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PPR336 is Associated with Polysomes in Plant Mitochondria

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The function of pentatricopeptide repeat (PPR) proteins has been associated with various post-transcriptional steps of organelle gene expression. Among them, translation and its regulation are essential processes. However, in plant mitochondria, they are also the steps of gene expression that are the least understood. In this study, PPR336 was identified as part of a high-molecular-weight complex in *Arabidopsis* mitochondria. PPR336 is an unusual representative of the large PPR family because it is relatively short and is characterised by a high expression level compared with other PPR proteins. PPR336 defines a small subgroup of eight class P PPR proteins that are similar in terms of motif organization. Among them, PPR336-like is the closest homolog of PPR336. Biochemical analysis has indicated that PPR336 is a strictly mitochondrial protein, extrinsically attached to the inner mitochondrial membrane and part of an RNase-sensitive complex. Sucrose gradients and polysome destabilisation experiments show that PPR336 is associated with ribosomes in plant mitochondria. Moreover, in *Ppr336/336-like* mutants, mitochondrial polysomes of lower molecular weight accumulate compared with wild-type plants. Polysome association and these unusual features suggest that PPR336 could be involved in a distinctive process, possibly translation in plant mitochondria.

Keywords: pentatricopeptide repeat proteins; ribosomes; translation; plant organelles; blue native

Introduction

Pentatricopeptide repeat (PPR) proteins are encoded by huge gene families in plant genomes (e.g., they are composed of 441 representatives in *Arabidopsis*).[1] The genes coding for PPR proteins are characteristic of eukaryotes because they are totally absent from prokaryotes. They are found in yeast and animals, albeit in very small numbers (i.e., on average, there are 80 times more PPR genes in plants).[2] The explosion of the number of PPR genes occurred during the evolution of terrestrial plants.[3] The discovery of PPR genes in the bryophyte *Physcomitrella patens* indicates that a large group of PPR genes already existed before the evolutionary separation of mosses and vascular plants.[4] As indicated by their name, PPR proteins are composed of 35 amino acid motifs repeated in tandem. These proteins have, on average, 12 PPR motifs. The sequences of these motifs are very degenerated, but their structure is likely to be conserved. Each PPR motif is thought to be configured as two antiparallel α-helices, and the tandem repeats of PPR motifs would form a superhelix enclosing a central groove involved in target binding. Moreover, PPR proteins have been divided into several categories based on the presence or absence of sequence variants of PPR motifs and various additional C-terminal domains.[1]

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Abbreviations used: PPR, pentatricopeptide repeat; MALDI–TOF, matrix-assisted laser desorption/ionization time of flight; T-DNA, transferred DNA; ORF, open reading frame; MnSOD, manganese superoxide dismutase; rRNA, ribosomal RNA.

PPR proteins are putative RNA binding proteins, and the vast majority are predicted to be targeted to organelles.[1] The expansion of PPR proteins in plants could have evolved to respond to plant-specific processes involving RNA in organelles. Indeed, genetic expression in plant mitochondria and that in chloroplasts have many distinctive processes for, as examples, the transcription of their genes, the splicing of group II introns, RNA editing, the maturation of transcript ends and a poly A-dependent RNA degradation system.[5–7] Similarly, the translation apparatus is original in plant organelles,[8] and translation initiation and its control are poorly understood. Particularly, in mitochondria, hardly anything is known. No Shine–Dalgarno-like sequence is present in the 5′-untranslated region of transcripts, and some genes are translated without any AUG initiation codon or classical termination codon.[5,9] PPR proteins are predicted to be involved in all the plant-specific gene expression processes.

