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Review

RNA editing in plants: Machinery and flexibility of site recognition☆

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ABSTRACT

In plants, RNA editing is a process that deaminates specific cytidines (C) to uridines (U). PLS subfamily members of PPR proteins function in site recognition of the target C. *In silico* analysis has predicted the code used for PPR motif–nucleotide interaction, and the crystal structure of a protein–RNA complex supports this model. Despite progress in understanding the RNA-binding mechanism of PPR proteins, some of the flexibility of RNA recognition observed in *trans*-factors of RNA editing has not been fully explained. It is probably necessary to consider another unknown mechanism, and this consideration is related to the question of how PPR proteins have managed the creation of RNA editing sites during evolution. This question may be related to the mystery of the biological function of RNA editing in plants. MORF/RIP family members are required for RNA editing at multiple editing sites and are components of the RNA editosome in plants. The DYW domain has been a strong candidate for the C deaminase activity required for C-to-U conversion in RNA editing. So far, the activity of this enzyme has not been detected in recombinant DYW proteins, and several puzzling experimental results need to be explained to support the model. It is still difficult to resolve the entire image of the editosome in RNA editing in plants. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. Introduction

RNA editing is a process that modifies the genetic information on RNA molecules. Different types of RNA editing have been reported in divergent organisms. Pioneer studies focused on *Trypanosoma*, in which numerous uridines (U) are inserted into or excised from RNA, drastically modifying the RNA sequence [1]. In flowering plants, RNA editing involves the deamination of specific cytidines (C) to U and is frequently observed in plastids and mitochondria [2]. In Arabidopsis, 43 sites are edited in the chloroplasts [3] and 619 in the mitochondria [4]. In many cases, amino acid alterations caused by RNA editing are essential for the expression of functional proteins [5,6], although we cannot ignore the many exceptions: plants still show site editing even when it is not necessary for the function of an encoded protein [7]. RNA editing also generates translational initiation or termination codons, which are often essential for gene expression [8,9]. The investigation of such a mechanism that is inconsistent with the central dogma has attracted many plant molecular biologists, but the machinery of RNA editing has still not been fully clarified and the biological function of RNA editing is still totally unclear. To avoid a serious overlap with the work reported in recent excellent reviews on RNA editing [2,10] and PPR proteins [11–13], and also in my own reviews [14–16], in this review article, I will focus on several issues, namely, the machinery, the flexibility of target recognition, and the biological function of RNA editing.

2. *Cis*-elements and *trans*-factors required for site recognition

A critical question in early works on plant RNA editing was how the RNA editing machinery recognizes RNA editing sites. Systems of plastid transformation [17] and *in vitro* RNA editing [18] were established by using *Nicotiana tabacum* (tobacco). Both techniques helped to clarify the *cis*-element required for defining the target site. In general, 20 to 25 nucleotides just upstream of the target C are sufficient for recognition by the RNA editing machinery. The hypothesis was that this *cis*-element was recognized by a *trans*-factor that recruited the RNA editing machinery to the site. It was also possible that this *trans*-factor was a component of the RNA editing machinery. Because many RNA editing sites are in protein coding regions, the sequences surrounding the sites are not highly conserved. One question was how *trans*-factors recognized *cis*-elements that did not have any consensus sequences. Short complementary RNA (guide RNA) to the *cis*-elements was a candidate *trans*-factor, as is the case in the *Trypanosoma* system [1], but this system had not been discovered in plants.

The *trans*-factor was finally discovered in Arabidopsis *chlororespiratory reduction 4* (*crr4*) mutants defective in activity of the chloroplast NADH dehydrogenase-like (NDH) complex [8]. *crr4* mutants are specifically

Abbreviations: MORF, multiple organellar RNA editing factor; NDH, NADH dehydrogenase-like (complex); PPR, pentatrico peptide repeat; RIP, RNA editing factor interacting protein; RRM, RNA recognition motif

