute were screened to look for T-DNA insertions in *Arabidopsis* PPR genes. We were unable to find any homozygous plants for two independent T-DNA insertion alleles for At5g60960 when we looked at progeny of a large number of heterozygous plants. We named this gene *PNM1* for PPR protein localized to the nucleus and mitochondria 1 (Figure 1A; see Supplemental Figure 1 online). The dissection of siliques from heterozygous mutant plants showed that about one-quarter of the embryos are aborted (Figure 1B), indicating that homozygous mutation of *PNM1* is lethal at an early stage of embryo development and, thus, that PNM1 has an essential function.

PNM1 Is Expressed in Young Tissues and Pollen Grains

The availability of numerous EST sequences confirms that *PNM1* is expressed. The *PNM1* 5′ transcript end was characterized by 5′ rapid amplification of cDNA ends. Transcript ends were exclusively located in a region between 26 and 40 nucleotides
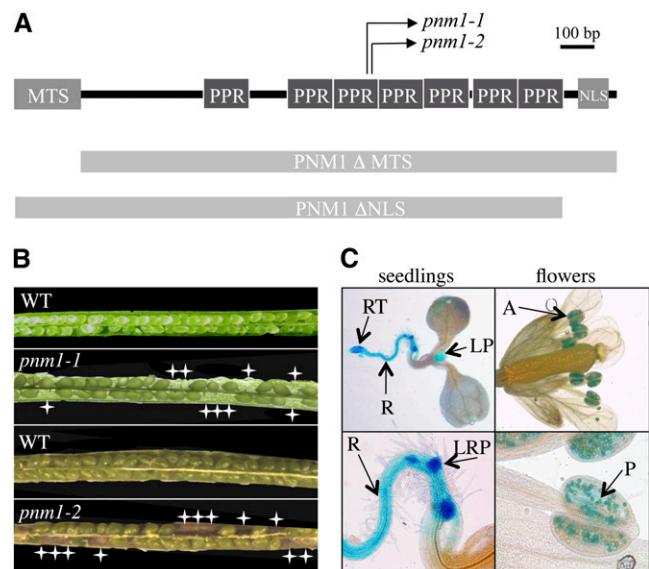
Figure 1. Molecular Characterization of *PNM1*.

(A) Diagram of the insertion positions of T-DNAs in *PNM1*. The different domains encoded by the gene are represented. Rectangles in the bottom panel represent the mutant *PNM1* genes cloned in binary vectors for functional complementation assays. *PNM1ΔMTS*, MTS truncated *PNM1*; *PNM1ΔNLS*, C-terminal domain truncated.

(B) *PNM1* is essential for embryo development. Phenotype of siliques from self-fertilized *PNM1/pnm1 Arabidopsis* plants for two independent T-DNA insertion alleles. Stars indicate aborted seeds with *pnm1/pnm1* embryos. WT, wild type.

(C) Histochemical localization of GUS activity in *Arabidopsis* plants transformed with the intergenic region upstream *PNM1* gene fused to the *GUS* reporter gene. Left: *GUS* staining of 8-d-old seedlings showing *PNM1* expression in the root (R), the root tip (RT), lateral root primordia (LRP), and leaf primordium (LP). Right: *GUS* staining of flower showing *PNM1* expression in pollen grains (P) within the anthers (A).

upstream of the *PNM1* initiation codon (see Supplemental Figure 2 online). It suggests that the *PNM1* transcript has neither alternative transcription start sites nor alternative splicing. To investigate the expression pattern of *PNM1* in different tissues, we used a promoter–β-glucuronidase (GUS) reporter system. The sequence between the start codon of *PNM1* and the stop codon of the upstream gene was used to drive the *GUS* reporter gene (P*PNM1*-*GUS* construct) and stably introduced in *Arabidopsis* by agroinfection. In five independent P*PNM1*-*GUS* plant lines, GUS activity was visible in root tips, lateral root primordial, and leaf primordia (Figure 1C). In addition, a high level of GUS activity was detected in the mature pollen grains. These data indicate that *PNM1* is mostly expressed in pollen grains and in actively dividing cells during vegetative growth.

