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# Determination of protein-only RNase P interactome in Arabidopsis mitochondria and chloroplasts identifies a complex between PRORP1 and another NYN domain nuclease

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## SUMMARY

**The essential type of endonuclease that removes 5′ leader sequences from transfer RNA precursors is called RNase P. While ribonucleoprotein RNase P enzymes containing a ribozyme are found in all domains of life, another type of RNase P called 'PRORP', for 'PROtein-only RNase P', is composed of protein that occurs only in a wide variety of eukaryotes, in organelles and in the nucleus. Here, to find how PRORP functions integrate with other cell processes, we explored the protein interaction network of PRORP1 in Arabidopsis mitochondria and chloroplasts. Although PRORP proteins function as single subunit enzymes *in vitro*, we found that PRORP1 occurs in protein complexes and is present in high-molecular-weight fractions that contain mitochondrial ribosomes. The analysis of immunoprecipitated protein complexes identified proteins involved in organellar gene expression processes. In particular, direct interaction was established between PRORP1 and MNU2 a mitochondrial nuclease. A specific domain of MNU2 and a conserved signature of PRORP1 were found to be directly accountable for this protein interaction. Altogether, results revealed the existence of an RNA maturation complex in Arabidopsis mitochondria and suggested that PRORP proteins cooperated with other gene expression factors for RNA maturation *in vivo*.**

**Keywords: RNase P, RNA maturation, mitochondrial nucleases, pentatricopeptide repeats.**

## INTRODUCTION

Similar to all other RNA molecules, transfer RNAs (tRNAs) undergo many maturation processes to become functional. One of the crucial steps in tRNA biogenesis is performed by an enzyme termed RNase P that removes the 5′ leader sequences from tRNA precursors. This endonuclease function, found in all domains of life, is essential to obtain usable tRNAs and therefore critical for translation (Altman, 2007). RNase P activity has attracted considerable attention over the last 30 years, in particular because the first characterized RNase P enzyme, in *Escherichia coli*, was found to be a ribonucleoprotein (RNP) particle containing a ribozyme (Guerrier-Takada *et al.*, 1983). Similar RNP RNase Ps were found in other Bacteria, in Archaea and in Eukarya, in particular in human nuclei and in both yeast nucleus and mitochondria (Hartmann and Hartmann, 2003). Despite major differences in the RNA subunit structure and in the RNP protein content (Hernandez-Cid *et al.*, 2012; Jarrous, 2017), all these RNPs are characterized by the presence of a conserved catalytic RNA. This finding led to the assumption that RNase P would occur universally as RNPs and that RNase P represented one of the rare conserved vestige of a prebiotic RNA world.

More recently, this view was contradicted and the interest in RNase P renewed with the identification of a second type of RNase P in eukaryotes that was composed only of protein (Holzmann *et al.*, 2008; Gobert *et al.*, 2010; Pinker *et al.*, 2013). This other type of RNase P, called PRORP for 'PROtein-only RNase P', was found in four out of five eukaryote supergroups, in organelles and/or in the nucleus (Lechner *et al.*, 2015). While some eukaryotes, such as

human, have retained PRORP specifically for mitochondrial RNase P activity (Holzmann *et al.*, 2008) and an RNP is present in the nucleus, other eukaryotes use PRORP in both organelles and the nucleus, with RNP RNase P being entirely absent. For instance, in Arabidopsis and *Trypanosoma*, multiple PRORPs perform specialized RNase P activities in the organelles and in the nucleus (Gutmann *et al.*, 2012; Täschner *et al.*, 2012), while, in *Chlamydomonas*, a single triple localized PRORP is responsible for RNase P activity in the nucleus, mitochondria and chloroplasts (Bonnard *et al.*, 2016). PRORP proteins belong to the large family of pentatricopeptide repeat (PPR) proteins, a eukaryote-specific family of RNA binders involved in numerous gene expression processes (Giegé, 2013; Hammani *et al.*, 2014). PRORP proteins are Λ shaped proteins, with an N-terminal PPR domain that is believed to confer substrate specificity to the enzyme, making one arm of the Λ. The other arm is made by a C-terminal catalytic domain belonging to the NYN (N4BP1, YacP-like nuclease) family (Anantharaman and Aravind, 2006). The apex of the Λ is a structural zinc-binding domain that connects the two main domains and appears to confer flexibility to the enzyme (Schelcher *et al.*, 2016; Pinker *et al.*, 2017). Other PRORP features are specific to certain phyla. For instance, in Streptophyta, a G-rich insertion as well as a PPPY motif are highly conserved (Lechner *et al.*, 2015). The comparison by several research groups of PRORP and RNP RNase P structures and modes of action has revealed that the two types of enzymes use a fundamentally similar catalytic mechanism and seem to share a similar RNA-binding strategy (Gobert *et al.*, 2013; Chen *et al.*, 2016; Mao *et al.*, 2016; Walczyk *et al.*, 2016; Klemm *et al.*, 2017; Pinker *et al.*, 2017), therefore demonstrating a remarkable case of convergent evolution. However, while rapidly growing numbers of studies have investigated the PRORP mode of action, reviewed by Schelcher *et al.* (2016), little information is known about their functional diversity, i.e. on their transcriptome-wide substrate spectra and on the integration of PRORP functions with other cellular processes.

Eukaryotic RNA-binding proteins act almost exclusively in complexes with few to hundreds of interacting partners (Smirnov *et al.*, 2017). Some of these complexes are well described, such as the spliceosome or the RISC complex but, in most cases, complexes and protein interaction networks are not characterized. For PPR proteins, including PRORP, interacting partners are largely unknown, although some PPR proteins have been found in high-molecular-weight fractions containing ribosomes (Uyttewaal *et al.*, 2008; Hammani *et al.*, 2011). More recently, these PPR proteins were found to be present as integral ribosomal proteins (Waltz *et al.*, 2019). PNM1/rPPR9 was also found to interact with two nuclear proteins, NAP1 and the transcription factor TCP8 (Hammani *et al.*, 2011) and different PPR proteins interact with each other or with multiple organellar RNA editing factor (MORF) proteins as part of the RNA editing machinery in plant organelles (Boussardon *et al.*, 2012; Takenaka *et al.*, 2012; Hartel *et al.*, 2013). Very little information is available on PRORP protein interactors. In human, PRORP requires TRMT10C and SDR5C1 (formerly known as MRPP1 and MRPP2) for RNase P activity (Holzmann *et al.*, 2008), possibly to help binding the non-canonical fold of human mitochondrial tRNAs, as discussed by Salinas-Giegé *et al.* (2015), and human PRORP is part of mitochondrial RNA granules (Antonicka *et al.*, 2013; Jourdain *et al.*, 2013). However, the integration of PRORP activity with other cell functions remains largely unexplored.

In this context, we investigated the PRORP1 protein interaction network in Arabidopsis mitochondria and chloroplasts. We found that PRORP1 can occur in protein complexes. In particular, we found that the PRORP1 PPPY motif is a protein/protein interaction motif used for direct interaction with mitochondrial nuclease MNU2 that is involved in mitochondrial RNA 5′ maturation (Stoll and Binder, 2016). We also showed that MNU proteins are involved in tRNA accumulation in Arabidopsis mitochondria. This finding suggested that the two nucleases might cooperate *in vivo* for tRNA 5′ processing.

## RESULTS

### Characterization of PRORP1 organellar protein interaction networks

As a pilot study, we searched for functional complexes involving PRORP1 in Arabidopsis mitochondria and chloroplasts. *Ab initio*, it could be expected that PRORP might occur in putative tRNA maturation complexes involving, for example, RNase Z, CCA nucleotidyl transferase or tRNA modification enzymes. To purify and characterize complexes containing PRORP1, an Arabidopsis *prorp1* knockout line was complemented with a construct expressing a C-terminal fusion of PRORP1 with a haemagglutinin (HA) affinity tag placed under the control of the *PRORP1* endogenous promoter. Plants from the PRORP1-HA line revealed a wild-type macroscopic phenotype and their molecular analysis showed that they expressed *PRORP1-HA* mRNA at higher levels when compared with wild-type Col-0 plants in both flowers and leaf tissues (Figure S1).