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defective in the RNA editing that generates the translational initiation codon of the *ndhD* gene (Table 1). Because of this defect, *crr4* mutants do not translate *ndhD* and consequently do not accumulate the chloroplast NDH complex. The defect was monitored as a specific alteration in chlorophyll fluorescence pattern [19]. The *CRR4* gene encodes a member of the PPR (pentatricopeptide repeat) family consisting of a tandem array of 35 degenerate amino acids [20]. CRR4 protein targets chloroplasts *in vivo* and binds the 36 nucleotides (−25 to +10) surrounding the target C (ndhD-1) in the translational initiation codon of *ndhD* [21]. When these findings were taken together with genetic evidence, the PPR protein CRR4 was concluded to be a *trans*-factor in RNA editing of the site. Subsequently, this was also shown to be true in mitochondria [22], and there were numerous subsequent discoveries of PPR proteins involved in RNA editing in both plastids and mitochondria [10–16]. The PPR family has an extraordinarily large number of members, especially in angiosperms. In Arabidopsis, the family contains approximately 450 members [20]. This answers the question of why *cis*-elements without consensus sequences are recognized by RNA editing machinery.

3. Function of PPR proteins

3.1. P subfamily

The PPR family is subdivided into P and PLS subfamilies (Fig. 1) [19]. Before focusing on the PLS subfamily, in which *trans*-factors for RNA editing are included, the general function of PPR proteins is discussed. In various RNA maturation steps supported by the P subfamily members, their protein function may be able to be generalized on the basis of the simple protein structure consisting of an array of PPR motifs. The P subfamily members bind to specific sequences of RNA and protect RNA molecules from the attack of endonucleases or modify the RNA secondary structure to recruit the general factors involved in RNA maturation process including translational machinery. Maize PPR10 is a member of the P subfamily and specifically binds to the *atpI-atpH* and *psaJ-rpl33* intergenic regions, which share similar sequences [23]. PPR10 is essential for the accumulation of RNAs with the 5′ or 3′ RNA ends close to its binding sites. To explain this mutant phenotype, a simple idea is that PPR10 tightly binds to the target site to protect the RNA from both 5′ → 3′ and 3′ → 5′ endonucleases [23]. Binding of PPR10 also remodels the RNA secondary structure of the ribosome-binding site, recruiting translational machinery [24]. This simple model might explain the function of majority of the P subfamily members [11]. Consistently, RNA footprints, probably reflecting the binding of PPR proteins, were detected in many processed RNA termini in chloroplasts [25,26].

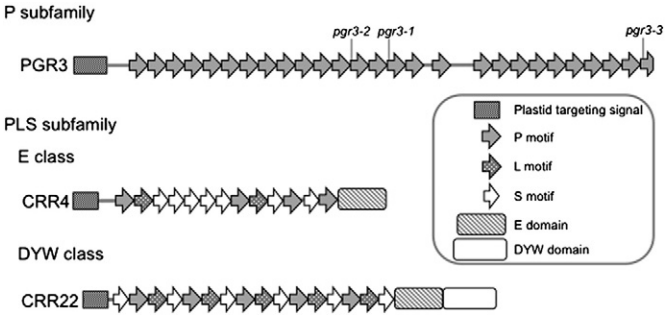

Fig. 1. Schematic model of representative PPR protein structures. Positions of the mutation in three *pgr3* alleles are indicated.