PNM1 Is Dual Localized in Mitochondria and the Nucleus

At5g60960 encodes a putative protein of 521 amino acids with an estimated molecular mass of 59 kD. Sequence analysis revealed that the protein harbors seven PPR motifs and two predicted localization signals (Figure 1), a mitochondrial target signal (MTS) at its N terminus, and a putative nuclear localization signal (NLS) rich in basic residues at its C terminus. Alignment of PNM1 with orthologs from numerous plant species shows that this basic region is highly conserved and thus might be functional (see Supplemental Figure 1 online). The presence of the two predicted localization signals suggested that PNM1 could be dual targeted to mitochondria and nuclei and led us to investigate the precise distribution of PNM1 in *Arabidopsis* cells. To analyze the subcellular localization of PNM1 in plant cells, we first transiently expressed in tobacco (*Nicotiana tabacum*) cells different green fluorescent protein (GFP)-tagged proteins under the control of a 35S promoter (Figure 2A). The C-terminal translational fusion of the full-length *PNM1* with GFP (PNM1_GFP) localizes to mitochondria of tobacco cells. The N-terminal fusion of GFP to PNM1 prevents its import into mitochondria, and the GFP_PNM1 protein shows a nuclear-cytosolic localization. Deletion of the mitochondrial target signal (PNM1ΔMTS_GFP) restricts the localization of the protein to the nucleus. These results suggest that PNM1 harbors a signal that can target the protein to the nucleus. To verify that this nuclear targeting property is indeed encoded by the C-terminal putative NLS signal, the last 174 bp of *PNM1* were fused in frame with *GUS* gene (GUS_NLS_GFP) encoding the cytosolic GUS protein. The GUS_NLS_GFP protein was localized exclusively to the nucleus. This shows that the putative NLS signal is active in plant cells. To confirm the mitochondrial and nuclear localizations of PNM1 in vivo, immunodetection was performed on *Arabidopsis* subcellular fractions using polyclonal antibodies generated against the full-length PNM1. The antibodies detected a unique 55-kD protein in the mitochondrial and nuclear fractions. The purity of the respective subcellular fractions was demonstrated with antibodies specific to each subcellular fraction (Figure 2B). To determine to which form of PNM1 the 55-kD signals correspond, the full-length and the predicted mature form (amino acids 60 to 521) of PNM1 were expressed and purified and their sizes compared with the signals detected. This analysis revealed that the signals correspond to the mature form of PNM1 (see Supplemental Figure 3 online).
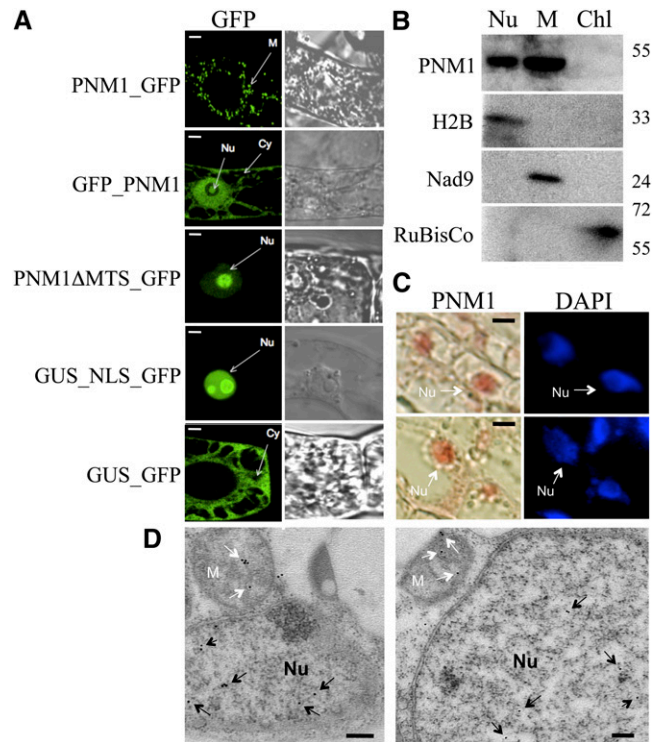
Figure 2. PNM1 Is Dual Localized to the Nuclei and Mitochondria of Plant Cells.

(A) BY-2 cells transiently expressing GFP fusion proteins were observed by confocal microscopy 6 h after bombardment. M, mitochondria; Nu, nucleus; Cy, cytosol. Bright-field images are shown in the right panels. Bars = 500 nm.

(B) Protein fractions from *Arabidopsis* were analyzed by immunoblots probed with antibodies directed against PNM1. Protein extracts of nuclei (Nu), mitochondria (M), and chloroplasts (Chl). The purity of the respective fractions was controlled with antibodies directed against the nuclear histone protein H2B, the subunit 9 of the mitochondrial complex I (Nad9), and the chloroplast large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo).

(C) Immunocytology experiments with PNM1 antibodies used on *Arabidopsis* seedling sections show a colocalization of PNM1 (red staining) with nuclei (4',6-diamidino-2-phenylindole [DAPI] staining). Bars = 1 μm.

(D) Immunogold labeling of PNM1 observed by electron microscopy. Black arrows indicate gold particles in the nucleus, whereas white arrows indicate gold particles in mitochondria within the same cells. Bars = 100 nm.

Localization was further investigated by immunohistochemistry. PNM1 antibodies were used to detect the protein in histochemical sections from *Arabidopsis* seedlings. Signal was observed in structures that correspond to nuclei as indicated by the colocalization with 4',6-diamidino-2-phenylindole staining (Figure 2C). Signal specificity was confirmed by comparing the PNM1 primary antibody with its preimmune serum. Localization was also investigated by immunogold labeling with PNM1-specific antibodies and electron microscopy (Figure 2D). This showed that PNM1 can be localized in mitochondria and the nucleus within the same cell. Altogether, the results show that PNM1 is a novel dual-localized PPR protein in *Arabidopsis* cells.

The Function of PNM1 Is Essential in Mitochondria

The dual localization of PNM1 in *Arabidopsis* cells reveals that the protein likely plays an essential role in either mitochondria or the nucleus or in both organelles. To determine which localization is essential for embryogenesis, heterozygous *pnm1-1* mutant plants were complemented with constructs driven by the endogenous *PNM1* promoter and expressing either the wild-type *PNM1* gene or truncated versions deleted of either the mitochondrial targeting signal or the NLS. The self-progeny of three independent transgenic and heterozygous *pnm1-1* plants (T1) for each construct were analyzed by genotyping. The wild-type transgene successfully complemented the *pnm1* mutation, indicating that the embryo-lethal phenotype was indeed caused by the loss of function of *At5g60960*. By contrast, the truncated gene lacking a MTS did not complement the *pnm1* mutation. Finally, the version deleted of the C-terminal part of the *PNM1* gene complemented the *pnm1* mutation and resulted in viable plants. These results indicate that PNM1 has an essential function in mitochondria and a distinct, although nonessential, role in the nuclei of plant cells.