To characterize the protein complexes involving PRORP1, mitochondria and chloroplasts purified from the *PRORP1-HA* line were lysed and solubilized complexes were purified by affinity to the HA-tag. Immunodetection analysis established that PRORP1-HA could indeed be pulled down by this approach (Figure S2). For the analysis of mitochondrial complexes, seven independent PRORP1-HA immunoaffinity experiments were performed, as well as six control experiments in which HA immunoaffinity purifications were performed with wild-type Arabidopsis

plants. For analysis of chloroplast complexes, three immunopurifications and four control experiments were performed. Proteins co-immunoprecipitated from both PRORP1-HA and control samples were digested with trypsin and identified by quantitative proteomics using nano liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). A statistical analysis based on spectral counts using a negative binomial GLM model (Chicois *et al.*, 2018) was performed to identify proteins that were significantly over-represented in PRORP1 immunoprecipitations compared with control experiments and therefore likely to represent PRORP1 interacting partners. Among the >1000 mitochondrial and >500 chloroplast proteins identified in the proteomic analysis (Tables S1 and S2), 49 proteins with adjusted *P*-values under 0.05 were considered *bona fide* PRORP1 mitochondrial interaction partners and 16 were retained as chloroplast interaction partners (Figure 1). It is noteworthy that mitochondrial protein partners could be pulled down in buffer conditions containing 150 mM NaCl, while chloroplast protein partners were only significantly enriched using less stringent 50 mM NaCl buffer conditions, i.e. immunopurifications performed with chloroplast extracts in 150 mM NaCl did not identify statistically significantly enriched protein. The retained mitochondrial proteins were mainly involved in gene expression processes and predicted to be localized to plant mitochondria. They includes a series of putative RNA-binding and/or DNA-binding proteins, 11 PPR proteins, nine ribosomal proteins, other translation-related proteins, as well as proteins of unknown function (Table 1a). The retained chloroplasts proteins also included some putative RNA-binding proteins such as the DEAD-box RNA helicase RH3, the RRM domain CP29B, translation initiation factor 2 and 3 ribosomal proteins (Table 1b).

The identification of many mitochondrial ribosomal proteins, including the novel ribosomal PPR proteins (Waltz *et al.*, 2019), led to the hypothesis that PRORP1 might be associated with the translation apparatus in Arabidopsis mitochondria. To test this hypothesis, mitochondrial complexes were separated using sucrose density gradients. Eight fractions representing the entire gradients were collected. Equivalent amounts of proteins from each fraction were reacted with HA-tag antibodies. The 60-kDa signal of PRORP1-HA was detected in fractions at the bottom of the gradients (Figure S3). Fractions were also reacted with antibodies specific for the mitochondrial ribosomal protein Rps4 and Nad9 from respiratory complex I. An Nad9 signal was only detected in the two top fractions of the gradients, suggesting that they contained complexes of sizes up to 1500 kDa. In contrast, Rps4 was detected in the same bottom fractions of the gradient as PRORP1, as well as in the top fractions. These bottom fractions corresponded to high-molecular-weight entities containing ribosomes, while the top fractions are likely to correspond to

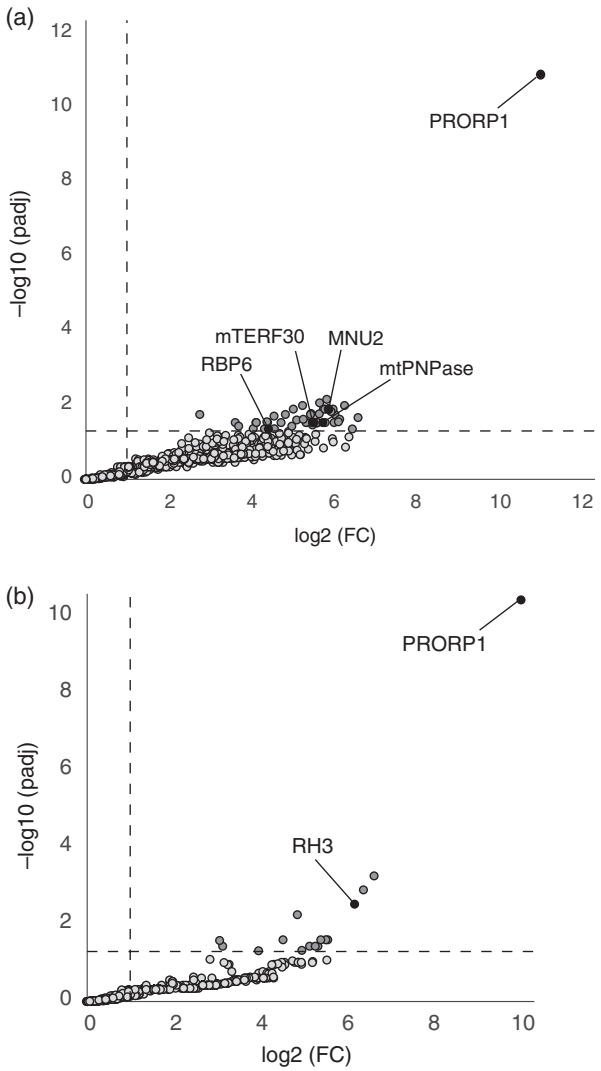


**Figure 1.** PRORP1 protein interaction network in Arabidopsis organelles. (a) Mitochondrial and (b) chloroplast PRORP1 interacting partners are displayed as a volcano plot according to their enrichment in PRORP1 IPs (*x*-axis, fold change (FC)) and to the statistical confidence of their enrichment (*y*-axis, adjusted *P*-value (padj)). The vertical dotted line indicates the threshold for a fold change of 2. The horizontal dotted line indicates the threshold for an adjusted *P*-value of 0.05. PRORP1 as well as the four mitochondrial proteins tested for direct interaction are indicated in (a). PRORP1 and a possible chloroplastic partner are indicated in (b).

dissociated ribosome subunits (Waltz *et al.*, 2019). This situation suggested that PRORP1 might be associated with the mitochondrial translation apparatus. To assess this, samples were treated with puromycin that specifically destabilizes ribosomes (Lu and Draper, 1994). In these assays, PRORP1, similar to Rps4, was no longer detected in bottom fractions (Figure S3). This result suggested that PRORP1 is indeed present in the high-molecular-weight complexes containing ribosomes, but not directly associated with one of the ribosome subunits. This finding is consistent with our recent characterization of Arabidopsis

 

**Table 1** PRORP1 protein interaction network in Arabidopsis organelles. (a) Mitochondrial and (b) chloroplast proteins over-represented in seven and three independent PRORP1-HA anti-HA immunoprecipitation (IP) fractions were identified by a statistical analysis on spectral counts using a negative binomial GLM model as described in Chicois *et al.* (2018). In total, 49 and 16 proteins with adjusted *P*-values 'adjp' of less than 0.05, and therefore considered as *bona fide* interacting partners, are listed here. Proteins were ranked according to increasing *P*-value 'rank' and sorted by functional categories. 'IP PRORP1' shows the average number of spectra for the respective proteins in PRORP1 IPs, 'IP WT' shows average numbers of spectra in control experiments in which IPs were performed with wild-type plants and 'LogFC' indicates the fold change over representation of proteins in PRORP1 IPs expressed in log2 scale. Protein names in bold are retained for further experiments.