PGR3 is a P subfamily member discovered in Arabidopsis *pgr3* mutants that show high chlorophyll fluorescence at high light intensity because of a partial defect in photosynthetic electron transport (Fig. 1) [27]. PGR3 is involved in three distinctive steps of RNA maturation in chloroplasts: 1) stabilization of *petL* operon RNA; 2) translation of *petL*; and 3) translation of *ndhA* [28]. Functions 1) and 2) are mediated by binding of PGR3 to the 5′ untranslated region (UTR) of *petL*. PGR3 has been shown to bind to the 5′UTR of *ndhA in vitro*, but a polysome analysis has not found any defect in the translation of *ndhA*, implying that this technique is limited in terms of the *in vivo* evaluation of translational activity [29]. Most probably, PGR3 is involved in the translation of *ndhA*, but I cannot eliminate the possibility that PGR3 is also involved in the translation of genes encoding other membrane-embedded subunits of the chloroplast NDH complex (NdhB to NdhG). Three mutant alleles of *pgr3* exhibit the different phenotypes [27]. In *pgr3-2*, function 1) is impaired but function 3) is not affected. On the other hand, *pgr3-3* is defective in functions 2) and 3) but function 1) is unaffected. In *pgr3-1*, all three functions are impaired but *pgr3-1* is still not a null allele because of the more severe growth phenotype of the recently identified knockout allele (unpublished results). In *pgr3-1* and *pgr3-2*, the same 4th threonine is replaced by isoleucine in the different PPR motifs, suggesting that this amino acid substitution specifically impairs the function of this PPR motif, with subtle effects on other regions (Fig. 1) [28]. We took advantage of this discovery by substituting the corresponding site in each PPR motif throughout PGR3 [29]. By doing this, we demonstrated the different contribution of each PPR motif to the recognition of two distinct targets. Another important discovery in this study was that the 11 C-terminal PPR motifs of PGR3 were dispensable for function 1) but were required for translation rather than RNA sequence recognition. Consistent with this discovery, the amino acid alteration occurred in the final incomplete PPR motif in *pgr3-3*, in which translation of *petL*, and also probably *ndhA*, was specifically inhibited (Fig. 1) [27]. We propose a two-component model of PGR3 function: the N-terminal 16 PPR motifs are involved in the recognition of target RNA, whereas the 11 C-terminal motifs are involved in translation, possibly by modifying the RNA structure to recruit translational machinery [29], as has been proposed in PPR10 [23]. Notably, this two-component model of PGR3 is similar to that proposed for PLS subfamily members involved in RNA editing [16]: the N-terminal PPR motifs of PLS subfamily

Table 1
RNA editing sites and *trans*-factors in Arabidopsis plastids. Editing sites in the non-translated region are not listed here.

Editing site[1)]	Editing site[2)]	Editing site[3)]	*trans*-factor
matK (2931)	C640		
atpF (12707)	C92		
rpoC1 (21806)	C488		FLV/DOT4
rpoB (23898)	C2432		
rpoB (25779)	C551	rpoB-3	CRR22
rpoB (25992)	C338		YS1
psbZ (35800)	C50		OTP84
rps14 (37092)	C149		
rps14 (37161)	C80		OTP86
accD (57868)	C794		RARE1, VAC1/AtECB2
psbF (63985)	C77		LPA66
psbE (64109)	C214		CREF3
petL (65716)	C5		
rps12 (69553)			
clpP (69942)	C559		CLB19
rpoA (78691)	C200		CLB19
rpl23 (86055)	C89		OTP80
ndhB (94999)	C1481	ndhB-9	OTP84
ndhB (95225)	C1255	ndhB-8	CREF7
ndhB (95608)	C872	ndhB-7	
ndhB (95644)	C836	ndhB-6	OTP82
ndhB (95650)	C830	ndhB-5	ELI1
ndhB (96419)	C746	ndhB-4	CRR22
ndhB (96579)	C586	ndhB-3	
ndhB (96698)	C467	ndhB-2	CRR28
ndhB (97016)	C149	ndhB-1	
ndhF (112349)	C290		OTP84, VAC1/AtECB2
ndhD (116281)	C887	ndhD-5	CRR22
ndhD (116290)	C878	ndhD-4	CRR28
ndhD (116494)	C674	ndhD-3	OTP85
ndhD (116785)	C383	ndhD-2	CRR21
ndhD (117166)	C2	ndhD-1	CRR4, DYW1
ndhG (118858)	C50		OTP82

Names of editing sites used in [3] [1)], [52] [2)], and in our studies[3)].

members recognize specific target RNA, whereas the C-terminal E and DYW domains have more general functions in RNA editing (see below) [5].

3.2. PLS subfamily

All the discovered *trans*-factors involved in RNA editing in plants belong to the PLS subfamily. Whereas the P subfamily members consist of a simple array of PPR motifs, PLS subfamily members consists of an array of triplets, namely, P (the canonical PPR motif), L, and S (Fig. 1). L and S motifs are related to the PPR motif but are 35 or 36 amino acids and 31 amino acids, respectively, with specific amino acid conservation patterns [11]. The PLS subfamily is further subdivided into three classes on the basis of the different conserved C-terminal domains (Fig. 1) [19]. E-class members have a C-terminal extension (E domain), which is related to the PPR motif. Approximately half of the PLS members with the E domain have an additional C-terminal extension (DYW domain) with highly conserved C-terminal ends consisting of aspartate (D), tyrosine (Y), and tryptophan (W). PLS-class members consist simply of P, L, and S motifs and have no C-terminal extension.