PPR proteins have been described as being able to bind RNA *in vitro* and *in vivo*,[1,10–14] although some of them were shown to bind DNA.[15] Genetic approaches have also identified PPR proteins as restorers of cytoplasmic male sterility (e.g., in petunia,[14] radish,[16] sorghum[17] and rice[18,19]). The genes *CRP1* and *PPR4* from maize and genes *HCF152*, *CRR2* and *PGR3* from *Arabidopsis* code for PPR proteins involved in the processing, stabilisation, splicing and translation of chloroplastic transcripts.[12,13,20,21] CRR4 and CCR21 are both E+-type PPR proteins essential for RNA editing in chloroplasts.[22,23] Interestingly, in *Trypanosoma brucei*, a number of PPR proteins were recently found to be essential for the biogenesis of mitochondrial ribosomes.[24]

Thus, studies on PPR proteins indeed connect these proteins with post-transcriptional processes in organelles. However, reports describing the biochemical properties and the mechanism of action of PPR proteins are still rare. Among them, HCF152 was described as forming a homodimer in chloroplasts.[10] PPR2 was found in a large heterogeneous protein complex in the stroma of chloroplasts.[25] Similarly, CRP1 was found in a multi-subunit complex but was not found associated with membranes and/or ribosomes.[20] In the mitochondria of petunia, a gel filtration approach identified PPR592 in fractions containing complexes of high molecular weight.[26]

Here we report a biochemical study on PPR336, an unusual representative of the PPR protein family. PPR336 defines a small subfamily of shorter PPR proteins. It is associated with polysomes on the inner membrane of *Arabidopsis* mitochondria and could thus play a role in mitochondrial translation.

Results

PPR336 is identified in a high-molecular-weight membrane complex

As part of a general survey of proteins present in membrane-associated complexes in *Arabidopsis* mitochondria, PPR336 (At1g61870) was found in a high-molecular-weight complex. For this analysis, the equivalent of 4 mg of *Arabidopsis* mitochondrial membrane proteins was treated with digitonin and the solubilised complexes obtained were separated on preparative blue native gels.[27,28] After migration, gel pieces covering the entire length of the blue native gels were excised, and the proteins they contained were electroeluted and concentrated. Proteins were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry. Numerous respiratory proteins were identified in gel bands corresponding to respiratory complexes IV, III, V as well as I and the supercomplex $I+III_2$ (data not shown), as already identified in previous studies.[29–31] The most interesting finding came from a gel band of 800 kDa, where no apparent band was visible on the stained blue native gels. In this band, PPR336 was identified (Fig. 1a). This protein is composed of conventional PPR repeats only and therefore belongs to the P subfamily of PPR proteins.[1] It is predicted to be localised in mitochondria by Predotar[32] and to be mitochondrial as well as chloroplastic by Mitoprot2[33] and TargetP.[34] PPR336 is an unusual representative of the PPR protein family because it is a relatively smaller protein that is composed of 408 amino acids as compared with the average 673 amino acids of the overall PPR family.[1] It also contains less PPR repeats, with 6 PPR repeats as compared with the average 12 PPR repeats observed for the proteins of the family. PPR336 is also characterised by its relatively high expression level, with, for example, 16 expressed sequence tags found for this gene as compared with the average 2 expressed sequence tags found for the other PPR genes.[1] A more precise investigation of microarray expression data, available from GENEVESTIGATOR‡, reveals that PPR336 is expressed at comparable levels throughout plant development (Supplementary Figure 1).[35] It is expressed at comparatively higher levels in inflorescences and roots, particularly in root tips and at lower levels in seedlings and rosettes (Supplementary Figure 2).

The subfamily of PPR336

Phylogenic analyses reveal that PPR336 defines a small subfamily of PPR proteins (Fig. 1b). In the global phylogenetic tree of class P PPR proteins,[1] a small subset of eight PPR proteins is composed of proteins resembling PPR336. They are all short (423 amino acids on average), have an average of six PPR repeats and share an unusually high percentage of sequence identity with PPR336 (average of 35%). Among them, At1g11630 is particularly similar to PPR336, with 70% of identical amino acids; we therefore named this protein PPR336-like. However, the eight proteins of the subfamily are not all expressed at high levels. GENEVESTIGATOR data indicate that expression levels vary 10-fold across the

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‡ https://www.genevestigator.ethz.ch