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REVIEW

Function of chloroplast RNA-binding proteins

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Abstract Chloroplasts are eukaryotic organelles which represent evolutionary chimera with proteins that have been derived from either a prokaryotic endosymbiont or a eukaryotic host. Chloroplast gene expression starts with transcription of RNA and is followed by multiple post-transcriptional processes which are mediated mainly by an as yet unknown number of RNA-binding proteins. Here, we review the literature to date on the structure and function of these chloroplast RNA-binding proteins. For example, the functional protein domains involved in RNA binding, such as the RNA-recognition motifs, the chloroplast RNA-splicing and ribosome maturation domains, and the pentatricopeptide-repeat motifs, are summarized. We also describe biochemical and forward genetic approaches that led to the identification of proteins modifying RNA stability or carrying out RNA splicing or editing. Such data will greatly contribute to a better understanding of the biogenesis of a unique organelle found in all photosynthetic organisms.

Keywords Chloroplast · RNA-binding protein · Post-transcriptional modification · Stabilization · Splicing · Editing

Introduction

In plants and algae, the compounds of the photosynthetic electron transport chain are located in the thylakoid membrane system of the chloroplast, a type of plastid that is responsible for conversion of light energy into chemical energy in the form of ATP [1]. Similar to mitochondria, chloroplasts contain their own genetic system comprising multiple, homogeneous, circular DNA molecules as well as their own transcriptional and translational machinery [2]. In the 1960s, Sagan [3] proposed in the endosymbiotic theory that both chloroplasts and mitochondria evolved from once free-living prokaryotic cells, which later endosymbiotically integrated into a eukaryotic host. Nowadays, it is generally accepted that mitochondria derived from an α-proteobacterial ancestor, while chloroplasts originated from a cyanobacteria-like prokaryote [4, 5]. In the case of chloroplasts, primary endosymbiosis led to the formation of three lineages: the Glaucophyta, the Rhodophyta, and the Chlorophyta. While the Glaucophyta represent the most basal lineage, Chlorophyta are the most derived primary plastids. Chloroplasts of these primary endosymbionts are characterized by the occurrence of two surrounding lipid bilayer membranes that originated from the outer and plasma membrane of the phagocytized gram-negative cyanobacteria-like ancestor [5]. The subsequent endosymbiotic integration of red and green algal cells into a free-living eukaryote finally resulted in the evolution of eukaryotic–eukaryotic chimera such as Euglenophyta, Chlorarachniophyta, and Chryptophyta to name a few [6]. In contrast to primary plastids, chloroplasts originating from secondary endosymbiosis can contain three or more surrounding membranes.

The endosymbiotic integration of once free-living prokaryotes into a eukaryotic host was accompanied by major modifications of the phagocytized cell. One of the most obvious events was a drastic reduction of the former prokaryote's genome coding capacity caused by loss or transfer of genetic material to the host genome [7]. Sequencing of

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plastid genomes showed that today's organelles encode between 20 and 200 proteins, while the genomes of the modern cyanobacteria *Synechocystis* sp. PCC6803 and *Nostoc punctiforme* comprise 3,268 and 7,432 protein-encoding genes, respectively [7–9]. However, proteome analyses and in silico studies of peptide leader sequences have shown that organelles comprise several thousand proteins [10, 11]. The differences in genome size and protein content indicate that the majority of the former prokaryote genes have relocated to the nuclear genome of the host, whereas only a minority of genes have remained under the control of the organelle. Genes belonging to the conserved core of open reading frames (ORFs) present in virtually all today's chloroplast genome sequences encode for subunits of the photosynthetic complexes, the ATPase, the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco), a bacteria-like polymerase, proteins of the ribosomal subunits as well as tRNAs and rRNAs [12]. Since plastids lost most of their genes during evolution, they are strongly dependent on nuclear-encoded proteins to maintain functionality. Thus, the import of nuclear gene products via specific translocation mechanisms, and the coordinated assembly of protein complexes composed of nuclear- and chloroplast-encoded proteins, is essential to sustain chloroplast metabolism [5, 7].

The evolution of the compartmentalized genetic system of the plant cell and the associated transfer of genetic material from the ancestral chloroplast genome to the eukaryotic nucleus required the development of elaborate strategies to control and regulate the interplay of nuclear and plastid gene expression to ensure plastid biogenesis and maintenance of the chloroplast's metabolism. Cross-talk between the two genetic systems is enabled by a variety of anterograde and retrograde signalling pathways [13]. Important mechanisms of anterograde signalling are the transcriptional, post-transcriptional, and translational regulation of chloroplast gene expression by means of nuclear-encoded *trans*-acting factors [13–15]. These nuclear-encoded proteins play key roles since they act as RNA-binding factors in crucial post-transcriptional processing steps such as RNA stabilization, splicing and editing (Fig. 1) [16, 17]. Here, we provide a brief overview focused on structural properties and function of RNA-binding proteins involved in the post-transcriptional modification of chloroplast RNAs.

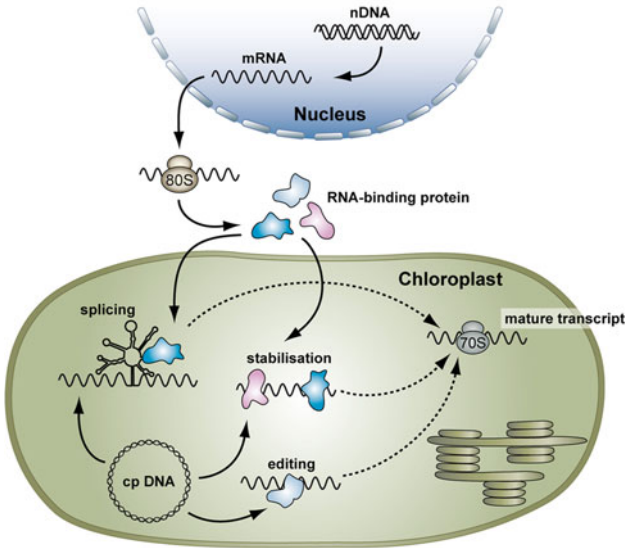

Fig. 1 RNA-binding proteins are involved in regulation of chloroplast gene expression The post-transcriptional modification of chloroplast RNAs represents an important step in the control of chloroplast gene expression. This process involves several steps, such as splicing, stabilization and editing. Various nuclear-encoded proteins participate in these plastid RNA processing events via direct binding to their target RNAs. Many of these RNA-binding proteins exhibit certain structural domains, such as the RRM, the CRM, or the PPR motif, that are thought to promote interaction between protein and RNA

Functional protein domains involved in RNA binding

To date, several well-defined protein domains involved in specific binding of RNA have been identified. The RNA-binding domains (RBD) described in this review represent only a small selection of the most common motifs found in eukaryotes [16, 18, 19]. Nevertheless, in particular RNA-recognition motifs (RRM), chloroplast RNA-splicing and ribosome maturation (CRM) domains and pentatricopeptide-repeat (PPR) motifs have been reported to play a crucial role in the RNA-binding capacities of proteins involved in chloroplast RNA processing [20–22].