The Arabidopsis genome encodes approximately 450 PPR proteins: the P and PLS subfamilies contain approximately 250 and 200 members, respectively [13]. The E motif is present in the majority of members of the PLS subfamily, and approximately 90 members also have the DYW motif [19]. As discussed below, the E and DYW motifs are considered to be related to RNA editing. Consistently, mutants defective in PLS subfamily members are generally defective in RNA editing at specific sites. The functions of the E and DYW motifs will be discussed later. Here, I introduce two examples of exceptions.

CRR2 is a member of the DYW class and was the first protein in this class to be characterized [30]. Arabidopsis *crr2* mutants were isolated by focusing on chloroplast NDH activity, as were *crr4* mutants. *crr2* mutants did not accumulate monocistronic *ndhB* mRNA, although its precursor RNA with the upstream *rps7* gene was detected, as in the wild type. On the basis of this mutant phenotype, we concluded that CRR2 was involved in intergenic RNA cleavage between *rps7* and *ndhB*. Consistent with this idea, endonuclease activity was detected in the recombinant protein of the DYW domain of CRR2 [6,31]. However, RNA footprints were detected in the region close to the 5′-end of monocistronic *ndhB* mRNA [25,26], implying that CRR2 functions in the stabilization of processed RNA rather than in intergenic RNA cleavage. The experimentally determined 5′-end of monocistronic *ndhB* mRNA did not exactly match the 5′-end of the footprint RNA. In general, PLS subfamily members do not produce any footprints on RNA, probably reflecting their transient interaction with target RNA. Additional research is needed before conclusions can be reached on the function of CRR2.

Another exception observed in DYW members was discovered in *Physomitrella patens*. PpPPR_43 is a DYW-class protein and is required for the splicing of intron 3 of the mitochondrial *coxI* gene [32]. Both the E motif and the DYW motif are dispensable to the function of PpPPR_43. This is in contrast to the fact that the E domain is essential for the function of PLS subfamily members in Arabidopsis [5,6]. Ichinose et al. [32] hypothesized that PpPPR_43 may have been involved in RNA editing and acquired the function to promote splicing by binding to a specific site. Probably because of the mutation in the mitochondrial genome, PpPPR_43 no longer functions in RNA editing and consequently the E and DYW domains became dispensable.

4. Mechanism to recognize the target site

Recently, there has been substantial progress in our understanding of how an array of PPR motifs recognizes an RNA sequence. The RNA–protein recognition code was clarified by *in silico* analysis focusing on several PPR proteins, the binding of which to RNA was precisely determined [33–35]. The strongest determinant was observed at position 6 of a PPR motif. The combination of this site with position 1 on the following PPR (position 1′) was also effective in determining binding preference. In addition to these two residues, position 3 was identified to have positive selective pressure in *Rf-PPR* [36]. The site was also incorporated into a three-code model [34]. In the PLS subfamily, the L motif has been suggested not to bind nucleotide bases, forming a gap at least at every three PPR motifs [33]. However, some L motifs may be involved in binding to targets via an L motif–specific PPR code [37].

The crystal structure of PPR10, consisting of 19 PPR motifs, was determined along with that of one of its target RNAs (18 nucleotides present in the *psaJ* to *rpl33* intergenic region) [38]. The 5′-end four nucleotides of the target RNA, 5′-GUAU-3′, were recognized by the 3rd to 6th PPR motifs, consistent with the *in silico* prediction and biochemical analysis [33]. The C-terminal end of PPR10 recognizes the 3′-end four nucleotides, 5′-UUUC-3′, of which only U15 and U16 strictly follow the PPR code. The central 10 nucleotides remain unincorporated by PPR10. The length of this central region is 1 nucleotide shorter in the target sequence present in *atpI* to *atpH*, suggesting that this gap region permits variation in nucleotide length. Among the 18/17 nucleotides required for recognition by PPR10, only six strictly follow the RNA code, and these sites are actually involved in binding to PPR10 in the crystal structure. Unexpectedly, PPR10 forms an antiparallel homodimer in the crystal [38,39]. Although monomeric PPR proteins bind to target RNA *in vitro*, the P subfamily member HCF152 has been shown to form a homodimer *in vivo* [40]. Even more unexpectedly, THA8, consisting of five PPR motifs, forms an asymmetric dimer with target RNA at the dimeric interface [41]. THA8 is a small PPR protein involved in splicing of the *ycf3* transcript. The structure of functional PPR proteins *in vivo* is critical information for resolving the RNA editosome.