PNM1 Is an RNA Binding Protein

PPR proteins were predicted to be RNA binding proteins (Small and Peeters, 2000), and studies have already shown that some PPR proteins can indeed directly bind RNA (Okuda et al., 2006). Here, we used Biacore technology based on surface plasmon resonance (Fivash et al., 1998) to establish whether PNM1 was also able to bind RNA or not. Recombinant PNM1 was captured on sensor chips, and RNA solutions were used as potential ligands. High-affinity binding was observed when *Arabidopsis* total mitochondrial RNA was used but not with total plastidial RNA. The release of RNA from PNM1 was slow, which suggests that the interaction with RNA is stable rather than transient. Mitochondrial RNA were fractionated by LiCl precipitation and used for interaction assays. Interaction was observed with mitochondrial RNA from the LiCl pellet containing mRNA as well as 26S and 18S rRNAs and not with the LiCl supernatant, which contains tRNAs and 5S rRNA (Giegé et al., 1998). Kinetic analyses were performed through consecutive injections of increasing concentrations of RNA. This enabled to determine that PNM1 is able to bind RNA with a K_d in the nanomolar range (see Supplemental Figure 4 online). When oligoribonucleotides representing poly A, C, G, and U were used, PNM1 was able to bind poly G with high affinity as already observed for other PPR proteins (Lurin et al., 2004), but not the other oligoribonucleotides. Thioredoxin or tobacco mosaic virus protein was used as a negative control to show that the affinity response observed was indeed due to RNA/protein interaction and not to artifactual binding of RNA to sensor chips. Altogether, the results indicate that PNM1 is able to bind RNA.

PNM1 Is Associated with Polysomes in Plant Mitochondria

PNM1 plays an essential function in *Arabidopsis* mitochondria. Its capacity to bind RNA suggests that its function could be related to an essential posttranscriptional process in mitochondria, as observed for a rapidly growing number of PPR proteins in plant organelles (Schmitz-Linneweber and Small, 2008). We first investigated the submitochondrial localization of PNM1. It was detected in mitoplasts (i.e., matrix and inner membrane) and in soluble and extrinsic membrane protein fractions (Figure 3A). These results indicate that PNM1 is a soluble protein in the mitochondrial matrix and a peripheral protein of the inner membrane as well. Subsequently, the association of PNM1 with complexes containing RNA in mitochondria was investigated through the immunodetection of PNM1 in complexes separated by sucrose density gradients. Fractions representing the entire gradients were collected. Equivalent amounts of proteins from
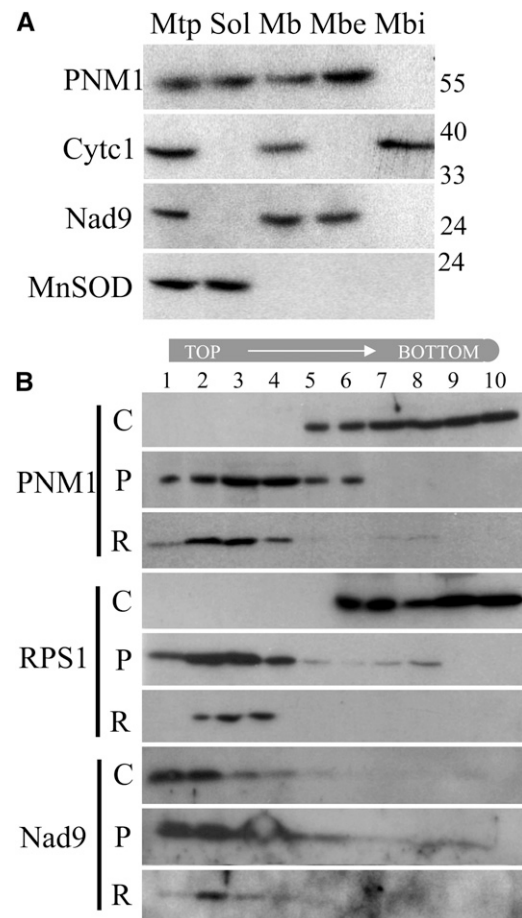
Figure 3. PNM1 Is Associated with Polysomes in Plant Mitochondria.

(A) Immunodetection of PNM1 on submitochondrial protein fractions. Mtp, mitoplasts; Sol, soluble proteins; Mb, membrane proteins; Mbi, intrinsic membrane protein; Mbe, extrinsic membrane proteins. Fractionations are controlled with antibodies specific for the intrinsic membrane protein cytochrome c1 (Cyt c1), complex I subunit 9 (NAD9), and the soluble MnSOD. Molecular masses are given in kilodaltons.

(B) Mitochondrial protein fractions were collected from sucrose gradients, separated by SDS-PAGE, blotted, and reacted with antibodies specific for PNM1, NAD9, or the ribosomal protein RPS1. Control (C) sample was untreated, whereas other samples were treated to destabilize polysomes with puromycin (P) or RNases (R).

each fraction were reacted with PNM1 antibodies. The 55-kD signal was detected for fractions at the bottom of the gradients (Figure 3B). Fractions were also reacted with antibodies specific for the ribosomal protein RPS1 and NAD9 from respiratory complex I. NAD9 was only detected in the top fractions of the gradients, suggesting that they contain complexes of sizes up to 2000 kD. On the other hand, RPS1 was detected in the same bottom fractions of the gradient as PNM1. These bottom fractions were proposed to contain polysomes (Delage et al., 2007). This suggested that PNM1 could be associated with polysomes. Mitochondrial samples were subjected to treatments leading to the destabilization of polysomes to demonstrate this assumption. Samples were treated with puromycin, which specifically destabilizes ribosomes (Lu and Draper, 1994), and with RNase, which results in the degradation of RNA necessary to maintain polysome integrity. In all cases, after treatment and separation on gradients, PNM1, similar to RPS1, was no longer detected in bottom fractions (Figure 3B). This confirms that PNM1 is indeed associated with polysomes in an RNA-dependent manner in *Arabidopsis* mitochondria, which is consistent with the capacity of PNM1 to bind RNA, although the precise nature of this association with polysomes is unknown.