One of the most widespread is the RRM, also referred to as the RBD or ribonucleoprotein (RNP) domain (Fig. 2). This motif was first discovered in nuclear heterogeneous RNP particles (hnRNPs) of eukaryotes; however, recent genomic surveys have shown that RRMs also occur in prokaryotes (predominantly cyanobacteria), and to a lesser extent in viruses [23–25]. A typical RRM consists of approximately 90 amino acids and folds into a conserved structure comprising a four-stranded β-sheet and two α-helices that form an α/β sandwich ($\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$) [26, 27]. The characteristic feature of the RRM are the two canonical consensus sequences [RK]-G-[FY]-[GA]-[FY]-[ILV]-X-[FY] and [ILV]-[FY]-[ILV]-X-N-L designated as RNP1 and RNP2, which are located in strand β_3 and strand β_1, respectively [23]. Binding of RNA is primarily mediated by hydrophobic and stacking interactions between three conserved aromatic side-chains located within RNP1 and RNP2 and the RNA [26]. Frequently, RRMs can be found in multiple copies or in combination with other protein domains. Multiple RRMs on a single polypeptide

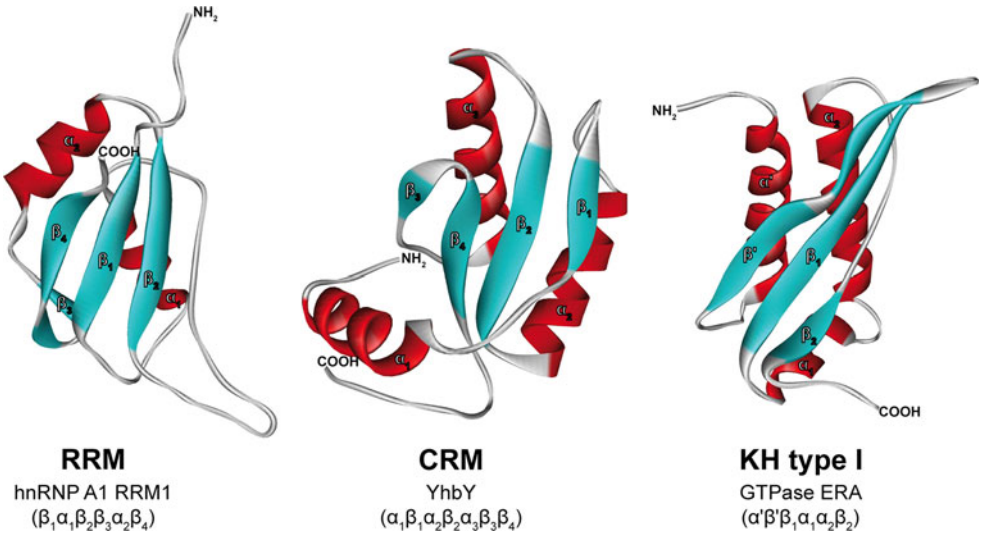

Fig. 2 Typical protein domains involved in RNA binding. The structures shown are secondary structures of RRM1 of hnRNP A1 [139], the CRM domain of YhbY, and the KH type I domain of GTPase ERA. The structures were generated with ViewerLite 4.2 (Accelrys)

chain enable the recognition of longer nucleotide sequences and contribute to RNA-binding affinity [23]. The combination of RRMs with other protein domains allows the modulation of RNA-binding properties, as well as protein–protein interactions or enzymatic activity [18]. Interestingly, certain RRMs are not involved in RNA binding but rather in protein–protein interaction.

A recently identified RBD that appears to be restricted to archea, bacteria and plants is the so-called CRM domain (Fig. 2) [28]. In archea and bacteria, CRM-containing proteins are represented by the orthologue YhbY, a small polypeptide harbouring a single CRM domain that binds to precursors of the 50 S ribosomal subunits [29]. However, in plants, CRM domains occur in a protein family having multiple copies of these domains. Structural analyses of bacterial YhbY proteins have shown that the CRM domain exhibits an $\alpha_1\beta_1\alpha_2\beta_2\alpha_3\beta_3\beta_4$ topology similar to the C-terminal fold of translation initiation factor 3 [30]. Several sites of the CRM domain seem to be involved in RNA binding, as was indicated by structural and mutational analyses [29, 31].

The sequence G-X-X-G is conserved in all bacterial YhbY orthologues and occurs in at least one of the CRM domains of multi-CRM-domain proteins. Interestingly, a G-X-X-G loop is involved in the RNA-binding properties of the nonrelated KH domain which was primarily identified in human hnRNP K (Fig. 2) [32]. Proteins harbouring KH domains have been detected in archea, bacteria and eukaryotes, where they most commonly occur in multiple copies [32, 33]. The KH domain is characterized by a motif of approximately 70 amino acids and has a conserved consensus sequence V-I-G-X-X-G-X-X-I [27]. Structural analysis of diverse KH domain-containing proteins revealed that there are actually two different variants of KH domains sharing the same consensus sequence [34]. Comparison of amino acids showed that both KH folds share a minimal motif that exhibits a $\beta_1\alpha_1\alpha_2\beta_2$ topology, but the two structures show different three-dimensional conformations. In addition, type I KH domains include a C-terminal extension composed of a β'-sheet and an α'-helix, while type II KH domains exhibit an N-terminal α'-helix and β'-sheet.