5. Flexibility of sequence recognition in *trans*-factors

Arabidopsis has approximately 650 RNA editing sites in two organelles. These sites are managed by roughly 200 members of the PLS subfamily, suggesting that a single *trans*-factor recognizes more than two sites on average. Indeed, CRR22 and SOL2 are involved in the RNA editing of at least three sites in plastids and at least six sites in mitochondria, respectively [6,42]. Similar cases have been observed in P subfamily members [23,27]. How does a single PPR protein recognize multiple target sequences? This can be explained by several mechanisms: 1) In the simplest case, the sequences are conserved between distinct target sites to some extent. PPR10 recognizes the 17 and 18 nucleotides present in the *atpI* to *atpH* and *psaJ* to *rpl33* intergenic regions, respectively [23]. Between the two target sequences, 13 nucleotides are conserved, although only six nucleotides that strictly follow the RNA code are involved in binding with PPR10 in the crystal structure [38]. This is the case because the target sequences are in the intergenic regions. However, many *cis*-elements recognized by RNA editing machinery are in the protein-coding region. 2) In the second case, a PPR protein utilizes different sets of PPR motifs to recognize different target RNAs. At least *in vitro*, the P subfamily member PGR3 binds to two target RNAs, namely, the 5′ UTRs of *petL* and *ndhA* [28]. Because the 5′ UTR of *ndhA* is in the coding region of *ndhH*, the two target sequences are not highly conserved. PGR3 consists of 27 PPR motifs, and each PPR motif of PGR3 contributes differently to binding to the two target sequences [29]. 3) In the third mechanism, some PPR motifs distinguish pyrimidines from purines and do not exactly recognize the nucleotide. This idea was originally proposed to explain the flexibility of sequence recognition in the PLS subfamily [43], but it is now clear that the rule is also applicable to the P subfamily [33–35]. However, this flexibility of the RNA code does not explain the fact that some *trans*-factors recognize unrelated sequences. It is also hypothesized that the L motif of the PLS subfamily does not recognize the nucleotide [33]. This mechanism permits the presence of gaps, which are not recognized by the PPR track in a *cis*-element, but it does not explain the flexibility of RNA sequence recognition. CRR22 is a member of the DYW class and is necessary

for RNA editing at three sites, ndhB-7, ndhD-5, and rpoB-3 [6]. The binding sites were finally narrowed down to the −20 to 0 region in the case of the ndhB-7 and ndhD-5 sites and the −17 to 0 region in the case of the rpoB-3 site; any consensus sequences were not found at these sites [44]. It is unlikely that, at some of these sites, CRR22 is needed to provide its DYW domain to editing machinery containing other PPR proteins as *trans*-factors. CRR22 is a genuine *trans*-factor for all three sites because the recombinant protein could bind to all of them [44]. It is probably necessary to consider additional mechanisms to explain the flexibility of sequence recognition, at least in the case of some *trans*-factors for RNA editing.

6. MORF/RIP family proteins

A forward genetic approach focusing on Arabidopsis mutants with impaired RNA editing at specific sites has identified the multiple organellar RNA editing factor 1 (*morf1*) locus [45]. RIP1 (RNA editing factor interacting protein 1) was independently identified by co-immunoprecipitation with a PPR protein, RARE1, which is required for RNA editing in the *accD* transcript [46]. Both proteins belong to the same MORF/RIP family consisting of 10 members in Arabidopsis [47]. Among them, MORF2/RIP2 and MORF9/RIP9 are targeted to plastids, whereas MORF8/RIP1 is dual-targeted to plastids and mitochondria. The remaining members are targeted to mitochondria, with the exception of RIP10, which may be encoded by a pseudogene. In plastids, a defect in either of two plastid-targeting proteins (MORF2/RIP2 and MORF9/RIP9) affected the majority of RNA editing sites (31 of 36 sites analyzed). Among these sites, editing was completely impaired at 12 sites in the single mutant. Two MORF/RIP proteins form a heterodimer, the function of which can be substituted for by homodimers at some sites. In plastids, some editing sites depend on the function of RIP1, forming a complex MORF/RIP family network in plastid RNA editing [47]. A similar network has been observed among MORF8/RIP1, MORF3/RIP3, and MORF1/RIP8, although a MORF8/RIP1 homodimer is likely involved in the majority of sites in mitochondria [47].