| Accession | Name | IP PRORP1 | IP WT | LogFC | *P*-value | adjp | Rank |
|---|---|---|---|---|---|---|---|
| a) *PRORP1 mitochondria interactome* | | | | | | | |
| AT2G32230 | **PRORP1** | 294.3 | 0.0 | 11.1 | 9.09E-15 | 1.31E-11 | 1 |
| RNA-binding proteins | | | | | | | |
| AT5G09840 | **MNU2** | 8.9 | 0.0 | 5.9 | 8.37E-05 | 1.29E-02 | 11 |
| AT5G14580 | **PNPase** | 8.1 | 0.0 | 5.8 | 5.63E-04 | 2.97E-02 | 28 |
| AT1G61980 | **mTERF30** | 7.0 | 0.0 | 5.6 | 7.77E-04 | 2.97E-02 | 29 |
| AT3G15000 | MORF8 | 20.1 | 0.7 | 4.4 | 7.11E-04 | 2.97E-02 | 30 |
| AT1G18630 | **mS85/Glycine-rich RBP6** | 3.0 | 0.0 | 4.5 | 1.44E-03 | 4.41E-02 | 48 |
| PPR proteins | | | | | | | |
| AT3G49240 | PPR | 15.7 | 0.0 | 6.6 | 3.03E-04 | 2.18E-02 | 20 |
| AT3G13150 | PPR | 6.3 | 0.0 | 5.4 | 6.44E-04 | 2.97E-02 | 27 |
| AT2G15690 | PPR | 10.7 | 0.0 | 6.1 | 7.43E-04 | 2.97E-02 | 34 |
| AT1G80270 | PPR | 34.3 | 1.5 | 4.1 | 1.32E-03 | 4.32E-02 | 44 |
| Ribosomal PPR proteins | | | | | | | |
| AT3G02650 | mS80/rPPR6 | 8.6 | 0.0 | 5.8 | 9.56E-05 | 1.29E-02 | 8 |
| AT2G37230 | mL102/rPPR5 | 25.9 | 0.5 | 5.1 | 7.30E-05 | 1.29E-02 | 9 |
| AT1G55890 | mS78/rPPR3a | 10.0 | 0.0 | 6.1 | 2.25E-04 | 1.80E-02 | 17 |
| AT4G36680 | mL103/rPPR7 | 9.1 | 0.0 | 5.8 | 6.02E-04 | 2.97E-02 | 26 |
| AT1G19520 | mS77/rPPR2 | 9.9 | 0.3 | 4.1 | 7.97E-04 | 2.97E-02 | 31 |
| AT3G13160 | mS79/rPPR3b | 14.3 | 0.5 | 4.2 | 8.24E-04 | 2.97E-02 | 32 |
| AT5G15980 | mS81/rPPR8 | 7.3 | 0.0 | 5.6 | 7.31E-04 | 2.97E-02 | 37 |
| Other mitochondrial ribosomal proteins | | | | | | | |
| AT3G59650 | mL43 | 8.3 | 0.0 | 5.9 | 9.78E-06 | 7.05E-03 | 2 |
| AT3G18240 | mS35 | 7.4 | 0.0 | 5.7 | 1.82E-05 | 8.76E-03 | 3 |
| AT1G53645 | mS84 | 11.1 | 0.0 | 6.3 | 3.58E-05 | 1.03E-02 | 5 |
| AT5G66860 | bL25m | 9.6 | 0.0 | 6.0 | 9.87E-05 | 1.29E-02 | 6 |
| AT3G49080 | uS9m | 8.9 | 0.0 | 5.9 | 6.08E-05 | 1.29E-02 | 7 |
| AT1G26750 | mS23 | 7.3 | 0.0 | 5.7 | 1.68E-04 | 1.73E-02 | 14 |
| AT2G07696 | rps7 | 3.9 | 0.0 | 4.9 | 2.13E-04 | 1.80E-02 | 15 |
| AT1G16870 | mS29 | 7.4 | 0.2 | 4.4 | 4.85E-04 | 2.83E-02 | 24 |
| AT5G46160 | uL14m | 8.7 | 0.2 | 4.8 | 7.15E-04 | 2.97E-02 | 35 |
| ATMG00080 | uL16m | 2.7 | 0.0 | 4.4 | 1.48E-03 | 4.41E-02 | 47 |
| Other translation-related proteins | | | | | | | |
| AT4G11160 | Initiation factor 2 | 13.6 | 0.2 | 5.3 | 3.39E-05 | 1.03E-02 | 4 |
| ATMG00560 | RPL2-like | 8.3 | 0.0 | 5.8 | 9.25E-05 | 1.29E-02 | 10 |
| AT3G18740 | RPL7-like | 7.9 | 0.2 | 4.6 | 2.62E-04 | 1.99E-02 | 19 |
| AT2G47610 | RPL7-like | 12.0 | 0.2 | 5.1 | 4.04E-04 | 2.53E-02 | 23 |
| AT3G58140 | Phe-tRNA synthetase | 10.9 | 0.0 | 6.2 | 4.91E-04 | 2.83E-02 | 25 |
| AT3G09200 | RPL10-like | 11.4 | 0.3 | 4.4 | 1.36E-03 | 4.35E-02 | 45 |
| Other proteins | | | | | | | |
| AT1G30680 | Toprim domain protein | 9.1 | 0.0 | 6.0 | 1.25E-04 | 1.50E-02 | 12 |
| AT3G15660 | Glutaredoxin 4 (GRX4) | 6.3 | 0.0 | 5.5 | 1.53E-04 | 1.70E-02 | 13 |
| AT4G37910 | HSP70 | 80.9 | 9.7 | 2.8 | 2.16E-04 | 1.80E-02 | 16 |
| AT5G62270 | Mucin-related protein | 6.6 | 0.0 | 5.5 | 2.18E-04 | 1.80E-02 | 18 |
| AT2G43360 | BIOTIN AUXOTROPH 2 | 10.6 | 0.0 | 6.2 | 3.68E-04 | 2.43E-02 | 22 |
| AT1G33360 | CLP protease regulatory SU | 6.4 | 0.0 | 5.7 | 5.46E-04 | 2.97E-02 | 33 |
| AT5G62530 | Aldehyde dehydrogenase 12A1 | 6.3 | 0.0 | 5.5 | 6.24E-04 | 2.97E-02 | 36 |
| AT2G37250 | Adenosine kinase (ADK) | 6.0 | 0.0 | 5.4 | 7.70E-04 | 2.97E-02 | 38 |
| AT5G26860 | Lon protease 1 (LON1) | 7.1 | 0.0 | 5.6 | 8.17E-04 | 2.97E-02 | 39 |
| AT4G20360 | RAB GTPase homolog E1B | 19.1 | 1.2 | 3.6 | 7.82E-04 | 2.97E-02 | 40 |
| AT3G07770 | HSP89 | 16.3 | 0.2 | 5.5 | 9.79E-04 | 3.44E-02 | 41 |
| AT5G65720 | Cysteine desulfurase (NIFS1) | 14.9 | 0.8 | 3.7 | 1.08E-03 | 3.71E-02 | 42 |

(continued)