A conserved motif shared by both KH folds is the above-mentioned loop connecting α_1 and α_2. This loop is characterized by the sequence G-X-X-G. Interaction with RNA involves a binding niche formed by previously mentioned secondary structures, and occurs through electrostatic and hydrophobic interactions and is also a result of shape complementary [33, 34]. However, in the case of the CRM motif, the sequence G-X-X-G is located in an unlooped region between the first β-strand and the second α-helix of the domain. Moreover, in the CRM domain of higher plants, where G-X-X-G is typically conserved as G-(R/I)-R-G, RNA binding seems to be mediated by the arginine residues, while in the KH domain the glycine residues have an important function [31]. Further conserved motifs of the CRM domain are a sequence of aromatic and positively charged amino acids (W-K-H-K) located in α_2 and the three amino acids Y-R-G (Y-R-P in bacterial CRM domains) at the C-terminal end of the domain [31]. It is assumed that RNA recognition of CRM domains is akin to that of RRM domains since both exhibit a β-strand RNA-binding platform characterized by aromatic residues and a similar secondary structure that is defined by a $\beta\alpha\beta\beta$ fold (RRM: $[\beta_1\alpha_1\beta_2\beta_3]$ $\alpha_2\beta_4$; CRM: $\alpha_1\beta_1\alpha_2$ $[\beta_2\alpha_3\beta_3\beta_4]$) [31].

A further sequence motif involved in RNA binding can be observed in one of the largest protein families of plants. The PPR proteins were initially identified in *Arabidopsis thaliana*, where they comprise a class of approximately 450 proteins [35, 36]. The characteristic feature of PPR proteins

is a consensus sequence encompassing 31–36 amino acids that is related to the tetratricopeptide repeat (TPR). This consensus sequence occurs in tandem repeats of 2–26 motifs per polypeptide [36]. The TPR motif folds into a pair of anti-parallel α-helices and is known to play a role in protein–protein interactions [37]. In contrast to this, the PPR motif exhibits certain structural variations that are consistent with a function in RNA binding [36]. Today, although the RNA-binding properties of several PPR proteins have been proven experimentally, details of the mechanics of RNA–protein binding are still vague [35, 38–40].

RNA-binding proteins modulate chloroplast RNA stability

In general, the abundance of mRNA depends on an elaborate interplay between transcription rates, RNA processing and decay. In chloroplasts, transcription is performed by at least two polymerases of different phylogenetic origin [41, 42]. One of them is the above-mentioned plastid-encoded plastid RNA polymerase (PEP). This polymerase exhibits homologies with the α, β, and β′ subunits of the *Escherichia coli* DNA-dependent RNA polymerase. In addition, the promoters specific for PEP resemble *E. coli* σ^{70}-type promoters including the −35 consensus element T-T-G-A-C-A and the −10 element T-A-T-A-A-T. The second polymerase is the nuclear-encoded phage-type RNA polymerase (NEP), which seems to be represented by a single polypeptide of approximately 110 kDa [43, 44]. In vitro studies revealed the presence of a small consensus sequence of the nucleotides C-R-T that appears to be sufficient for initiation of transcription [45]. Regulation of transcriptional activity of PEP, as well as NEP, is dependent on, among other regulatory elements, a set of nuclear-encoded σ-factors [46]. Interestingly, both RNA polymerases seem to be responsible for the transcription of different sets of genes. Thus, it is proposed that PEP drives transcription of genes encoding elements of the photosynthetic machinery, whereas NEP transcribes genes encoding components of the plastid transcriptional and translational system [41, 47].

Nevertheless, one study has shown that transcriptional activity mostly does not correspond to chloroplast transcript levels, in particular during developmental processes [48]. This finding indicates that transcriptional regulation plays a minor role in modulation of chloroplast transcript abundance [13, 49, 50]. In contrast, an elemental factor for regulation of chloroplast mRNA accumulation appears to be the control of transcript stability by RNA-binding factors [17, 51].

Several RNA-binding proteins involved in stabilization of chloroplast RNAs form high molecular weight RNP particles with ribosome-free RNAs (Table 1) [52]. They belong to a group of extremely abundant proteins with a size of approximately 30 kDa. Such RNA-binding proteins have been identified in various species including *Nicotiana tabacum*, *Zea mays*, *Pisum sativum*, *Spinacia oleocera*, *Hordeum vulgare* and *A. thaliana* [53–58]. A characteristic feature within the structure of these proteins is the occurrence of two RRMs and an acidic N-terminal domain. Based on the similarity of their RRM sequences, they can be subdivided into three phylogenetic groups [55]. In the case of the five *N. tabacum* proteins, the classification is as follows: cp29A and cp29B belong to group I, cp28 and cp31 to group II, and cp33 to group III. Their function in stabilizing chloroplast transcripts was demonstrated in experiments with *N. tabacum* chloroplast extracts depleted of all five cpRNPs, which showed a rapid degradation of in vitro synthesized *psbA* mRNA, while subsequent supplementation with heterologously expressed cpRNPs resulted in the restoration of *psbA* mRNA half-life. UV crosslinking showed that the observed stabilizing effect is mediated by direct binding of cpRNPs to *psbA* transcripts [52]. Since no distinct binding sites for cpRNPs have so far been detected within the sequences of chloroplast RNAs, it was proposed that they represent a kind of "general" RNA-binding

Table 1 RNA-binding proteins involved in stabilization of chloroplast RNAs

Protein	Organism	Function	Target	RBD	Reference
cp28/cp29A/cp29B/cp31/cp33	*N. tabacum*	Stabilization	Non-ribosome-bound mRNAs	RRM	[58]
cp31A/B	*A. thaliana*	Stabilization and editing	See *N. tabacum* orthologue, editing of several sites	RRM	[21]
28RNP	*S. oleocera*	Stabilization	See *N. tabacum* orthologue	RRM	[54]
33RNP	*P. sativum*	Stabilization	See *N. tabacum* orthologue	RRM	[53]
Mca1	*C. reinhardtii*	Stabilization	*petA* mRNA	PPR	[140]
Mrl1	*C. reinhardtii*	Stabilization	*rbcL* mRNA	PPR	[71]
PPR5	*Z. mays*	Stabilization	*trnG* UCC precursor	PPR	[38]
PPR10	*Z. mays*	Stabilization	*atpI*, *atpH*, *psaJ*, *rpl33* mRNA	PPR	[80]

protein akin to the nuclear hnRNPs. Moreover, it is thought that both RNA-binding proteins are phylogenetically related and that cpRNPs, similar to hnRNPs, may associate nonspecifically with any ribosome-free RNA to mediate stability and ribonuclease resistance [59]. Actually, cpRNPs also appear to possess additional functions; for instance in pre-tRNA splicing, since they were reported to co-immunoprecipitate with chloroplast intron-containing precursor RNAs [58]. Recent findings also indicate a further role for cpRNPs in editing of chloroplast transcripts; this function is probably not a catalytic one as the small proteins consist almost entirely of the two RRMs and the acidic N-terminus, lacking functional protein domains [21]. In contrast to the above-described chloroplast RNA-binding protein family that interacts rather nonspecifically with chloroplast transcripts, several proteins have been described that have specific target mRNAs.