Organelle RRM protein 1 (ORRM1) has a duplicated set of MORF/RIP domains and is also involved in multiple RNA editing events in plastids [48]. Despite its functional similarity to MORF/RIP members, ORRM1 contains an RNA recognition motif (RRM) at its C-terminal end. Because ORRM1 associates with some PLS-class members, probably via its MORF/RIP domains, it was surprising that the RRM domain was sufficient to complement the mutant in a transient assay system using protoplasts at several RNA editing sites, including ndhB C467. It was hypothesized that the high copy number of the RRM-domain protein complemented the function of the full-length protein because the RRM domain could bind to at least some target RNA *in vitro* [48]. Sun et al. [48] further hypothesized that MORF/RIP domains may be needed for more efficient targeting of ORRM1 to the site via interaction with PLS-class members *in vivo*. Unfortunately, they did not perform an *in vitro* RNA binding assay at the ndhB C467 site. This explanation is likely but is also puzzling, considering the function of the RRM domain *in vivo*. The ndhB C467 site is recognized by a PPR protein, CRR28 [6]. This is consistent with the fact that the MORF/RIP domain of ORRM1 interacts with CRR28 in yeast [48]. In the putative ternary complex consisting of CRR28, ORRM1, and the target RNA, the *cis*-element at the ndhB C467 site is occupied by CRR28. ORRM1 interacts with CRR28 via the MORF/RIP domain. With which region of target RNA does the RRM domain of ORRM1 interact? The *cis*-element is the minimum sequence for recognition by the RNA editing machinery including ORRM1 at this site. If the binding sites of CRR28 and the RRM domain of PRRM1 were to overlap (i.e. if there were competition between CRR28 and ORRM1 for binding to the site), then it would be necessary to consider the transient interaction of CRR28 with this site. This may be consistent with the fact that RNA footprints have not been found at the binding sites of *trans*-factors [25,26]. It may be necessary to reevaluate the function of RRM proteins, including CP31, in RNA editing [18,49].

Protoporphyrinogen IX oxidase 1 (PPO1) mediates the final step of the common pathway shared by chlorophyll and heme biosynthesis. Unexpectedly, its mutant is defective in plastid RNA editing at multiple sites, as in the case of *morf*/*rip* mutants [50]. The phenotype is unlikely caused secondarily by the albino phenotype because PPO1 interacts with three MORF/RIP proteins present in plastids. Furthermore, the mutations in the region responsible for the interaction with MORF/RIP proteins impaired RNA editing but did not impair chlorophyll biosynthesis. To function in RNA editing, however, PPO1 does not require its FAD or substrate binding sites. PPO is essential for stabilizing MORF/RIP proteins via physical interaction. It is still difficult to resolve the total picture of RNA editing.

7. Editing enzyme

Mutations in the RNA editing enzyme that catalyzes C-to-U conversion likely lead to editing defects at multiple sites. On the basis of protein structure, however, MORF/RIPs or ORRM1 are unlikely to have C deaminase activity. The central question of C deaminase (RNA editing enzyme) activity remains. The DYW domain present at the C-terminal end of DYW-class members has been the sole and strong candidate for association with C deaminase activity since the concept was first proposed [51]: the DYW domain contains a motif similar to the C deaminase signature, and its occurrence is phylogenetically correlated with the presence of C-deamination-type RNA editing in organisms. Despite this focus of interest, C deaminase activity has not been detected in recombinant proteins of the DYW domain [6,31]. In contrast, endonuclease activity has been detected in some DYW domains, including CRR2. As discussed above, however, careful discussion is still needed regarding the function of the DYW domain in CRR2.