To identify the precise RNA targets of PNM1 in mitochondria, extensive efforts were deployed to coimmunoprecipitate PNM1 in its native form bound to RNA partners from a solubilized *Arabidopsis* mitochondrial extract with either anti-PNM1 serum or purified anti-PNM1 polyclonal antibodies as described previously (Schmitz-Linneweber et al., 2005). Surprisingly, we were unable to immunoprecipitate PNM1 presumably because the epitopes recognized by the antibodies in denaturing conditions are hidden when the protein is in its native form.

PNM1 Interacts with Nuclear Proteins

The identification of protein partners should give clues to understanding the function(s) of PNM1. For this, a screen using a system derived from yeast two-hybrid was performed. The DUAL hunter system (DualsystemsBiotech) enables the use of a nuclear protein as bait. Briefly, a bait protein of interest is inserted in frame with the membrane protein Ost4p, the C-terminal half of ubiquitin, and the transcription factor LexA-VP16 (X-Cub constructs). A second protein of interest (the prey) is fused at the C terminus of a mutated version of the N-terminal domain of ubiquitin (Nub-X constructs). If bait and prey interact, Cub and NubG complement to form split-ubiquitin. Then, ubiquitin-specific proteases release LexA-VP16, which migrates to the nucleus and activates the transcription of reporter genes (Möckli et al., 2007). We screened an *Arabidopsis* seedling cDNA library for proteins that interact with PNM1. Of 10^7 transformants screened, 11 positive clones were identified. Eight different cDNA clones contained sequences of *At4g26110* encoding NAP1, the Nucleosome Assembly Protein 1. The cDNAs rescued from the three other clones represented a nuclear transcriptional factor TCP8 (AT1G58100) and two proteins of unknown functions (AT5G24680 and AT1G44920). TCP8 belongs to the TCP transcription factor family known to bind site II elements in promoter regions (Martín-Trillo and Cubas, 2010). AT5G24680 contains a ubiquitin fold modifier-specific peptidase domain (IPR012462),

suggesting that the protein could interact with the ubiquitin protein fused to PNM1 rather than with PNM1 itself. AT5G24680 and AT1G44920 do not contain any predictable localization or function in mitochondria and/or the nucleus and were thus not further investigated at this stage. The full-length mature PNM1, NAP1, and TCP8 cDNA sequences were subsequently cloned in the respective DUAL hunter vectors to confirm protein interactions (Figure 4A). The results showed that PNM1 is indeed able to interact directly with both NAP1 and TCP8, two proteins involved in gene expression and localized in plant nuclei.
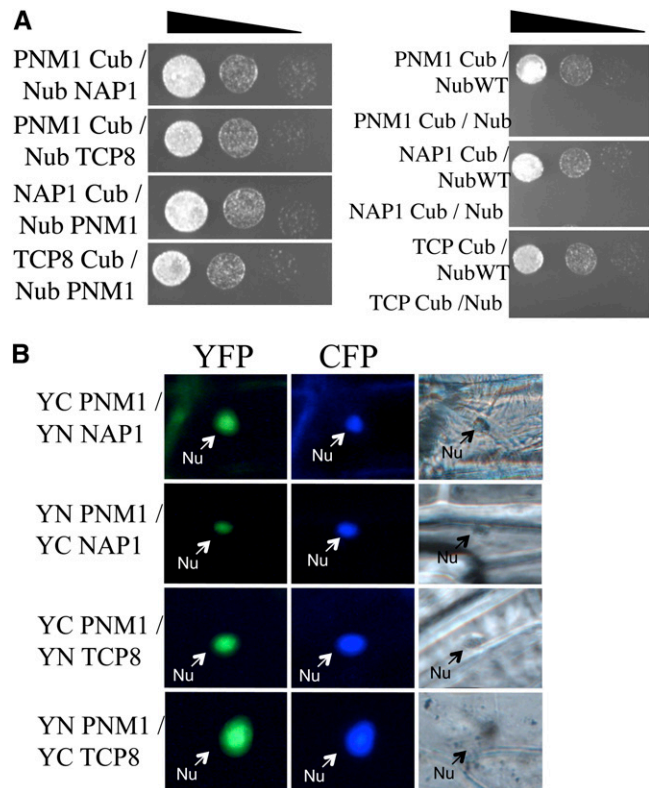
**Figure 4.** PNM1 Interacts with Nuclear Proteins.

(A) Yeast two-hybrid–like assays show that PNM1 interacts with NAP1 and TCP8. Yeast cells were cotransformed with X-Cub and Nub-X constructs. Liquid cultures of double transformants are plated at DO600 = 0.1 and at increasing 10× and 100× dilutions of the cultures (black triangles) on synthetic dropout selective medium that lacked Leu, His, adenine and Trp supplemented with 10 mM 3-amino-1,2,4-triazole. NubWT represents the nonmutated form of the N-terminal part of ubiquitin and was used here as a positive control for interactions with Cub constructs.

(B) BiFC visualization of PNM1 interaction with NAP1 and TCP8 in nuclei (Nu) of onion epidermal cells. Left, reconstituted YFP fluorescence image of cells cotransfected with the split-YFP constructs and the control CPRF2-CFP mainly localized in the nucleus, CFP signal in the center panel. The bright-field images of the onion cells are shown on the right. As a control, YC or YN PNM1, NAP1, and TCP8 fusion constructs were also cotransfected with the corresponding YC or YN alone. In this case, no YFP fluorescence was observed.