In the scope of chloroplast transcript stabilization, specific sequence elements located mainly in the 5′ and 3′ untranslated regions (UTR) of transcripts have been reported to be of importance. These elements involve, for instance, an inverted repeat within the 3′ UTR of most chloroplast transcripts that can fold into a typical stem-loop structure. It is assumed that these structural elements are necessary for accurate RNA maturation and contribute to RNA stability by impairing exonuclease activity [60–62]. However, even though no general stem-loop structures can be found in the 5′ UTR of chloroplast transcripts, the deletion or mutational changes within certain elements of plastid 5′ UTRs can also lead to alterations in transcript stability, and as a result of this, to a decrease in transcript accumulation and protein synthesis [63–67].

To date, several nuclear factors have been examined, mainly in *Chlamydomonas reinhardtii*, that bind to 5′ and 3′ UTRs and thereby contribute to transcript stability. One well-characterized example is the nuclear-encoded protein Mca1 of *C. reinhardtii* that belongs to the PPR protein family [68]. Mca1 is required for expression of *petA*, encoding cytochrome *f*, the largest subunit of the cytochrome b_6 *f* complex that is part of the photosynthetic electron transport chain. *C. reinhardtii* strains in which *mca1* is affected fail to accumulate any *petA* transcripts. Pulse labelling experiments showed that this effect results from an increase in *petA* transcript instability since transcriptional rates remain unchanged [69]. Moreover, functional complementation analysis of *mca1* mutants demonstrated that Mca1 protein abundance is a rate-limiting factor for *petA* transcript levels [70]. In addition, analysis of chimeric 5′ UTRs showed that the target of Mca1 lies within the first 21 nucleotides of the *petA* UTR. The insertion of a poly G tract at the beginning of the *petA* 5′ UTR inhibits the degradation of its transcript in the absence of Mca1 protein, indicating that Mca1 functions as a protective factor against 5′–3′ ribonucleolytic degradation [68].

Recently, a further PPR protein that is required for stabilization of the *rbcL* mRNA in *C. reinhardtii* and *A. thaliana* was identified. In the *C. reinhardtii* mutant strain *mrl1* photosynthetic activity is affected due to a lack of rubisco, the CO_2-fixing enzyme of the Calvin cycle. It was shown that this effect relies on instability of the *rbcL* transcript since transcription rates remain unchanged. Finally, the target region of Mrl1 was reported to be the 5′ UTR of the *rbcL* transcript [71].

A factor involved in stabilization of *psbD* transcript in *C. reinhardtii* is Nac2 (Mbd1). This factor was identified in the photosynthetic mutant *nac-26* that exhibits an unstable photosystem II (PSII) since it is not able to synthesize the PSII core protein D2. No mature *psbD* transcript can be detected in the mutant strain, although transcription rates of *nac2* are unaltered, as seen by pulse-labelling and northern blot experiments [72]. Nac2 has a molecular weight of 140 kDa and belongs to the family of TPR proteins since it contains nine TPR-like motifs [22]. Sedimentation analysis demonstrated that Nac2 is part of a high molecular weight complex, as it was seen to interact with further proteins. Since there is no direct evidence to show that Nac2 binds directly to *petD*, it is proposed that it acts as a scaffold protein, promoting the assembly of a protein complex that binds to the 5′ UTR of *petD* and leads to protection against 5′–3′ ribonucleolytic degradation [64]. It was later shown that Nac2, together with the protein RPB40 (RB38) is part of a 550 kDa RNP complex that is involved in translational control of *psbD* [73].

A further polypeptide that is suggested to mediate the assembly of a RNA-binding complex involved in transcript stabilization is Mbb1. Mbb1 is required for stable accumulation of *psbB*, *psbT* and *psbH*, with *psbB* encoding the core antenna CP47 (PsbB) of PSII. This polypeptide was identified in the PSII-deficient *C. reinhardtii* strain 222E [74]. Analysis of chimeric genes consisting of the *psbB* promoter, 5′ UTR, and a reporter gene allowed the identification of the target site in the 5′ UTR of *psbB* [75]. Mbb1 is part of a 300 kDa RNP complex, exhibits ten TPR domains, and displays no known RNA-binding motifs [76].

Finally, Mcd1 is required for steady-state accumulation of *petA* transcripts encoding subunit IV of the cytochrome b_6 *f* complex, since it protects the mRNA from 5′ to 3′ exonuclease activity. Interestingly, Mcd1 does not resemble any other regulatory chloroplast proteins and exhibits no discernible RBDs [77].

In higher plants, only a few factors involved in transcript stability have been described so far [78–80]. One of them is HCF145, which was identified in an *A. thaliana* mutant affected in PSI activity due to a lack of the two PSI core proteins [79]. This defect appears to be a result of increased

instability of the tricistronic *psaA*, *psaB*, and *rps14* transcript, encoding the two core proteins of PSI and the ribosomal protein S14. Nevertheless, the *hcf145* gene product is still unknown.

Another protein is the PPR protein PPR10 identified in a nonphotosynthetic *Z. mays* mutant strain, lacking a functional *ppr10* gene. *ppr10* mutants fail to accumulate specific transcripts derived from the polycistronic transcription units *atpI-atpH-atpF-atpA* and *petL-petG-psaJ-rpl33-rps18* [80]. All transcripts mapping in the intergenic region of *atpI-atpH* or *psaJ-rpl33* show decreased levels, indicating a function of PPR10 in the stabilization of these RNAs. In vivo and in vitro studies demonstrated that PPR10 binds to specific consensus sequences within the overlapping 5′-and 3′-termini of the transcripts. It is proposed that PPR10 acts as "protective cap" blocking 5′–3′ and 3′–5′ exonuclease activity.