Despite the lack of definitive evidence, accumulating information supports the idea that the DYW domain is responsible for C deaminase activity. ELI1 belongs to the DYW class and is required for RNA editing of the ndhB C830 site in chloroplasts. Recombinant protein of the ELI1 DYW domain binds two zinc atoms per polypeptide [52]. A similar conclusion has been reported in regard to the involvement of DYW1 in RNA editing at the ndhD-1 site, although one zinc atom per polypeptide was detected [53]. As observed in the case of many DYW members, however, truncation of the DYW domain from ELI1 does not affect complementation of the mutant *in vivo*. This finding may be explained by the hypothesis that the DYW domain is supplied from other molecules. If a PLS family member forms a heterodimer with another PPR protein, then it would be possible for the DYW domain to be supplied by that other molecule. However, the DYW domains of CRR28 and OTP85 interact with the −3 to 0 region of the target site [54]. The mutant version of CRR28 truncated at its DYW domain complements RNA editing at all sites, indicating that the DYW domains are dispensable [6]. On RNA molecules, *cis*-elements (−13 to −4 for both the ndhB-2 site and the ndhD-3 site) are occupied by the mutant version of CRR28. How could another protein with a DYW domain gain access to the −3 to 0 region?

The above question can be answered simply in the specific case of the ndhD-1 site. The Arabidopsis *dyw1* mutant is specifically defective in RNA editing at this site in chloroplasts [55]. The phenotype is identical to that of the *crr4* mutant defective in an E-class member of the PLS subfamily [8]. Because CRR4 lacks the DYW domain, the domain should be supplied from another protein if the domain is really responsible for editing activity. The fusion protein CRR4-DYW1 complements the phenotype of the *crr4 dyw1* double mutant, suggesting that the two proteins interact *in vivo* [55]. A remaining question is how the other E-class members, such as CRR21, are involved in RNA editing without the DYW domain. Because DYW1 is the specific partner of CRR4, it cannot be the source of DYW domains for other E-class members. The same question can be raised regarding the complementation of RNA editing with the mutant version of PPR protein lacking the DYW domain.

The DYW domain is dispensable for RNA editing *in vivo* in *trans*-factors, but there are some exceptions. In MEF1, truncation of

the DYW motif impairs complementation of the mutants in mitochondria [56]. The DYW domain is also essential for CRR2 protein function in chloroplasts [6]. The DYW domain of CRR2 has endonuclease activity *in vitro* and is involved in the production of the 5′-end of monocistronic *ndhB* mRNA [6,30]. Exchange of the DYW domain of CRR2 for those of CRR22 and CRR28 impairs RNA editing activity, although the domain is exchangeable between CRR22 and CRR28 and is even dispensable for their function in RNA editing [6]. It is still possible that mutation in the DYW domain affects the stability of PPR protein, but it is more likely that the function of the DYW motif depends on PPR proteins—even those involved in RNA editing.

In contrast to the DYW domain, the E domain is generally essential for protein function [5,52]. It is not required for binding target RNA and is exchangeable between two E-class members, CRR4 and CRR21 [5,6]. On the basis of this finding, we proposed a two-component model of RNA editing machinery [5]. It is clear now that the machinery is more complicated than our original image. The exact function of the E domain is unclear, but it may form a gap in a DWY protein because deletion of the domain does not affect binding to target RNA. In the *cis*-elements at the ndhB-2 and ndhD-3 sites, the −17 to −4 region is recognized by the PPR track of CRR28 and the −3 to 0 site was probably occupied by its DYW domain [54]. Takenaka et al. [2] proposed an attractive model of RNA editing machinery in which a dimer of MORF/RIP proteins bridged the *cis*-elements (upstream beyond −4) and the target site (0).

8. Why do plants edit RNA?

At many sites, RNA editing is essential for expressing functional proteins, but this fact does not explain why plants do not correct their genomic information. Why do plants edit RNA? RNA editing may regulate gene expression. RNA editing of the ndhD-1 site generates a translational initiation codon and potentially regulates the efficiency of translation. Indeed, the efficiency of editing at this site depends on the tissue and developmental stage [57]. However, even the low level of RNA editing at this site does not limit the accumulation of chloroplast NDH complex in *Nicotiana tomentosiformis* [58]. Plants appear be fine even though many sites are encoded by T in the genome. In monocots, the translational initiation codon of *ndhD* is encoded by ATG, instead of by ACG as in many eudicots, including Arabidopsis and tobacco, suggesting that the efficiency of RNA editing does not necessarily require regulation.