The protein interactions observed in yeast were investigated in planta. For this, we used the bimolecular fluorescence complementation approach. This method relies on expression of the two proteins of interest as translational fusions either to the non-fluorescing N-terminal (YN) or C-terminal (YC) halves of yellow fluorescent protein (YFP). Only when the YFP halves are brought together by interaction of the fused proteins can they form a functional YFP fluorophore, which can be detected with an epifluorescence microscope (Hu et al., 2002). A set of vectors was generated to express PNM1, NAP1, and TCP8 fused with the N-terminal (YN) or the C-terminal (YC) part of the YFP after transient transformation of onion epidermal cells using particle bombardment. YFP fluorescence was observed in the nucleus of plant cells when the vectors encoding PNM1 were cobombarded in combination with the vectors expressing NAP1 and TCP8, respectively (Figure 4B). These results show that PNM1 interacts physically with NAP1 and TCP8 in the nuclei of living plant cells.

PNM1 mRNA Level Is Higher in Mutants Complemented with PNM1ΔNLS

To get insights into the function of PNM1 in plant nuclei, we analyzed both macroscopic and molecular phenotypes of complemented *pnm1* plants. Three independent complementation lines were analyzed for both heterozygous PNM1ΔMTS and homozygous PNM1ΔNLS. PNM1 proteins levels were first checked in both mitochondria and the nucleus. PNM1 levels were unchanged in the heterozygous PNM1ΔMTS lines, whereas it was absent from the nucleus in PNM1ΔNLS plants (Figure 5A). When grown on half-strength Murashige and Skoog medium, PNM1ΔNLS plants showed a delay in root elongation. Plants from the three lines had 26% shorter roots after 10 d on average (see Supplemental Figure 5 online). Because PNM1 interacts with TCP8 in plant nuclei and because the *PNM1* gene has site II elements (DNA elements known to be recognized by TCP transcription factors) in its promoter region (see Supplemental Figure 6 online), we investigated transcript levels of *PNM1* in PNM1ΔNLS plants. A 2.8-kb transcript was detected in wild-type plants, whereas a slightly smaller RNA, which corresponds to PNM1 sequence lacking the 171 nucleotides of the NLS region, was detected in mutants. In addition, PNM1 transcript levels were on average 25% higher in PNM1ΔNLS compared with the wild type for the three independent mutant lines analyzed (Figure 5B). To verify that transcript level variations were indeed due to the absence of PNM1 from plant nuclei, we also looked at RNA levels of exemplary chosen nuclear genes also containing site II elements in their promoter sequence (i.e., *ATP2*, *COXVc*, *MPPα*, *SDH1*, and the complex I 23 kD subunit) that all encode proteins of the mitochondrial oxidative phosphorylation machinery. Similar to *PNM1*, transcript levels for these genes were on average 29% higher in mutants (Figure 5B). These gene expression variations were statistically significant (i.e., with Student's *t* test P values < 0.05). By contrast, when genes that do not contain site II elements in their promoter region were used (i.e., the mitochondrial *AOX1a* and *NDB2* as well as *ACTIN2*), unchanged expression levels were observed in the mutant lines (Figure 5C).
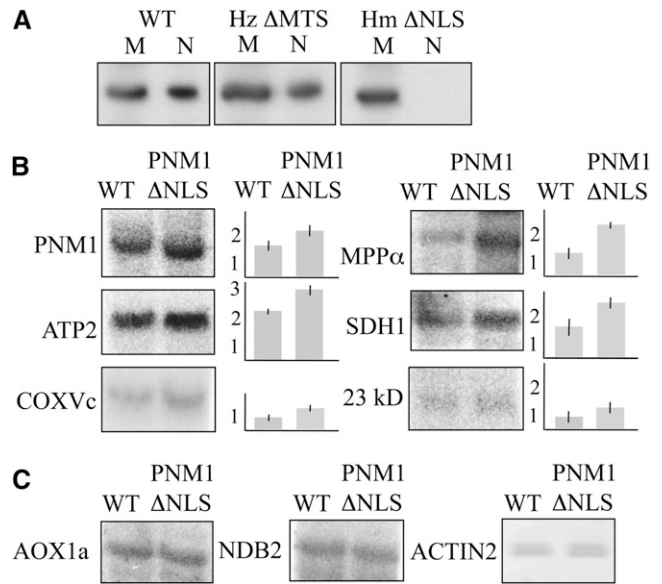
**Figure 5.** Molecular Analysis of Complemented *pnm1* Mutants.

(A) PNM1 protein levels were monitored by immunoblot analysis in mitochondria (M) and nuclei (N) from wild-type (WT), heterozygous PNM1ΔMTS, and homozygous PNM1ΔNLS plants.

(B) Transcript levels were compared by RNA gel blot analysis in wild-type and PNM1ΔNLS plants. Identical amounts of RNA were hybridized with radiolabeled probes specific for genes encoding mitochondrial proteins with site II elements in their promoter region. Signal was quantified and normalized according to local background. Average signals obtained for the three independent complementation lines and three different wild-type plants are represented as histograms. Error bars show signal SD between the individual lines.

(C) Similar transcript levels analyses were performed for genes that do not contain site II elements in their promoter region (i.e., genes encoding the mitochondrial proteins AOX1a and NDB2 as well as ACTIN2).