RNA-binding proteins are involved in chloroplast splicing

Several chloroplast genes encoding proteins, as well as tRNAs, are disrupted by introns belonging either to group I or to group II [81–83]. Both intron classes are characterized by their structural features and splicing mechanisms [83, 84]. The typical secondary structure of a group I intron exhibits approximately ten paired regions, P1 to P10, that fold into three domains. Splicing occurs via two transesterification reactions and includes the nucleophilic attack of an external guanosine nucleotide on the 5′-splice site, and the subsequent attack of the newly generated free 3′-OH of the 5′-exon on the 3′-exon, resulting in the ligation of the 5′- and 3′-exons and release of the intron [83]. Group II introns fold into a typical secondary structure comprising six partly helical domains, D1 to D6, extending from a central wheel. Depending on their structure, they can be further subdivided into three different main families: IIA, IIB, and the more recently identified IIC [81, 85]. The splicing mechanism of group II introns, also referred to as the lariat pathway, differs from that of group I introns, and is similar to that of nuclear spliceosomal introns. Instead of an external guanosine nucleotide, the 2′-OH of a bulged adenosine, also called the branch point adenosine, is used as the nucleophile. The reaction pathway includes attack of the 5′-splice site by the 2′-OH of the branch point adenosine, leading to release of the 5′-exon and formation of the intron lariat 3′-exon. Subsequently, the 3′-splice site is attacked by the 3′-OH of the released 5′-exon, resulting in ligation of the 5′- and 3′-exons and release of the lariat intron [84, 86].

Initially, some group I and group II introns, mainly discovered in fungal mitochondrial genomes, were shown to splice in vitro and therefore were referred to as ribozymes [87, 88]. In photoautotrophic organisms, the first autocatalytically spliced group II intron (rI1) was discovered in a mitochondrial gene from the green alga *Scenedesmus obliquus*, encoding the large subunit rRNA [89]. For efficient in vivo splicing, however, these autocatalytically spliced introns are dependent on accessory protein machineries and factors. Many of these are obviously conserved between organelles and species since, for example, rI1 splices correctly in vivo in chloroplasts of *C. reinhardtii* as well as in *E. coli* [90, 91]. Moreover, mutations of conserved intron nucleotides leads to the same effect on in vivo chloroplast splicing as on in vitro splicing reactions [90]. Besides their ribocatalytic activity, both intron groups have the ability to act as mobile genetic elements. Thus, they are able to insert site-specifically into intronless alleles, a process known as homing. Alternatively, they are able to move to other ectopic sites [87, 88, 92].

In many cases, splicing and mobility of group I and II introns is facilitated by a protein that is encoded by an ORF within the intron itself and which is therefore denoted as intron-encoded protein (IEP) [83, 85, 88]. In group I introns, the location of the IEP-encoding ORF varies, but mostly it can be found within a peripheral loop of the intron structure, thereby avoiding impairment with core structural elements. Most group I IEPs have endonuclease activity to facilitate intron movement by generation of double-strand DNA breaks and maturase activity to promote splicing. Group I IEPs comprise four different classes, displaying specific-sequence motifs. These include proteins with the LAGDLIDADG, the GIY-YIG and the HNH motifs, and the His-Cys box [93]. Endonucleases exhibiting the first three motifs also exist as free-standing ORFs not associated with an intron. In group II introns, ORFs are located in the loop of domain D4 [88]. The IEPs of group II introns are multifunctional proteins, which generally comprise four conserved domains for reverse transcriptase (RT) activity, splicing or maturase function, as well as DNA binding and endonuclease activity. As with group I introns, group II IEPs facilitate in vivo intron splicing and intron mobility [94]. A well-characterized IEP is RT which was shown by an in vitro assay to have RNA-directed DNA polymerase activity with a sensitivity to RT inhibitors [95]. Later, experiments with yeast demonstrated the transposition of an mtDNA intron by a target DNA-primed reverse transcription [96]. The first chloroplast intron encoding a RT was discovered in the *petD* gene from the green alga *S. obliquus* and encodes a subunit of the cytochrome b_6 *f* complex [97].

However, most group I, and almost all organellar group II introns, lack IEPs [83, 92]. Nevertheless, all introns need to be spliced correctly in order to retain gene functionality.

Hence, in many cases, host-encoded proteins are recruited for splicing of group I and II introns. A genetic screen in *Z. mays* led to the identification of two proteins, CRS1 and CRS2, that are involved in splicing of group II introns [98]. *crsI* mutants have a reduced level of chloroplast ATP synthase, which is due to a defect in splicing of the subgroup IIA intron of the *atpF* transcript, whereas, *crsII* mutants are deficient in splicing of a whole set of transcripts, including the group IIB introns of *petD*, *rpl16*, *rps16*, *trnG*, *ndhB*, *petB*, *rps12-1*, *ndhA* and *ycf3-1* [28, 98, 99]. Molecular cloning of the *crsI* gene allowed further analysis of the putative gene product and revealed the presence of a novel protein domain, the above-described CRM domain, which was found to be conserved in further plant proteins and is represented as a free-standing ORF in archea and bacteria [100]. Moreover, co-immunoprecipitation analysis revealed that CRS1 harbours three CRM domains and binds to *atpF* transcripts in vivo [28]. Further, the heterologously expressed *crs1* was shown to form a dimer and to bind to *atpF* intron RNA in vitro with a k_D of 3 n*M* (Crs1 monomer concentration) [30]. The CRS1 binding sites were identified by hydroxyl-radical footprinting experiments and lie in domain D1 and D4 of the *atpF* intron. It was suggested that CRS1 is required for correct folding of the *atpF* intron. Nevertheless, CRS1 appears not to be sufficient for splicing, but to require additional factors [20]. CRS2 (23 kDa) displays similarities to bacterial peptidyl-tRNA hydrolase, an enzyme responsible for cleaving the ester bond, linking tRNA and emerging protein in abortive translation products [101]. It is found in large RNP complexes in the chloroplast stroma.