Unexpectedly, plants often edit their RNA, even if the resulting amino acid alterations are not required for protein function. OTP82 is a DYW-class protein required for RNA editing at the ndhB-9 and ndhG-1 sites. Although *otp82* mutants completely lack editing activity of these sites, NDH accumulation, activity, or supercomplex formation with photosystem I is unaffected [7]. In the wild type, these sites are partially edited, suggesting that NdhB and NdhG subunits originating from both edited and unedited transcripts accumulate in the NDH complex. Plants edit some sites even without strict selection pressure for the protein translated from the edited transcripts. Because of the large set of PLS subfamily members in their genomes and the flexibility of their RNA sequence recognition, plants potentially manage large numbers of RNA editing sites—probably more than the number of actual RNA editing sites present in organelle genomes. With this ability, plants have the potential advantage of maintaining the diversity of genome information. During the evolution of land plants, RNA editing may have repaired numerous T-to-C mutations accumulating in the organelle genomes. Many mutations would have been finally corrected to T in the genome, but at some sites, the residues may have been fixed to C to code for different amino acids.

In plastids, there is a biased distribution of RNA editing sites (Table 1). Among 33 RNA editing sites present in the coding regions of plastid genes in Arabidopsis, 16 are found in four *ndh* genes (*ndhB*, *ndhD*, *ndhF*, and *ndhG*) encoding membrane subunits of the chloroplast NDH complex. *ndhB* and *ndhD* contain 9 and 5 editing sites, respectively. In the plastid genome, 11 *ndh* genes encode NDH subunits [59]. Four subunits—NdhH to NdhK—form the core of subcomplex A, which mediates electron transport from ferredoxin to plastoquinone. Chloroplast NDH is a ferredoxin-dependent plastoquinone reductase rather than an NAD(P)H dehydrogenase [60]. Notably, editing sites are not present in *ndhH* to *ndhK* genes in Arabidopsis, and this trend is conserved in other flowering plants [61]. Coupled with the electron transport from ferredoxin to plastoquinone, membrane subunits, including NdhB, D, F, and G, probably translocate protons across the thylakoid membrane, forming ΔpH. The chloroplast NDH complex experienced drastic changes in structure during the evolution of land plants. In evolution, the chloroplast NDH complex has acquired novel subunits that form subcomplex B, which is not conserved in the cyanobacterial NDH complex, and the lumen subcomplex, which is not conserved in *Physcomitrella patens* [62,63]. During the evolution of flowering plants, the NDH complex became associated with photosystem I to form a supercomplex for stability of the NDH complex [62,64,65]. The membrane subunits are involved in binding to at least some of these novel subunits and, for this purpose, they may have required several critical amino acid changes during evolution. In cyanobacteria, multiplication and subsequent diversification of *ndhD1* to *ndhD6* and *ndhF1* to *ndhF3* are key steps in the evolution of two or three distinct types of NDH complexes involved in respiration or photosytem I cyclic electron transport and inorganic CO_2 concentration [66]. It is also possible that the subunits were selected during evolution because they are more tolerant to the accumulation of amino acid alterations. This may also be the reason for the biased accumulation of RNA editing sites.

9. Concluding remark

The mechanism by which RNA sequences are recognized via tracks of PPR motifs has become clear. Some components of the RNA editing machinery have been discovered. These discoveries are leading to technologies for manipulating RNA *in vivo* [67]. The remaining central question is the nature of the RNA editing enzyme. Despite progress, especially in regard to the mechanism of target recognition, the fundamental question remains as to the biological function of RNA editing in plants. To understand the puzzling nature of RNA editing in plants, it will probably be necessary to put evolution into perspective. If this is done, then the critical questions will be how the flexibility of site recognition by *trans*-factors was acquired and how plants utilized this mechanism to deal with the creation of RNA editing sites.

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