**Table 1** (continued)

| Accession | Name | IP PRORP1 | IP WT | LogFC | *P*-value | adjp | Rank |
|---|---|---|---|---|---|---|---|
| AT1G06130 | Glyoxalase 2-4 (GLX2-4) | 4.6 | 0.0 | 5.0 | 1.11E-03 | 3.72E-02 | 43 |
| AT3G08530 | Clathrin heavy chain | 12.1 | 0.0 | 6.5 | 1.50E-03 | 4.41E-02 | 49 |
| Unknown proteins | | | | | | | |
| AT5G24165 | Unknown protein | 5.3 | 0.0 | 5.3 | 3.70E-04 | 2.43E-02 | 21 |
| AT5G49210 | Unknown protein | 3.4 | 0.0 | 4.6 | 1.39E-03 | 4.36E-02 | 46 |
| b) *PRORP1 chloroplast interactome* | | | | | | | |
| AT2G32230 | PRORP1 | 166.3 | 0 | 10.1 | 5.22E-14 | 3.58E-11 | 1 |
| RNA-binding proteins | | | | | | | |
| AT5G26742 | RH3 | 12.0 | 0 | 6.2 | 1.71E-05 | 2.94E-03 | 4 |
| AT2G37220 | CP29B | 22.7 | 1.75 | 3.2 | 7.39E-04 | 3.64E-02 | 11 |
| Ribosomal proteins | | | | | | | |
| AT1G78630 | RPL13 | 14.3 | 0.25 | 4.6 | 3.13E-04 | 2.48E-02 | 6 |
| AT3G25920 | RPL15 | 25.7 | 2 | 3.1 | 3.77E-04 | 2.59E-02 | 10 |
| AT3G52150 | PSRP2 | 6.7 | 0 | 5.4 | 7.44E-04 | 3.64E-02 | 13 |
| Translation-related protein | | | | | | | |
| AT1G17220 | FUG1 | 4.7 | 0 | 5.0 | 1.02E-03 | 4.67E-02 | 15 |
| Other proteins | | | | | | | |
| AT5G49910 | HSP70-7 | 16.3 | 0 | 6.7 | 1.57E-06 | 5.40E-04 | 2 |
| AT4G24280 | HSP70-6 | 14.0 | 0 | 6.4 | 5.46E-06 | 1.25E-03 | 3 |
| AT5G09660 | PMDH2 | 25.3 | 0.5 | 4.9 | 4.02E-05 | 5.52E-03 | 5 |
| AT1G34430 | EMB3003 | 8.3 | 0 | 5.6 | 2.52E-04 | 2.48E-02 | 7 |
| AT4G16155 | LPD2 | 7.7 | 0 | 5.6 | 3.17E-04 | 2.48E-02 | 8 |
| ATCG00500 | ACCD | 7.3 | 0 | 5.4 | 3.25E-04 | 2.48E-02 | 9 |
| AT5G25980 | TGG2 | 7.0 | 0 | 5.3 | 6.76E-04 | 3.64E-02 | 12 |
| AT3G16950 | LPD1 | 5.7 | 0 | 5.2 | 7.42E-04 | 3.64E-02 | 14 |
| AT2G38040 | CAC3 | 16.0 | 0.5 | 4.0 | 1.12E-03 | 4.78E-02 | 16 |

mitoribosome (Waltz *et al.*, 2019) that did not identify PRORP1 as a core ribosome protein. The association with the translation apparatus observed here must therefore be indirect, and the precise nature of the high-molecular-weight complexes containing both PRORP1 and mitoribosomes is unknown.

### PRORP1 directly interacts with the mitochondrial nuclease MNU2

As a next step, a yeast two-hybrid-like approach was used to try to identify proteins that might directly interact with PRORP1. Sucrose gradient experiments described above had suggested that interactions with ribosomal proteins are indirect. Similarly, a previous study had suggested that PPR proteins and PRORP can interact indirectly via RNA. Indeed, PRORP and PPR proteins can cooperate for the maturation of the same transcript, as shown for RFL2 and PRORP1 that are both required for processing the *orf291* transcript in Arabidopsis mitochondria (Fujii *et al.*, 2016). Although the two proteins functionally interact, they do not appear to interact directly (Fujii *et al.*, 2016). In this light, the direct interaction of PRORP1 with PPR proteins identified here was not explored. Direct protein associations were therefore investigated for the other putative RNA-binding proteins identified in this analysis. Selected proteins were therefore MNU2, a putative nuclease containing an NYN-like domain, and shown to be involved in mRNA 5′ maturation in Arabidopsis mitochondria (Stoll and Binder, 2016), PNPase, a 3′ to 5′ exonuclease involved in RNA 3′ maturation and decay in plant mitochondria (Holec *et al.*, 2006; Hammani and Giegé, 2014), mTERF30 a putative RNA- and/or DNA-binding protein of unknown function (Kleine, 2012) and a glycine-rich RNA-binding protein associated with mitoribosomes (Waltz *et al.*, 2019). The identification of these proteins in immunopurifications of complexes suggested that RNase P activity held by PRORP1 might be connected with other 5′ and/or 3′ RNA maturation processes in Arabidopsis mitochondria.

Direct protein interaction was monitored using the 'DUAL hunter' yeast two-hybrid-like system. The absence of auto-activation of the PRORP1 Cub construct (the bait construct) was investigated through its interaction with the mutated Nub (Nub), used for prey constructs and the control prey Δp53 (Figure 2a). Then, PRORP1 Cub interactions with MNU2, glycine-rich RBP6, mTERF30 and PNPase were investigated. The activation of reporter genes *ADE2* and *HIS3* could only be observed with the MNU2 construct, therefore showing that only MNU2 can physically interact with PRORP1 (Figure 2a).

The occurrence of a mitochondrial complex containing both PRORP1 and MNU2 was then investigated *in planta* using a blue native (BN) PAGE approach. Mitochondrial

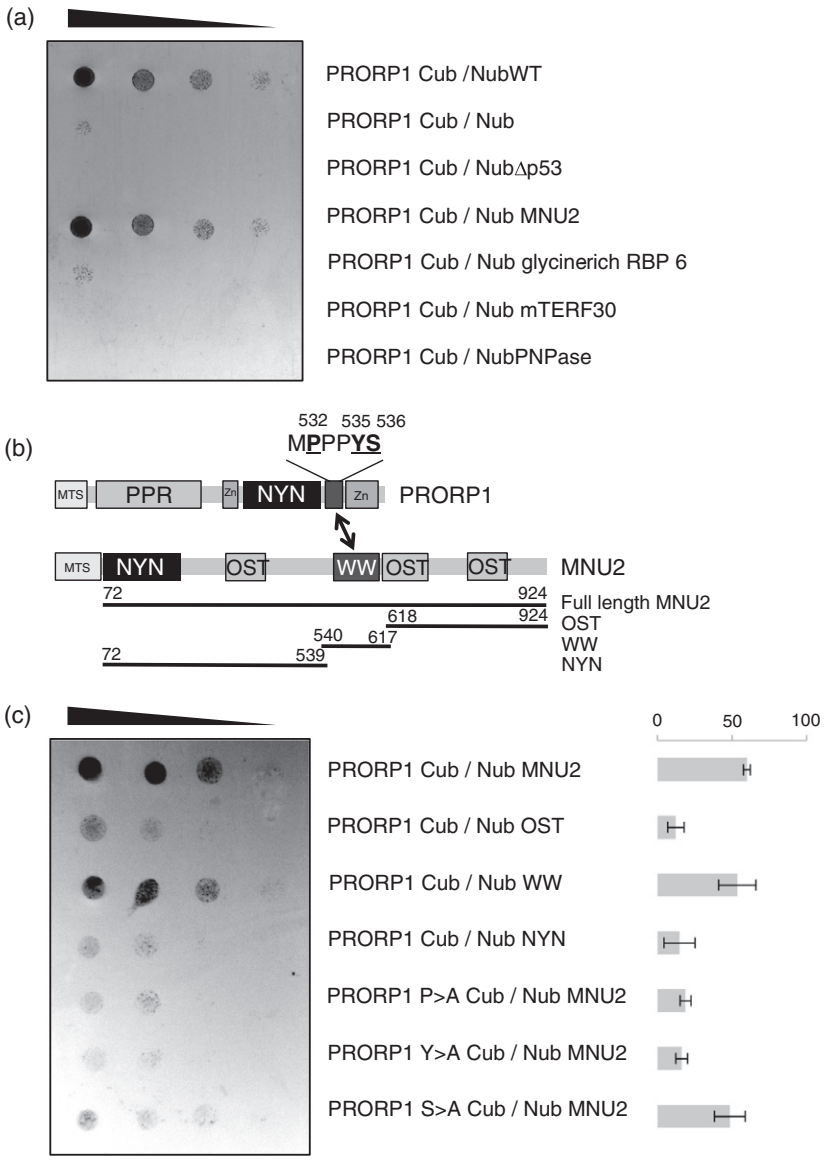


**Figure 2.** PRORP1 direct interaction with MNU2 is evidenced by yeast split-ubiquitin assays. (a) The upper panel shows interaction assays of the bait construct PRORP1 Cub with positive control NubWT and negative controls Nub and Δp53. The lower panel shows interaction assays of PRORP1 with MNU2, glycine-rich RBP6, mTERF30 and PNPase. Drops representing four- to five-fold serial dilutions of yeast cultures of double transformants were deposited on plates with minus leucine, tryptophan, adenine and histidine media supplemented with 10 mM 3-aminotriazol. Black triangles indicate decreasing cell concentrations in the yeast drops. (b) Schematic representation of PRORP1 and MNU2, highlighting their representative domains. Positions of the MPPPYS motif of PRORP1 that were mutated to alanine are underlined and black bars represent the individual sub-constructs of MNU2 termed 'NYN', 'WW' and 'OST' that were used in interaction assays. Amino acid positions are also indicated for the mutated positions of PRORP1 and the MNU2 domains. (c) The four-first lanes show interaction assays of PRORP1 Cub with MNU2 mutants, while the lower lanes show interactions of full-length MNU2 with PRORP1 point mutants. Histograms indicate interaction quantifications measuring the accumulation of a β-galactosidase reaction product measured in nm/min. Error bars represent standard deviations for experiments performed with three independent yeast double transformants.