Using a yeast two-hybrid screening approach, two proteins, CAF1 and CAF2, which also interact in vivo with CRS2 were detected [28]. Interestingly, these proteins are also members of the CRM protein family since each possesses two CRM motifs. Analysis of *caf1* and *caf2* mutants demonstrated that all introns spliced depending on CRS2 also require CAF, indicating a high degree of interdependence between the different polypeptides. Indeed, the CRS2-dependent introns can be subdivided according to their need for either CAF1 or CAF2. Thus, *petD*, *rpl16*, *rps16* and *trnG* require CAF1, *ndhB*, *petB* and *rps12-1* require CAF2, whereas *ndhA* and *ycf3-1* require both CAF proteins [28]. Since binding of CRS2 to its intron is weak and nonspecific, it is proposed that the CAF proteins generate an RNA-binding platform, which in turn recruits CRS2 to specific introns [28, 102].

In *A. thaliana*, orthologues of the *Z. mays* splicing factors have been detected [103]. The splicing functions and target transcripts of AtCRS1, AtCAF1 and AtCAF2 were shown to resemble the *Z. mays* counterparts, indicating that these proteins adopted group II intron splicing functions prior to the evolution of mono- and dicotyledons. Moreover, AtCAF1 functions in splicing of two introns, *rpoC1* and *clpP-1*, which are not present in *Z. mays* [103].

Besides CRS2, CAF1 and CAF2 co-immunoprecipitate with a protein called RNC1, which exhibits similarities to ribonuclease III [104]. However, RNC1 lacks endonuclease activity and seems to promote splicing of several introns belonging to subgroup IIA and subgroup IIB, including *ndhB*, *trnG*, *petB* and *petD* as well as *trnI*, *trnA*, *trnK* and *trnV*, and possibly *rps12-2* and *atpF* [104]. Co-immunoprecipitation and pull-down assays showed that WTF1, a stromal protein harbouring a novel RBD, DUF860 (domain of unknown function 860), strongly interacts with RNC1. RNA-binding capacities of WTF1 and DUF860 were demonstrated via electrophoretic mobility shift assays (EMSAs). DUF860 shows weak similarities to ubiquitin hydrolases (UBHs) and lacks amino acids essential for catalytic activity. In contrast to UBHs, which are distributed in all eukaryotic branches, proteins harbouring DUF860 domains are restricted to plants. Moreover, structural models show distinct variations between DUF860 and UBHs, with DUF860 exhibiting similarities to the surface structure of RRMs. Therefore, it was suggested that DUF860 be renamed as plant organelle RNA-recognition (PORR) domain [105].

Recently, two further proteins interacting with CRS2/CAF particles have been identified [106, 107]. CFM2 is related to CRS1, but harbours an additional CRM domain at the C-terminus. It binds to the group II introns *ndhA* and *ycf3-1* as well as to the group I intron of the *trnL*-UAA precursor. Hence, CFM2 is one of the few proteins identified to date that is involved in splicing of group I introns [88]. The *A. thaliana* orthologue AtCFM2, in addition to the above-mentioned introns, is also involved in splicing of the *clpP-2* group II intron not present in *Z. mays* [106]. In contrast, CFM3 associates with *petB*, *petD*, *ndhB*, *rpl16*, *rps16* and *trnG* transcripts. CFM3 also belongs to the "CRS1" subfamily and harbours three CRM domains. Interestingly, it appears to be dual-targeted and localizes to chloroplasts as well as mitochondria. Taken together, all *cis*-spliced introns in *Z. mays* that are dependent on CRS2/CAF complexes (*petD*, *rpl16*, *rps16*, *trnG*, *ndhB*, *petB*, *ndhA* and *ycf3-1*) also require either CFM2 or CFM3. An exception is the *trans*-spliced intron *rps12-1*, which seems to be spliced without the need for CFM2 or CFM3 [107].

In contrast, the stroma-localized PPR protein PPR4 that besides its 16 tandem PPR repeats comprises an N-terminal RRM domain, is involved in *trans*-splicing of *rps12-1*. As mentioned above, the CRS2/CAF2 complex is also required for splicing of *rps12-1*. Yet it is still unclear whether CRS2/CAF2 and PPR4 act simultaneously or independently of each other [39].

Another PPR protein involved in chloroplast splicing is OTP51. OTP51 harbours seven PPR domains and two

C-terminal LAGLIDADG domains, with LAGLIDADG domains being typical for endonucleases. The LAGLIDADG domains of OTP51 though lack certain critical residues responsible for endonucleolytic activity. Thus, it is proposed that OTP51 lacks endonuclease activity, but possibly retains maturase activity. *otp51* mutants fail to splice intron 2 of *ycf3* transcript, and since YCF3 is important for correct assembly of PSI, they exhibit a strongly decreased content of PSI, but interestingly also of PSII. In addition to its function in splicing of *ycf3-2* transcript, OTP51 appears to contribute to splicing of *atpF*, *trnK* and *trnV*.

The above-described examples demonstrate that the chloroplast splicing machinery of higher plants is very complex with several proteins acting together in large RNP complexes. This is also true for splicing of *C. reinhardtii psaA*, which encodes the core protein of the photosystem I reaction centre. The *psaA* gene is separated into three exons, which are widely distributed over the plastom and flanked by consensus sequences typical for group II introns belonging to subgroup IIB [108]. The exons are transcribed individually and the major transcript is then assembled in *trans*. Genetic analyses have shown that *trans*-splicing of *psaA* depends on at least 14 nuclear-encoded factors [109]. Mutations in these genes block splicing of either intron 1 (class B mutants) or intron 2 (class A mutants), or both introns (class C mutants). Furthermore, class C mutants can be blocked either in the splicing of intron 1 or in the processing of the small chloroplast RNA *tscA* from the polycistronic precursor *tscA/chlN* [109–111]. *tscA* RNA is essential for formation of the tripartite intron 1 of *psaA* since it contributes domains D1 to D4 [112]. Seven nuclear-encoded proteins affecting splicing of *psaA* have been identified [113–119].

By genetic analyses Rat1 and Rat2 proteins crucial for 3′-end processing of *tscA* RNA were identified. Rat1 displays significant homologies with the NAD^+-binding domain of poly(ADP-ribose) polymerases, but mutations in this domain have no impact on correct *tscA* processing. Rat2, on the other hand, apart from a short glycine-rich region, shows no homologies with known proteins [118].