TCP8 Can Bind the *PNM1* Promoter Region

The interaction of PNM1 with TCP8 and the transcript level upregulation for genes such as *PNM1* containing site II elements in their promoter region in PNM1ΔNLS mutants led us to investigate whether TCP8 could directly bind the *PNM1* promoter. This was analyzed by electromobility shift assays with recombinant TCP8 and double-stranded DNA probes representing two regions in *PNM1* promoter (i.e., the SII probe containing two site II elements and the Pr probe without such elements) (see Supplemental Figure 6 online). Two shifted bands were observed with TCP8 and the SII probe (Figure 6). When PNM1 was added (lane 5 in Figure 6), no additional shifted band was observed but the relative amount of band 1 increased (as compared with lane 3). However, no shift was observed between PNM1 alone and the SII probe. No shift was observed with the Pr probe, and no interaction was observed between single-stranded DNA probes and TCP8. The two shifted bands could be due to the presence of two site II elements in the SII probe. Alternatively, the two bands could reflect the association of either monomers or dimers of TCP8 to the probe. Indeed, TCP proteins were already shown to be able to dimerize (Aggarwal et al., 2010). In this case, the
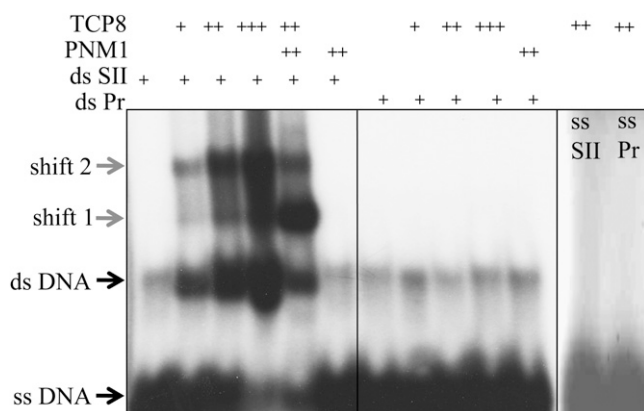
Figure 6. TCP8 Is Able to Bind the Promoter Region of *PNM1*.

Electromobility shift assays performed with TCP8 and PNM1 and two 40-nucleotide-long DNA probes representing sequences present in the promoter region of PNM1 as shown in Supplemental Figure 6 online. The SII probe contains two site II elements, and the Pr probe does not contain SII elements. Assays were performed with increasing amounts of TCP8 and/or PNM1 and end-labeled DNA. Black arrows show the DNA probes present as single-stranded (ss) or double-stranded (ds) DNA. Gray arrows show the shifted bands that were only observed with the SII double-stranded probe when TCP8 was added. As a control, we showed that TCP8 was unable to shift single-stranded DNA probes.

addition of PNM1 to the binding reaction might have resulted in a competition with TCP8 for dimer formation. Altogether, these results suggest that TCP8 can specifically bind site II elements in the promoter region of *PNM1*.

DISCUSSION

Through genetic studies, we have shown that the loss of *PNM1* is lethal for the embryo in *Arabidopsis*. *PNM1* encodes a PPR protein. PPR proteins play crucial functions in plant organellar gene expression associated with RNA cleavage, processing, splicing, RNA editing, and translational activation (Schmitz-Linneweber and Small, 2008). Several reports have shown that loss of function of PPR genes in *Arabidopsis* often causes an embryo-lethal phenotype (Lurin et al., 2004; Schmitz-Linneweber et al., 2006; Gobert et al., 2010).

TargetP (Emanuelsson et al., 2000) and Predotar (Small et al., 2004) programs predict three-quarters of PPR proteins to be targeted to mitochondria or plastids leading to the possibility that some PPR proteins could function outside of organelles (Lurin et al., 2004; O'Toole et al., 2008). GRP23 is the only PPR protein so far reported to be located in the nucleus in plants (Ding et al., 2006). Here, we showed that PNM1 is dual localized to both nuclei and mitochondria. Functional analysis of *PNM1* knockout plants with site-specific accumulation of PNM1 showed that only the mitochondrial localization of PNM1 is required for embryo development. This result suggests that PNM1 is a novel dual-targeted PPR protein playing an essential function for embryogenesis in mitochondria and a distinct role in the nucleus. PNM1 dual localization is an unexpected and intriguing feature. Only a

very limited number of plant proteins have been clearly demonstrated to localize to both the nucleus and organelles (Krause and Krupinska, 2009). Determining the significance of these multiple localizations and their potential effects on organelle biogenesis will be an important challenge for the future.

In mitochondria, PNM1 is associated with ribosomes in an RNA-dependent manner. It is possible that the association of PNM1 with polysomes is indirect, that PNM1 is part of an RNA maturation complex that could be attached to polysomes. Alternatively, it is possible that PNM1 interacts directly with ribosomes or other components of the translation apparatus, thus having a direct role in mitochondrial translation or its control.

In the nucleus, PNM1 can interact with both TCP8 and NAP1, two nuclear factors involved in the control of gene expression. Nucleosome assembly proteins (NAPs) are conserved from yeast to human and facilitate the in vitro assembly of nucleosomes as histone chaperones. They shuttle histones from the cytosol into the nucleus, assemble nucleosomes, and promote chromatin fluidity, thereby affecting the transcription of many genes (Park and Luger, 2006). In higher plants, several NAP1 homologs have been identified (Dong et al., 2003; Liu et al., 2009). In *Arabidopsis* and tobacco, NAP1 proteins bind histones, thus suggesting a conserved function of these proteins among eukaryotes (Dong et al., 2005; Liu et al., 2009). The functional reason for the association of PNM1 with NAP1 is unknown. However, PNM1 might be part of a chromatin-remodeling complex involved in gene expression control in the nucleus. Alternatively, PNM1 might associate with NAP1 for its transit from the cytosol to the nucleus.