complexes extracted from the *PRORP1-HA* line were separated according to size on a first dimensional BN gel and subunits of the respective complexes were resolved in the second dimension by SDS-PAGE. Immunodetection analysis with HA-tag-specific antibodies revealed signals corresponding to PRORP1-HA (Figure 3). The major signal is most likely to correspond to a monomeric form of PRORP1. However, other signals were also observed at sizes corresponding to complexes ranging from >100 to >500 kDa. While multiple signals were sometimes observed, a 150 kDa complex signal was consistently detected (Figure 3b). This complex size was estimated to be 150 kDa, because it migrates slightly below the respiratory complex IV (identified with its Cox2 subunit), that migrates at an apparent 160 kDa position in BN analyses (Senkler *et al.*, 2017). To identify whether MNU2 also occurs in the 150 kDa complex, MNU2-specific antibodies were raised and used in the BN analysis. An MNU2-specific signal was detected at an apparent molecular weight of about 100 kDa that also corresponded to a 150 kDa complex. This size is coherent with the calculated sizes of a complex that would contain the mature forms of PRORP1 and MNU2 at a 1:1 stoichiometry.

While MNU2 was already shown to participate in the 5′ maturation of some mitochondrial mRNAs (Stoll and Binder, 2016), it was not clear whether MNU2 localization was restricted to mitochondria or whether it was dual targeted to mitochondrion and chloroplast proteins, as found for PRORP1. Western blot analysis performed on purified chloroplasts and mitochondria fractions unambiguously showed that MNU2 antibodies were specific and that MNU2 was only present in mitochondria (Figure S4),

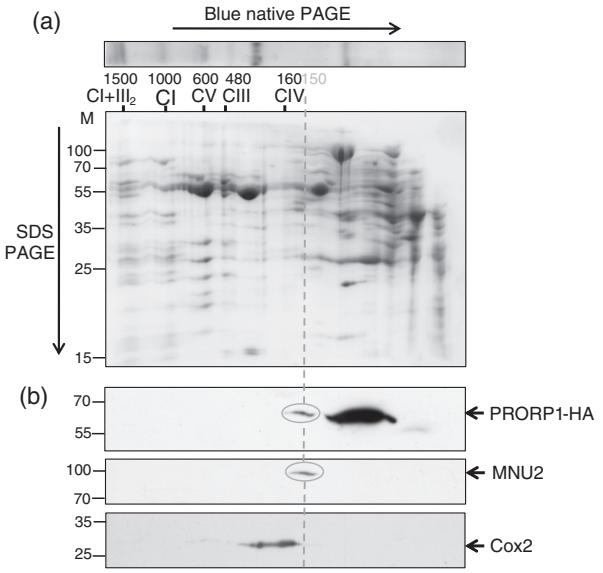


**Figure 3.** Blue native analysis identifies PRORP1 and MNU2 in a mitochondrial protein complex. (a) Stained membrane representing Arabidopsis mitochondrial complexes prepared from a plant line expressing PRORP1-HA separated in a first dimension by Blue Native PAGE and as a second dimension by SDS-PAGE. CI to CV indicate respiratory chain complexes I to V assigned according to the literature (Giegé *et al.*, 2003; Senkler *et al.*, 2017) and their respective sizes in kDa. M indicates molecular weight markers in kDa. (b) Western blot analysis of this exemplary chosen membrane reacted with HA-specific antibodies, revealing the 60 kDa signal of PRORP1-HA. The same membrane was also reacted with MNU2-specific antibodies, revealing the 100 kDa signal of MNU2 and with COX2 antibodies revealing the 30 kDa signal of COX2. Signals corresponding to a 150 kDa complex are detected with both HA and MNU2 antibodies.

therefore showing that the PRORP1/MNU2 interaction was restricted to mitochondria. Altogether, these results suggested that PRORP1 can directly interact with MNU2 in a 150 kDa complex that occurs in mitochondria.

### PRORP1 PPPY motif is a protein interaction platform

Subsequently, the PRORP1/MNU2 interaction was characterized in detail, i.e. to identify domains or motifs of the two proteins responsible for protein interaction. Bioinformatics analyses of the MNU2 sequence features revealed that it contained a mitochondrial targeting signal, an N-terminal NYN-like putative nuclease domain, three N- and C-terminal OST-like putative RNA-binding domains and a central WW protein/protein interaction domain (Macias *et al.*, 1996; Anantharaman *et al.*, 2010; Stoll and Binder, 2016). The WW domain was defined by two sequence signatures containing tryptophans and phenylalanines conserved among spermatophytes and separated by 30–135 amino acids according to species (Figure S5). MNU2 cDNA was therefore divided into three constructs containing the separate NYN, WW and two OST domains, respectively (Figure 2b), and cloned in frame with Nub. Interactions with PRORP1 were visualized though the activation of *ADE2* and *HIS3* and quantified by β-galactosidase assays through the activation of the *lacZ* reporter gene. This approach revealed that the WW construct interacted with PRORP1 at a level close to that of wild-type MNU2, whereas interaction levels with the OST and NYN constructs decreased by 80% and 76%, respectively, compared with wild-type MNU2 (Figure 2c). This result strongly suggested that the WW domain of MNU2 was responsible for the interaction with PRORP1. Functional and mechanistic analyses of WW domain-containing proteins have revealed that WW domains specifically interact with proline-rich motifs, most of the time characterized by a PPPY signature (Yagi *et al.*, 1999). Interestingly a proline-rich domain is conserved in PRORP sequences, localized in the PRORP-connecting domain and facing towards the outside of the PRORP Λ shape (Figure 4). While a PxxY signature is fairly conserved in distantly related PRORP sequences, i.e. in Opisthokonta, Excavata, SAR and Archaeplastida, an MPPPYS motif is particularly well conserved in Streptophyta, including Arabidopsis (Figure S6). To test the importance of the PRORP1 motif MPPPYS for the interaction with MNU2, point mutants of the PRORP1 Cub construct were generated, i.e. including mutations of the first proline, of the tyrosine and of the serine in the motif. Interaction assays of the point mutants with wild-type MNU2 revealed that the serine mutant interacts with MNU2 at a level close to that of wild-type PRORP1, while the proline and tyrosine mutants interaction levels decreased by 69% and 73%, respectively (Figure 2c). This result is in accordance with previous analyses of the interaction of the PEB2 PPPY motif with yes-associated protein (YAP) WW domains that had shown that the first proline and the tyrosine of the motif were the most important residues for the interaction with WW domains (Yagi *et al.*, 1999). Altogether, results strongly suggested that the WW domain of MNU2 and the PPPY motif of PRORP1 were responsible for the PRORP1/MNU2 interaction in Arabidopsis mitochondria.