Raa1 is a quite large polypeptide of approximately 206 kDa exhibiting only minor similarities to other proteins. Interestingly, it contains several repeated amino acid stretches which are thought to represent counterparts to the well-known PPR or TPR motifs. Mutant analyses showed that Raa1 is not only involved in correct processing of the *tscA*/*chlN* precursor transcript, but also facilitates splicing of *psaA* intron 2. Thus, Raa1 is regarded as a multifunctional factor [115]. Just as for Raa1, Raa2 was shown to promote splicing of the second *psaA* intron. Raa2 has a much smaller size of about 40 kDa, and displays similarities to pseudouridine synthases, a protein family known for modification of specific uridine residues in tRNAs, rRNAs and snRNAs. However, *raa2* mutants lacking specific amino acids that are known to be required for catalytic activity of pseudouridine synthases are not affected in intron 2 splicing [116]. Raa3 is involved in splicing of *psaA* intron 1 and shows only minor homologies with other proteins within a small amino acid stretch harbouring similarities to pyridoxamine 5′-phosphate oxidases. Nevertheless, as is the case for Raa2, it is currently unclear whether this domain contributes to Raa3 functionality [114].

Besides the above-described proteins, which were revealed via analyses of *psaA trans*-splicing deficient mutants, two proteins were identified by biochemical approaches. Using the yeast three-hybrid system and *tscA* RNA as a bait, a protein of approximately 35 kDa designated as cNAPL was isolated. cNAPL shares similarities to the nucleosome assembly protein (NAP) family that is known for its multifunctional role in nucleosome assembly, remodelling of chromatin, and transcriptional and cell cycle regulation. The RNA-binding properties of cNAPL were verified via EMSAs that showed that the protein binds specifically to *psaA* intron 1 and also, albeit with a lower affinity, to other U-rich chloroplast transcripts [113]. Integration of the mitochondrial group II intron rI1 from the green alga *S. obliquus* led to the isolation of a 61 kDa protein later identified as α-subunit of the chaperonin Cpn60. Cpn60 is related to members of the bacterial GroEL family known to function in folding and assembly of proteins. EMSAs confirmed RNA-binding capacity of recombinant Cpn60, which interacts specifically with domains D5 and D6 of *psaA* intron 1 [117, 120].

As for the chloroplast-splicing machinery in higher plants, the above-described *C. reinhardtii* proteins also form part of large multiprotein and RNP complexes. Raa1 and Raa2, for example, associate in a large membrane-bound protein complex of 400–500 kDa, whereas Raa3 was identified in a soluble ribonucleoprotein complex of approximately 1,700 kDa [114, 116]. The organization of the above-described chloroplast-splicing factors in RNP particles shows striking resemblances to the spliceosome of the nucleus [121]. Thus, these multiprotein complexes could be regarded as chloroplast spliceosomes.

Interestingly, several of the above-described proteins involved in chloroplast splicing show homologies with proteins that are known to have functionality in other cellular processes. Examples include RNC1 that exhibits homologies with ribonuclease III and Raa2 that exhibits homologies with pseudouridine synthases [104, 116]. However, functional analyses of the catalytic activity typical for these two enzymes demonstrate that they have mostly lost their functional competence. This finding

therefore indicates that preexisting cellular proteins may have been recruited during evolution to play a role in splicing because of their RNA-binding capacity.

RNA-binding proteins facilitate RNA editing

RNA editing refers to the post-transcriptional modification of individual nucleotides within an mRNA molecule. RNA editing takes place in nearly all land plants except the Marchantiales, an order of liverworts, whereas this process is apparently absent in algae and cyanobacteria. Since *Marchantia* branched early on during the evolution of land plants, it has been proposed that RNA editing developed in the early stage of land plant evolution [122]. In chloroplasts, most RNA editing events involve cytidine (C) to uridine (U) exchanges, while the conversion of U to C, at least in angiosperms, occurs rarely [123, 124]. Generally, RNA editing sites are found in ORFs, but in some cases, sites can also be found in noncoding regions located mostly upstream of the start codon [15, 17]. There are notable differences in editing frequencies between different species, e.g. the transcriptome of the hornwort *Anthoceros formosae* has 947 editing sites whereas *Z. mays* has only 27 sites [125, 126]. Molecular and biochemical experiments in *Z. mays* have shown that RNA editing occurs directly after transcription and is independent of splicing and intercistronic processing [127, 128]. No distinct consensus sequence surrounding the RNA editing site has yet been found; however, there are experimental data that demonstrate that certain *cis*-elements close to the target nucleotide are essential for editing activity [17].

There is evidence that nuclear-encoded factors are involved in plastid-editing processes, just as was shown for stabilization and splicing of chloroplast RNAs (Table 2). Several of these factors have been identified recently. In a genetic study of photosynthetic electron transport in *A. thaliana*, a protein involved in specific editing of *ndhD* transcript that encodes a subunit of the chloroplast NAD(P)H dehydrogenase complex (NDH) functioning in cyclic electron flow was identified [129]. Plant mutants affected in *crr4* are defective in editing site 1 of the *ndhD* (ndhD-1) transcript which generates the codon needed for translational initiation. *crr4* encodes a PPR protein comprising 11 PPR repeats. Since heterologously expressed *crr4* binds to nucleotides surrounding ndhD-1, it is proposed that CRR4 acts as a platform for recruitment of a putative RNA editing enzyme such as a cytidine deaminase. A second PPR protein CRR21 is implicated in editing of a further RNA editing site of the *ndhD* transcript, ndhD-2, which leads to conversion of serine-128 of NdhD protein to leucine [40]. Analysis of