By contrast, the TCP class of transcription factors is found only in plants. Members belonging to this class are important regulators of plant growth and development and control multiple traits in diverse plant species (Martín-Trillo and Cubas, 2010). TCP proteins share an ~60-residue homologous region called the TCP domain (Cubas et al., 1999), common to all the members. The TCP domain is responsible for the binding of TCP proteins to GC-rich DNA sequence motifs in vitro (Kosugi and Ohashi, 1997). These motifs, called site II elements, have been identified as *cis*-elements in many plant promoters (Martín-Trillo and Cubas, 2010). Welchen and Gonzalez (2005, 2006) have observed the prevalence of site II elements in the promoters of nuclear genes encoding components of the oxidative phosphorylation (OxPhos) machinery from both *Arabidopsis* and rice (*Oryza sativa*) and have thus proposed that TCP transcription factors might be involved in the coordinated expression of the nuclear-encoded subunits of the mitochondrial OxPhos machinery.

Biogenesis of the OxPhos complexes requires the expression of two genomes within one cell. This is because some of their subunits are encoded within the organelle genome, while the rest are encoded in the nucleus (Mackenzie and McIntosh, 1999). It is generally assumed that the expression of genes encoded in these genomes must be somehow coordinated to ensure correct complex assembly. A global study of this coordination suggests that it occurs at the posttranslational level (Giegé et al., 2005). Regarding the coordination of nuclear genes themselves, it is more likely that regulation takes place at the transcriptional level, perhaps through the interaction of common sets of transcription factors like TCP with cognate binding sites present in the

respective promoters (Welchen and Gonzalez, 2006). If this is true, the activity of the nuclear transcription factors should be modulated by a retrograde signal coming from mitochondria.

It has been hypothesized that proteins that are dual targeted to both the nucleus and an organelle play a role in this coordinating mechanism (Krause and Krupinska, 2009). Thus, we propose that PNM1 represents a novel PPR protein and a potential regulator of the expression of two distinct genomes through its association with the ribosomes in mitochondria and transcriptional factors in nuclei of plant cells. The analysis of *pnm1* mutants complemented with PNM1ΔNLS revealed that *PNM1*, which has site II elements in its promoter regions, has increased transcript levels when PNM1 is absent from the nucleus and that TCP8 is able to bind the promoter region of *PNM1*. It is thus tempting to speculate that PNM1 might act as a negative regulator for its own gene expression and probably also for the expression of a yet unknown number of other nuclear genes. Accordingly, it is imaginable that PNM1 function might be associated to the fine-tuning of transcript levels in the nucleus for mitochondrial biogenesis.

METHODS

Genetic and Complementation Analysis

Arabidopsis thaliana ecotype Columbia (Col) was used. The T-DNA insertion mutant lines were obtained from the ABRC Stock Center. Total cellular DNA was isolated as described by Edwards et al. (1991). Plants were genotyped by PCR, and the insertion position was confirmed by sequencing with a T-DNA left border primer. *pnm1-1* is SAIL_868_D10 (insertion site +958) and *pnm1-2* is SAIL_224_H01 (insertion site +971). For PNM1ΔMTS, the first 177 nucleotides were omitted, whereas for PNM1ΔNLS, the last 171 nucleotides were absent. For complementation, products were cloned in fusion with *PNM1* endogenous promoter into pGWB1 binary vector and introduced into *pnm1-1* heterozygote mutants via *Agrobacterium tumefaciens* GV3101.

Promoter-GUS Fusion Analysis

The 652 nucleotides upstream from the *PNM1* initiation codon were cloned in fusion with GUS. Tissues from promoter-GUS fusion plants issued from five independent lines were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (Biosynth) as described by Jefferson et al. (1987).

Protein Expression and Antibody Production

The *PNM1* full-length coding region without the termination codon was cloned into pBAD/Thio TOPO (Invitrogen) in fusion with an N-terminal thioredoxin and C-terminal polyhistidine tags. Proteins were expressed for 2 h at 37°C in LMG194 *Escherichia coli* induced with 0.2% arabinose (p/v) and purified by affinity to Ni according to the manufacturer's instructions. Purified PNM1 protein was used to immunize rabbit to produce polyclonal antibodies. For RNA binding assay, the fragment encoding PNM1 without the predicted mitochondrial transit peptide (amino acids 60 to 521) was cloned into the same vector expressed and purified with the same procedure.

PNM1 Localization Experiments

PNM1 localization was investigated by GFP fusion in tobacco (*Nicotiana tabacum*) BY-2 cells and confocal microscopy as described previously (Carrie et al., 2009), by immunodetection using PNM1-specific antibodies on equivalent amounts (30 μg) of *Arabidopsis* subcellular fractions. Sera were used at dilutions of 1:5000 for PNM1 antibodies, 1:100,000 for wheat (*Triticum aestivum*) NAD9 (Lamattina et al., 1993), 1:10,000 for wheat RPS1, of 1:10,000 for tobacco MnSOD (Bowler et al., 1989), 1:50,000 for yeast $Cytc_1$ (G. Schatz, Basel University, Basel, Switzerland), 1:10,000 for ribulose-1,5-bisphosphate carboxylase/oxygenase (B. Camara, Strasbourg, France), and 1:2000 for histone H2B antibodies (W.H. Shen, Strasbourg, France). PNM1 localization was also investigated by immunohistochemistry as described previously (Mundel et al., 2000). Immunogold labeling was performed as described previously (Erhardt et al., 2005). Briefly, *Arabidopsis* seedlings were fixed overnight in 4% glutaraldehyde and treated for 2 h with 10% (w/v) picric acid and 2 h with 2% uranyl acetate and stained with 0.1% (v/v) osmium tetroxyde. Samples were dehydrated with ethanol and infiltrated with EPON812 medium-grade resin. Sections (90 μm) were cut with an Ultracut E microtome (Reichert) and collected on grids coated with formvar (EMS). For PNM1 localization, a 1:500 dilution of PNM1 antibodies was used followed by incubation with goat-anti-rabbit antibodies coupled to 15-nm colloidal gold particles (Aurion EM Reagents). Samples were visualized with a Hitachi H-600 electron microscope operating at 75 kV. The specificity of immunogold labeling was verified in control experiments where secondary antibodies alone were used.