### MNU proteins are required for tRNA accumulation in Arabidopsis mitochondria

Because the primary function of PRORP1 is to perform tRNA maturation, we wondered whether MNU2 might be involved in tRNA biogenesis as well. Arabidopsis expresses MNU1 and MNU2, two proteins that were proposed to have redundant functions (Stoll and Binder, 2016). Previous work has strongly suggested that MNU1 and MNU2 are non-essential nucleases involved in the maturation of mRNA 5′ ends in plant mitochondria (Stoll and Binder, 2016). To analyze their possible involvement for tRNA biogenesis, mitochondrial RNAs from *mnu1/mnu2* knock-out lines and from control wild-type plants were compared using RNA blot hybridizations (Figure 5). This approach revealed that the chosen mitochondrial

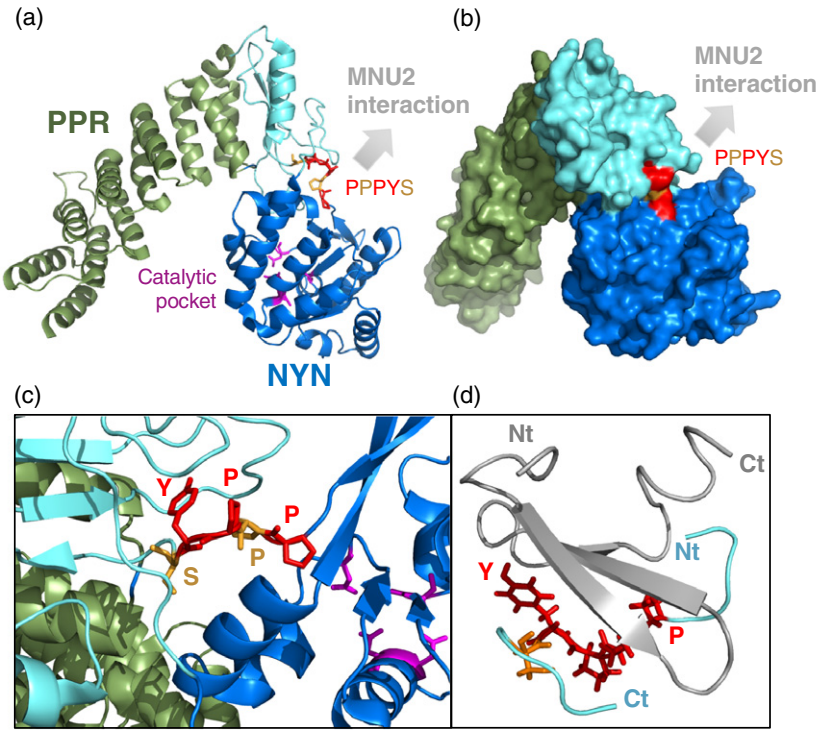


**Figure 4.** Structural model of the interaction between PRORP1 motif PPPY and MNU2 WW domain. (a) Crystal structure of Arabidopsis PRORP1 (Howard *et al.*, 2012), indicating the location of the PPPY motif shown in red, highlighting its accessibility for protein interaction. (b) Structural envelope of Arabidopsis crystal structure shown in (a). (c) Close-up of the PRORP1 PPPY motif showing the orientation of individual residues, in particular the accessibility of the first proline and tyrosine that were shown to be important for protein/protein interactions, and inaccessibility of the second proline and serine (orange) not required for interaction. (d) Nuclear magnetic resonance (NMR) structure of the WW domain of Smurf2 (grey) in interaction with the PPPY motif (red) of Smad7 (PDB, 2LTZ) (Aragón *et al.*, 2012) shows the orientation of the WW domain interaction with the proline-rich motif. Extrapolation to PRORP1/MNU2 interaction enables the proposal that the catalytic domain of MNU2 situated in Nt of the WW domain might be oriented toward the catalytic pocket of PRORP1.

tRNA$^{Ser}$ (GCU), tRNA$^{Lys}$ (UUU) and tRNA$^{Gly}$ (GCC) levels decreased by 22%, 41% and 42%, respectively, in three biological replicate experiments (i.e. with RNAs extracted from independent mitochondrial purifications). As a control, mitochondrial 5S rRNA levels were measured and did not vary in *mnu1/mnu2* mutants when compared with the control plants. Although of moderate effect and non-essential (contrary to PRORP1 that is essential for tRNA maturation), the function of MNU proteins does appear to be related directly or indirectly to the accumulation of mitochondrial mature tRNAs *in vivo* (Figure 5).

## DISCUSSION

With this study, the functional network of PRORP1 in Arabidopsis mitochondria and chloroplasts begins to be unravelled. The presence of PRORP1 in high-molecular-weight fractions containing ribosomes, while not directly bound to mitoribosomes, suggests that it is indirectly associated with the translation apparatus in plant mitochondria. This interaction could be mediated by RNA, i.e. PRORP1 similar to other PPR proteins (Hammani and Giegé, 2014) might be involved in the maturation of mRNAs already loaded on mitochondrial ribosomes. Beyond tRNA maturation, PRORP1 has already been shown to be involved in the maturation of mitochondrial mRNAs at the level of tRNA-like structures, as observed *in vivo* for *nad6* 3′ end maturation (Gutmann *et al.*, 2012) and *orf291* processing (Fujii *et al.*, 2016). Such an association would suggest that the maturation of mRNAs and translation are tightly coupled processes in plant mitochondria.

The analysis of immunoprecipitated protein complexes has revealed a number of potential protein partners for PRORP1. With the exception of MNU2, other mitochondrial candidates did not show direct interaction with PRORP1. This situation suggests that, similar to the translation apparatus, PRORP1 is indirectly associated via proteins and/or RNAs to gene expression regulators such as RBP6, mTERF30 or PNPase. Similarly, in chloroplasts, the association with an RNA helicase such as RH3, involved in intron splicing (Gu *et al.*, 2014), and with CP29B, proposed to be involved in the control of chloroplast RNA levels (Nakamura *et al.*, 2001), suggests that RNase P activity might be coupled with other chloroplast gene expression pathways. Finally, as in mitochondria, the identification of ribosomal proteins and a translation initiation factor might point towards an indirect association with the chloroplast translation apparatus. Nonetheless, PRORP1 interactions with chloroplast proteins are probably more transient and/or indirect than with mitochondrial proteins, as they could only be detected in less stringent salt conditions.

The characterization of the PRORP1/MNU2 interaction has revealed that a specific domain of MNU2 and a proline-rich motif of PRORP1 were directly accountable for protein interaction. A phylogenetic analysis of 388 PRORP sequences representing the diversity of PRORP across eukaryotes (Lechner *et al.*, 2015) has revealed that the