Table 2 RNA-binding proteins involved in splicing of chloroplast RNAs

Protein	Organism	Function	Target	RBD	Reference
CRS1	*Z. mays*	Splicing	*atpF* mRNA	CRM	[20]
AtCRS1	*A. thaliana*	Splicing	See *Z. mays* orthologue	CRM	[103]
CAF1	*Z. mays*	Splicing	*petD*, *rpl16*,*rps16*, *ndhA*, *ycf3-1*, *trnG* mRNA	CRM	[102]
AtCAF1	*A. thaliana*	Splicing	*rpoC1*, *clpP-1* mRNA, see *Z. mays* orthologue	CRM	[103]
CAF2	*Z. mays*	Splicing	*ndhB*, *pet*, *rps12-1*, *ndhA*, *ycf3-1* mRNA	CRM	[102]
AtCAF2	*A. thaliana*	Splicing	See *Z. mays* orthologue	CRM	[103]
CFM2	*Z. Mays*	Splicing	*ndhA*, *ycf3-1* mRNA, *trnL*-UAA precursor	CRM	[106]
AtCFM2	*A. thaliana*	Splicing	*clpP-2*, see *Z. mays* orthologue	CRM	[106]
CFM3	*Z. mays*, orthologues in *A. thaliana* and *O. sativa*	Splicing	*petB*, *petD*, *ndhB*, *rpl16*, *rps16* mRNA, *trnG* UCC precursor	CRM	[107]
WTF1	*Z. mays*	Splicing	*ndhB*, *petB*, *rpl16*, *rps16*, *petD*, *trnG*, *atpF*, *rpl2*, *rps12-2*, *trnI*, *trnA*, *trnK*, *trnV* mRNA	DUF860	[105]
HCF152	*A. thaliana*	Splicing or stabilization	*petB* mRNA	PPR	[141]
OTP51	*A. thaliana*	Splicing	*ycf3-2* mRNA	PPR, LAGLIDADG	[142]
PPR4	*Z. mays*	*Trans*-splicing, biogenesis of ribosomes	*rps12-1* mRNA	PPR/RRM	[39]
PPR531-11	*P. patens*	Splicing	*clpP* mRNA	PPR	[143]
Raa1	*C. reinhardtii*	*Trans*-splicing, processing	*psaA*, *tscA* mRNA	Putative PPR protein	[115]

chlorophyll fluorescence indicates that the NDH activity of *A. thaliana crr21* strains is impaired. Interestingly, CRR4 and CRR21 lack significant homologies except for their C-terminal domains. These domains show similarities to the E/E$^+$ domain, which belongs to the E$^+$ subgroup of the so-called PLS subfamily of PPR proteins. This subfamily is restricted to plants and can be subdivided into four subgroups based on their C-terminal motifs: PLS, E, E$^+$ and DYW [130]. Domain-swapping experiments showed that the C-terminal domain is exchangeable between CRR4 and CRR21. Nevertheless, EMSAs demonstrated that the E/E$^+$ domain does not play a role in RNA binding. A PPR protein sharing similarities with CRR4 and CRR21 is CLB19 [131]. This PPR protein is involved in editing of two transcripts, namely *rpoA*, encoding the alpha subunit of PEP, and *clpP*, encoding a subunit of the ATP-dependent ClpP serine protease.

Recently, several further PPR proteins belonging to the DYW subgroup have been reported to function in plastid RNA editing, one of which is OTP82 [132–138]. OTP82 is required for editing of ndhB-9 and ndhG-1 leading to conversion of serine-279 of NdhB to leucine and conversion of serine-17 of NdhG to phenylalanine, respectively [133]. Remarkably, mutants affected in *otp82* do not show a significant visible phenotype. OTP82 consists of 741 amino acids and contains 14 PPR repeats. It has been shown that the amino acid conversions generated by OTP82 are not required for NDH activity nor for NDH stability and complex formation. Functional complementation of an *otp82* mutant with a truncated version of OTP82 lacking the DYW domain leads to restoration of the wild-type editing rates. This finding indicates that the DYW domain has no apparent function regarding in vivo editing and is in accordance with previous functional analyses of the DYW domain of the RNA-editing factors CRR22 and CRR28 [132, 133]. OTP82 was identified among six other editing factors in a reverse genetic approach using a high-resolution melting screen [134]. Strains *otp80*, *otp81*, *otp85* and *otp86* were shown to be affected in editing of *rpl23*, *rps12*, *ndhD* and *rps14*, respectively. *otp84* is impaired in editing of three transcripts, *ndhF*, *ndhB* and *psbZ*. To elucidate the question as to how editing factors might recognize multiple target sites, a systematic bioinformatic analysis was applied: the 15 nucleotides immediately upstream the editing site of *A. thaliana* editing targets were examined regarding possible consensus sequences. This approach indeed allowed the identification of an unambiguous 15-nucleotide consensus in the multiple target sites operated on by the same factor. Furthermore, the composition of the consensus indicates that the targeting of editing factors rests upon the recognition of conserved nucleotide and purine/pyrimidine positions within the target sites.

Conclusion

This article has reviewed the function of RNA-binding proteins in chloroplasts. In addition to protein domains involved in RNA binding, many studies have provided insight into the biological function of these proteins during the post-transcriptional modification of chloroplast RNAs (Table 3). With the availability of whole genome

Table 3 RNA-binding proteins involved in editing of chloroplast RNAs

Protein	Organism	Function	Target	RBD	Reference
cp31A/B	*A. thaliana*	Stabilization and editing	See *N. tabacum* orthologue, editing of several sites	RRM	[21]
CRR4	*A. thaliana*	Editing	*ndhD* mRNA	PPR	[129]
CRR21	*A. thaliana*	Editing	*ndhD* mRNA	PPR	[40]
CRR22	*A. thaliana*	Editing	*ndhB*, *ndhD*, *rpoB* mRNA	PPR	[132]
CRR28	*A. thaliana*	Editing	*ndhB*, *ndhD* mRNA	PPR	[132]
CLB19	*A. thaliana*	Editing	*rpoA*, *clpP* mRNA	PPR	[131]
YS1	*A. thaliana*	Editing	*rpoB* mRNA	PPR	[134]
RARE1	*A. thaliana*	Editing	*accD* mRNA	PPR	[135]
LPA66	*A. thaliana*	Editing	*psbF* mRNA	PPR	[136]
AtECB2	*A. thaliana*	Editing	*accD* mRNA	PPR	[137]
OTP80	*A. thaliana*	Editing	*rpl23* mRNA	PPR	[138]
OTP81	*A. thaliana*	Editing	*rps12* mRNA	PPR	[138]
OTP82	*A. thaliana*	Editing	*ndhB*, *ndhG* mRNA	PPR	[133, 138]
OTP85	*A. thaliana*	Editing	*ndhD* mRNA	PPR	[138]
OTP84	*A. thaliana*	Editing	*ndhF*, *ndhB*, *psbZ* mRNA	PPR	[138]
OTP86	*A. thaliana*	Editing	*rps14* mRNA	PPR	[138]

sequences from diverse organisms, future genetic approaches will be directed towards deciphering the complete functional capacity of chloroplast RNA-binding proteins. For example, we will learn more about further interacting partners besides RNA, and most probably will explore the composition of protein RNA complexes within the chloroplasts. Finally, the main question waits to be answered: What directs the specificity of RNA-binding proteins which at the end will define its biological function?

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