Arabidopsis Fractionation and Mitochondrial Polysome Analysis

Mitochondria, submitochondrial fractions, and chloroplasts were prepared as described previously (Spielewoy et al., 2001; Giegé et al., 2003). Nuclei were extracted from protoplasts. Protoplasts (10^6) were resuspended in 1 mL of NIB buffer (10 mM MES KOH, pH 5.5, 0.2 M sucrose, 2.5 mM EDTA, and 2.5 mM DTT). After protoplast lysis, the homogenate was loaded on 2.3 M sucrose, 1× NIB, and 40% Percoll (v/v) gradients. Gradients were centrifuged for 30 min at 3200*g*, and nuclei were collected at the Percoll/sucrose interphase and washed and resuspended with 0.35 M sucrose and 0.5 mM $MgCl_2$. Polysome-enriched fractions were prepared as described previously from cauliflower (*Brassica oleracea*) mitochondria (Uyttewaal et al., 2008), and for polysome destabilization experiments, lysates were treated with 10 mM puromycin or 600 units/mL of RNase A.

RNA Binding Assays

Experiments were performed with a Biacore 2000 optical biosensor instrument (GE Healthcare, Biacore). The running buffer was composed of 10 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, and surfactant P20 0.005%. PNM1 was bound to anti-His antibodies immobilized on a CM5 sensor surface using standard amine-coupling chemistry. Briefly, the antibodies at 10 μg/mL diluted in 10 mM sodium acetate, pH 4.0, were injected on the surface. Remaining activated groups were blocked with 1 M ethanolamine, pH 8.5. His-tagged proteins diluted in the running buffer at 30 ng/μL were injected and captured on the flow cells. For molecular binding assays, RNA samples at 17, 35, 70, 140, and 280 nM were injected consecutively for 1 min at a flow rate of 20 μL/min. Responses were XY-zeroed and double-referenced (i.e., the sensorgrams were corrected for signals in the reference flow cell that contains the control tobacco mosaic virus protein or thioredoxin as well as for the blank buffer data). To facilitate data comparison, responses were normalized relative to the density of captured His-tagged PNM1. Results were interpreted using the BiaEvaluation 4.1.1 software (GE Healthcare). Binding curves for PNM1 and mitochondrial total or LiCl pellet RNA were fitted to a 1:1 interaction model describing the interaction, which allowed the estimation of K_d values.

Protein Interaction Analysis

PNM1 protein interactions were investigated by screening an *Arabidopsis* cDNA library in yeast with the DUAL hunter system (DualsystemsBiotech). Briefly, PNM1 cDNA was cloned with *Sfi*I technology in pDHB1. The bait vector obtained was transformed in the yeast strain NMY51. The bait strain was transformed according to the manufacturer's instructions with a commercially available library expressing Nub fused to cDNAs. The library was constructed with total RNA from a mixture of tissues from 6-d-old *Arabidopsis* seedlings (DualsystemsBiotech). Protein interactions were confirmed in planta by bimolecular fluorescence complementation experiments in onion epidermal cells as described previously (Marrocco et al., 2006).

RNA Gel Blot Assays

Total RNA was isolated from *Arabidopsis* seedlings using the TRIzol method. RNA samples were separated on agarose denaturing gels, blotted on Hybond-NX membranes (Amersham), and hybridized using standard methods (Sambrook et al., 1989). Equal loading of lanes was verified by methylene blue blot staining. Specific PCR products used to probe the blots were P^{32} labeled with a DECA-Prime II kit (Ambion) following the manufacturer's instructions. Signal was acquired with a FLA-7000 phosphor imager (Fujifilm), normalized according to local background and quantified with the software Quantity One (Bio-Rad).

Electromobility Shift Assays

Electromobility shift assays were performed essentially as described for *Arabidopsis* TCP4 (Aggarwal et al., 2010). Briefly, 10 fmol of γATP end-labeled oligonucleotides were annealed with equimolar amounts of unlabeled antisense oligonucleotides slowly cooled to room temperature for 30 min. The resulting double-stranded DNA probes were incubated with 1, 3, or 10 ng of purified recombinant proteins in buffer containing 20 mM HEPES-KOH at pH 7.8, 100 mM KCl, 1 mM EDTA, 0.1% BSA, 10 ng salmon sperm DNA, and 10% glycerol for 30 min and loaded on an 8% native polyacrylamide gel. Electrophoresis was performed at 4 V/cm for 45 min with 0.5× Tris-borate buffer.

Accession Numbers

The genes described in this article correspond to the following Arabidopsis Genome Initiative codes: At5g60960 (*PNM1*), At1g58100 (*TCP8*), and At4g26110 (*NAP1*). A complete list of oligonucleotides used in this study is available in Supplemental Table 1 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of the PNM1 Protein Sequence with Exemplary Chosen Orthologs from *Vitis vinifera* and *Oryza sativa*.

Supplemental Figure 2. Determination of *PNM1* Transcript 5′ End.

Supplemental Figure 3. Size Estimation of the PNM1 Signals Detected in Mitochondria and the Nucleus.

Supplemental Figure 4. PNM1 Binds Mitochondrial RNA.

Supplemental Figure 5. Macroscopic Phenotype of *pnm1* Mutants Complemented with PNM1ΔNLS.

Supplemental Figure 6. Promoter Region of PNM1.

Supplemental Table 1. Complete List of Oligonucleotides Used in This Study.

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