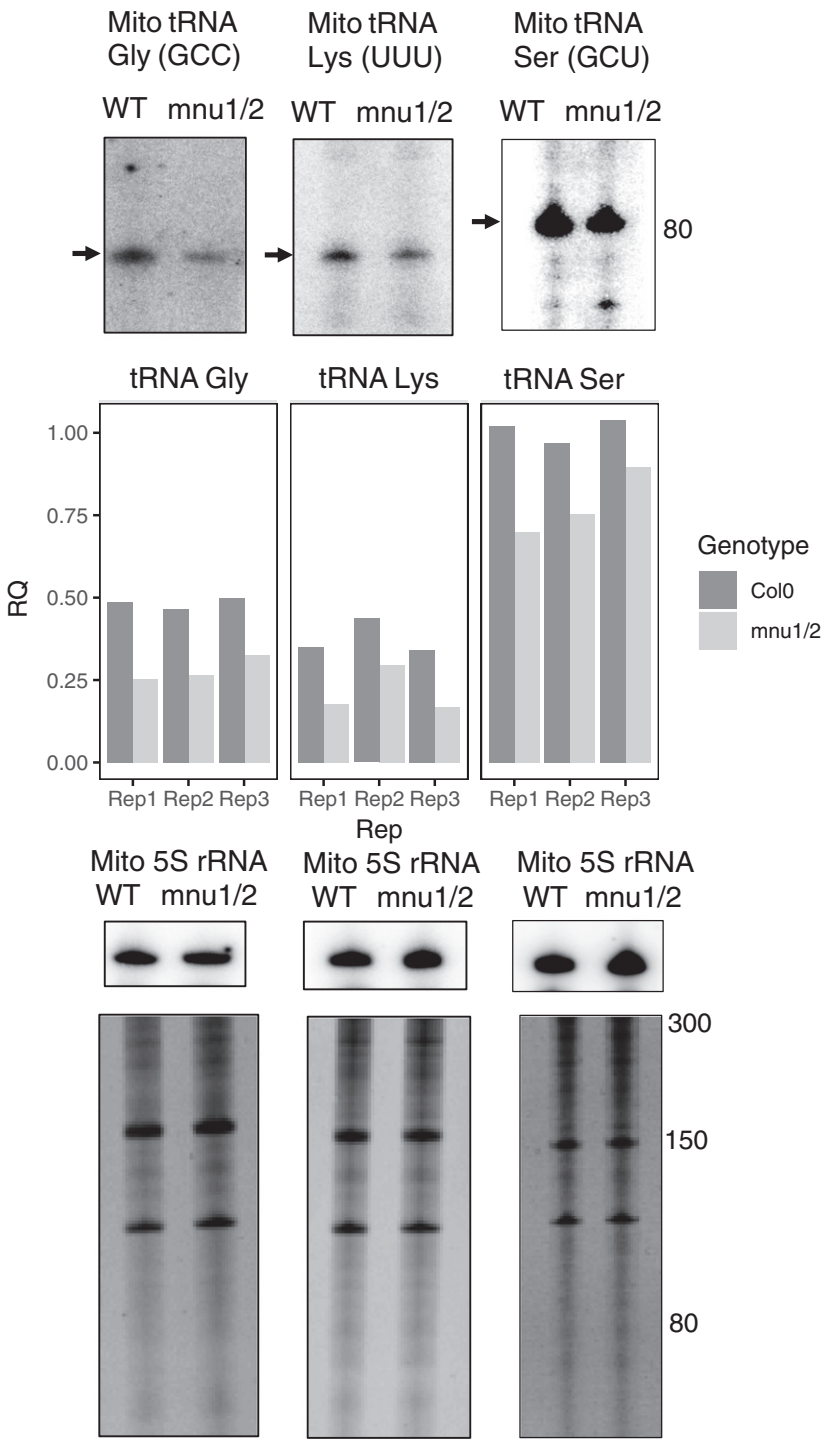


**Figure 5.** MNU proteins are involved in mitochondrial tRNA accumulation. Total mitochondrial RNAs from control (WT) and double knock-out lines (mnu1/2) were separated on denaturing acrylamide gels, blotted and analysed by hybridizations using specific probes representing the Arabidopsis mitochondrial tRNAGly (GCC), tRNALys (UUU) and tRNASer (GCU). Arrows show the main signals corresponding to the sizes of mature tRNAs (76, 76 and 91 nucleotides respectively). Control hybridizations were performed with 5S rRNAs. Intensities of PhosphorImager signals were quantified for mature tRNA and 5S rRNAs using ImageJ software. Quantities of mature tRNAs relative to 5S rRNAs (RQ) are shown for three replicate experiments (Rep1 to Rep3) as histograms with dark grey bars showing WT samples and light grey bars, mutant samples. Stained blots used in the respective hybridizations are shown in the bottom panels. Molecular weight markers are indicated in ribonucleotides.

proline-rich motif is very well conserved in Streptophyta (Figure S6). However, a degenerate motif, in which the first proline and the tryptophan are relatively well conserved, is also present in other distantly related eukaryote branches (Figure S6). This finding suggests that the proline-rich motif is ancient and might have already been present in an ancestral PRORP, in an organism at the root of the major modern eukaryote groups. While the conserved PPPY motifs in Streptophyta are most probably all involved in protein interactions, the functionality of the degenerate motifs in other eukaryote groups is questionable, but cannot be ruled out. Similarly, the distribution of MNU2 orthologues across eukaryotes was examined. The analysis of genomic data available identified 109 MNU2-like

 

sequences, specifically in Spermatophyta. All these proteins are predicted to be localized to mitochondria and/or plastids similar to PRORP1. Beyond seed plants, no other protein bearing all the hallmarks of MNU proteins (i.e. NYN, OST and WW domains) could be identified (Figure S6). This result suggest that PRORP/MNU interactions are specific to seed plant organelles. The conservation of the proline-rich domain in nuclear PRORP proteins, while MNU2-like proteins are strictly organellar, suggests that other WW domain proteins might interact with nuclear PRORPs in Embryophyta nuclei. Similarly, other yet unidentified WW domain proteins might interact with PRORP proteins in other eukaryote clades.

The major question remains to understand the functional reason for the interaction between PRORP1 and MNU2 in Arabidopsis mitochondria. A published report showed that the function of MNU proteins is required for the maturation of some Arabidopsis mitochondrial mRNAs (Stoll and Binder, 2016) and results obtained here suggested that they might also be involved in the biogenesis of mitochondrial tRNAs, directly or indirectly. However, the MNU mode of action is unknown. For instance, it is unclear whether MNU proteins act as endonucleases or an exonucleases. In the PRORP1/MNU2 complex, a possible function of MNU2 could be to degrade tRNA 5′ leader sequences after PRORP1 cleavage. Alternatively, MNU2 might directly participate in pre-tRNA and/or mRNA maturation. For example, it might trim long leader sequences, while PRORP1 would perform the final maturation of shortened leader sequences. This would be in agreement with the observations that pre-tRNAs are often transcribed with long leader sequences in plant mitochondria (Hammani and Giegé, 2014), while *in vitro* PRORP cleaves pre-tRNAs with very short leader sequences with higher efficiency (Howard *et al.*, 2016). Such a cooperation between two nucleases for RNA maturation would be reminiscent of the concerted action of RNase II and PNPase for the 3′ end maturation of mRNAs in plant mitochondria (Perrin *et al.*, 2004; Stoll and Binder, 2016).

The fast growing amount of data on PRORP protein mode of action, i.e. the determination of their catalytic constants, has surprisingly revealed *in vitro* that PRORP enzymes are not as good catalysts as ribonucleoprotein RNase P enzymes (Schelcher *et al.*, 2016). The identification of interaction partners, such as MNU2, enabled us to propose that protein partner functions might enhance or regulate PRORP activity *in vivo*. In the presence of additional factors, PRORP proteins might turn out to be better catalysts. Such hypotheses will have to be investigated through the functional and mechanistic characterization of complexes involving PRORP proteins. In the longer term, complete interaction networks of PRORP enzymes in the nucleus will reveal the full extent of their integration among gene expression and other cellular processes.

## EXPERIMENTAL PROCEDURES

### Plant material

*Arabidopsis thaliana* wild-type (WT) and mutant lines were on a Col-0 ecotype/background. The PRORP1-HA line used in the study was constructed as follows. The full-length coding sequence of *PRORP1* was placed under the control of a *PRORP1* endogenous promoter and the overall sequence was cloned into the pEarleyGate301 vector, resulting in an HA epitope downstream of the construct (Earley *et al.*, 2006). Heterozygous Arabidopsis PRORP1 (At2g32230) insertional mutants Col-0 *GK-385G09* (Gobert *et al.*, 2010) were transformed by agroinfiltration with the resulting vector. Plants homozygous for wild-type *prorp1* knock-out mutation and complemented with HA-tagged PRORP1 were selected for subsequent analyses. Knock-out lines for *mnu1*, *mnu2* and the double knock-out *mnu1/mnu2* were the lines used by Stoll and Binder (2016).

### Mitochondria purification, chloroplast purification and blue native analysis

Arabidopsis mitochondria were prepared from Arabidopsis inflorescences by differential centrifugation and step density gradients, as described previously (Giegé *et al.*, 1998). Chloroplasts were prepared from 6-week-old Arabidopsis leaves by differential centrifugation and discontinuous Percoll gradients as described previously (Seigneurin-Berny *et al.*, 2008). Mitochondrial complexes were resolved by BN PAGE. For this, 500 μg of mitochondrial proteins were resuspended in ACA750 buffer containing 750 mM amino dicaproic acid, 50 mM bis-Tris and 0.5 mM $Na_2EDTA$, pH 7.0. Protein complexes were solubilized with digitonin, 5/1 detergent/protein (w/w) for 30 min on ice, centrifuged at 100 000 ***g*** for 15 min at 4°C and then 5% (v/v) Serva blue solution (750 mM ACA750 solution, 5% (w/v) Serva Blue G250) was added to the supernatant. Complexes were separated on 5–13% acrylamide gradient gels in 0.5 M amino di-caproic acid, 50 mM bis-Tris pH 7.0 buffer, with 50 mM bis-Tris pH 7.0 anode buffer and 50 mM tricine, 15 mM bis-Tris, 0.02% (v/v) Serva Blue G250, pH 7.0 cathode buffer. Electrophoresis was carried out overnight at 5 mA. Gel lanes were cut out and denatured for 1 h at room temperature in 50 mM Tris-HCl pH 6.8, 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol. For the second dimension, components of the various complexes were resolved by SDS-PAGE, as described previously (Giegé *et al.*, 2003).

### Ribosome association analysis

Ribosome-enriched fractions were prepared, as described previously (Uyttewaal *et al.*, 2008), with purified Arabidopsis mitochondria. For ribosome destabilization experiments, lysates were treated with 10 mM puromycin for 30 min on ice before sucrose gradient separation.

### Immunoprecipitations of protein complexes

Immunoprecipitation experiments were performed using the μMACS™ system (Miltenyi Biotech). In brief, 1 or 2 mg of purified Arabidopsis mitochondria or chloroplasts, respectively, was solubilized in 1 ml buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl for mitochondria and 50 mM NaCl for chloroplasts, 1% Triton X-100, and protease inhibitors for 30 min on ice. The solubilized extract was centrifuged for 10 min at 100 000 ***g*** and the supernatant incubated with 50 μl of microbeads conjugated with anti-HA antibodies for 30 min on ice and applied to μMACS columns placed in the magnetic field of a μMACS separator at 4°C. Columns were washed four times with 200 μl 50 mM Tris-HCl pH 8.0,

150 mM NaCl, 1% Triton X-100 and once with 200 µl 20 mM Tris-HCl pH 7.5 for mitochondria and five times with 400 µl 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 0,1% Triton X-100 for chloroplasts. Complexes were eluted with 95°C preheated elution buffer containing 50 mM Tris-HCl pH 6.8, 50 mM dithiothreitiol (DTT), 1% (w/v) sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.005% (w/v) bromophenol blue and 10% (v/v) glycerol.

### Proteomic analysis of protein complexes

Mass spectrometry analyses of the eluted complexes were performed at the Strasbourg-Esplanade proteomic platform. In brief, proteins were trypsin digested, mass spectrometry analyses and quantitative proteomics were carried out by nano LC-ESI-MS/MS analysis on AB Sciex TripleTOF mass spectrometers and quantitative label-free analysis was performed through in-house bioinformatics pipelines. The MS analyses showing more than 100 spectra for the bait PRORP1-HA were considered successful co-immunoprecipitations (Co-IP) and retained for further statistical analysis. To identify significantly enriched proteins, a statistical analysis based on spectral counts was performed using a home-made R package as described in Chicois *et al.* (2018) except that the size factor used to scale samples was calculated according to the DESeq normalization method (Gregori *et al.*, 2013). The R package uses a negative binomial GLM model based on EdgeR (Robinson *et al.*, 2010) to calculate, fold-change, *P*-value and Benjamini–Hochberg corrected *P*-value. Proteins that were over-represented in PRORP1 immunoprecipitates (IP) were visualized as a volcano plot that displayed log2-fold change and −log10-adjusted *P*-value on the x and y axes, respectively. Graphs were plotted using the ggplot2 package (v.2.2.1) in R , with colour scale adjusted according to statistical significance.

### Yeast split-ubiquitin assays

PRORP1 direct protein interactions were investigated in yeast using the DUAL hunter system, (DualsystemsBiotech®), a versatile yeast two-hybrid-like genetic system based on the 'split-ubiquitin' system that enables monitoring of interactions between any combination of soluble and/or membrane proteins. Briefly, PRORP1 was inserted in frame with the membrane protein Ost4p, the C-terminal half of ubiquitin and the transcription factor LexA-VP16 (PRORP1 Cub bait construct). Then, cDNAs encoding the proteins of interest were fused at the C-terminal of a mutated version of the N-terminal domain of ubiquitin (Nub X prey constructs). If bait and prey interact, Cub and Nub complement to form split-ubiquitin. Then, ubiquitin-specific proteases release LexA-VP16 that migrates to the nucleus and activates the transcription of reporter genes (Mockli *et al.*, 2007). The capacity of the PRORP1 Cub construct to activate reporter genes was investigated through the interaction with the wild-type form of Nub (NubWT) that is able to interact with Cub and reconstitute functional ubiquitin without protein interaction with the bait. *PRORP1* coding sequence without the nucleotide sequence coding the mitochondrial targeting signal was cloned into pDHB1at *Sfi*I restriction enzyme sites. The bait construct obtained was transformed into the yeast strain NMY51. The bait strain was co-transformed according to manufacturer's instructions with constructs representing mature forms of glycine-rich RBP6, mTERF30, PNPase and MNU2 (residues 72–924) cloned into the prey vector pPR3N. Nucleotide sequences of parts of MNU2 corresponding to amino acids 72–539, 540–617 and 618–924, termed NYN, WW and OST, were also cloned into the pPR3N vector. Transformation into yeast was controlled by growth on minus leucine and tryptophan medium. Protein interaction was monitored by the expression of the reporter genes *ADE2*, *HIS3* and *lacZ*. The expression of *ADE2* and *HIS3* was visualized by the growth on minus adenine and histidine medium supplemented with 10 mM 3-aminotriazol. Expression of *lacZ* was followed by measurement at $OD_{420}$ for the accumulation of the product metabolized by β-galactosidase with 2.2 mM 2-nitrophenyl β-D-galactopyranoside (*o*-NPG, Sigma, St. Louis, MO, USA) as a substrate.

### Protein expression and antibodies production

A nucleotide sequence corresponding to the C-terminal part of MNU2 (A539 to V924) was cloned into p0GWA to express recombinant protein fused to a C-terminal poly-histidine tag in *E. coli* BL21 cells. Proteins were purified under denaturing conditions by HisTrap affinity chromatography. Proteins were injected into rabbits to raise polyclonal antibodies. The serum was used at 1/20 000 dilution for western analysis.

### RNA blot analyses

RNA blot analyses of MNU2 knock-out plants were performed as described previously (Gutmann *et al.*, 2012). Blots were hybridized with radiolabelled gene-specific oligonucleotides and signal revealed using a FLA-7000 PhosphorImager (Fujifilm, Tokyo, Japan) and quantified with ImageJ.

### Bioinformatics analyses

Subcellular localization predictions of protein identified in immunopurification experiments were determined with TargetP (http://www.cbs.dtu.dk/services/TargetP/) and Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html). Structure models were determined using the Phyre2 algorithm (Protein Homology/analogY Recognition Engine V2.0) in the intensive modelling mode (Kelley and Sternberg, 2009). Molecular docking and related figures were obtained using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0, Schrödinger, LLC). PRORP and MNU-like sequences were retrieved using the BLAST tool in NCBI and Phytozome1.2. Proteins were aligned using Muscle software (Edgar, 2004). WebLogo 3 was used to highlight conserved residues (Crooks *et al.*, 2004).

## DATA AVAILABILITY

All the data presented, the plant lines and biological material used here are available upon request to authors.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

PG, AG, GB and FW designed experiments, analysed results and wrote the manuscript. HZ analysed results. AB, FW, GB, MA, PH, LK, CS, AG and PG performed experiments.

## SUPPORTING INFORMATION

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

**Figure S1.** Construction of Arabidopsis plants expressing a PRORP1-HA transgene.

**Figure S2.** PRORP1 can be immunopurified with extracts from Arabidopsis lines expressing PRORP1-HA.

**Figure S3.** PRORP1 is associated with high-molecular-weight complexes containing ribosomes in Arabidopsis mitochondria.

**Figure S4.** MNU2 is a mitochondrial protein.

**Figure S5.** Description of the tryptophan-rich domain present in MNU2 sequences.

**Figure S6.** Phylogenetic distribution of the PRORP1 PPPY motif and MNU2-like sequences.

**Table S1.** PRORP1 protein interaction network in Arabidopsis mitochondria.

**Table S2.** PRORP1 protein interaction network in Arabidopsis chloroplasts.

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