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Review

RNA editing in plant organelles: machinery, physiological function and evolution

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Abstract. In plants, RNA editing is a process for converting a specific nucleotide of RNA from C to U and less frequently from U to C in mitochondria and plastids. To specify the site of editing, the *cis*-element adjacent to the editing site functions as a binding site for the *trans*-acting factor. Genetic approaches using *Arabidopsis thaliana* have clarified that a member of the protein family with pentatricopeptide repeat (PPR) motifs is essential for RNA editing to generate a translational initiation codon of the chloroplast *ndhD* gene. The PPR motif is a highly degenerate unit of 35 amino acids and appears as tandem repeats in proteins that are involved in RNA maturation steps in mitochondria and plastids. The *Arabidopsis* genome encodes approximately 450 members of the PPR family, some of which possibly function as *trans*-acting factors binding the *cis*-elements of the RNA editing sites to facilitate access of an unidentified RNA editing enzyme. Based on this breakthrough in the research on plant RNA editing, I would like to discuss the possible steps of co-evolution of RNA editing events and PPR proteins.

Key words. *Arabidopsis*; chloroplast; mitochondria; photosynthesis; PPR protein; RNA editing.

Introduction

RNA editing is the process of altering an RNA sequence from that encoded by the genome. The phenomenon was first reported in the mitochondria of protozoa [1], in which insertion or, less frequently, deletion of U residues takes place in mitochondrial mRNAs (reviewed in [2]). It was later discovered in the mitochondria [3–5] and plastids [6] of plants. The plastid is an organelle specific to plants and develops into a chloroplast, where photosynthesis occurs in green tissues like leaves. The plastid contains its own genome encoding genes responsible for photosynthesis and housekeeping functions. In contrast to the protozoa system, organellar messenger RNAs (mRNAs) are subject to C-to-U and less frequently U-to-C conversions in plants.

Despite the several hundred examples of editing events in plant or protozoa organelles, only a few are known in mammalian mRNAs (reviewed in [7]). C-to-U editing in apolipoprotein B (apoB) mRNA generates a stop codon, resulting in a translation of premature protein [8, 9]. This RNA editing is tissue-specifically regulated and thus contributes to the expression of two versions of protein, apoB48 and apoB100, from a gene. APOBEC-1 (apoB editing catalytic subunit-1) contains a zinc-dependent deaminase domain that is conserved in C deaminase and is essential for editing apoB mRNA as an editing enzyme [10]. APOBEC-1 interacts with ACF (APOBEC-1 complementation factor) for site-specific editing. ACF contains three copies of single-stranded RNA binding motifs (RRMs) and binds the mooring sequence located at the 3′ end of the edited C, facilitating access of APOBEC-1 to the editing site [11].

We have recently identified a member of the PPR family, CRR4, as being an essential factor in a specific RNA editing event in plastids of higher plants, *Arabidopsis thaliana* [12]. PPR proteins are believed to be involved in RNA maturation steps in plastids or mitochondria and

form an extraordinarily large family, especially in higher plants [13]. Instead of ACF with RRMs, PPR proteins are likely to facilitate access of the editing enzyme to the editing site by binding the editing site. Although the process of RNA editing is similar between plant organelles and mammals, the machineries are likely to be divergent. In this review, I focus on the mechanism of RNA editing in plants, which has up to now been poorly understood, and discuss the possible physiological functions of RNA editing and the co-evolution of PPR proteins and RNA editing sites. Some reviews complement the missing topics on plant RNA editing here [14, 15].

The character of RNA editing in plants

In plants, RNA editing is the process of altering a specific C residue to U and less frequently from U to C. Exceptionally, the reverse editing of U to C is abundant in the plastids of hornwort, *Anthocerous formosae* [16]. In many editing events, especially in plastids, RNA editing is essential for expressing functional proteins by modifying the amino acid sequences [17, 18], or generating a translational start codon [6, 12] and a stop codon [19]. Therefore, many RNA editing sites have to be processed with high efficiency so as not to express mutant versions of proteins. However, especially in the mitochondria, transcripts are edited with various degrees of efficiency. For example, analysis of more than 100 complementary DNAs (cDNAs) to the mitochondrial *nad3* gene in *Oenothera* failed to detect an RNA in which all the possible editing sites had been processed [20]. The most variable editing sites are usually in the third-codon position, where editing does not alter the corresponding amino acid (silent editing sites). However, even in the non-silent editing sites, they are heterogeneously edited, suggesting that the *nad3* gene may encode a series of proteins with minor sequence variations. Curiously, although the RNA sequences from mitochondrial *atp6* gene are variable, a single homogeneous protein accumulates [21]. In contrast, both edited and unedited *rps12* transcripts are translated, but only the edited translation products accumulate in mitochondrial ribosomes [22]. These results indicate that translation of unedited transcripts is somehow suppressed and/or protein products originating from partially edited RNAs are rapidly turned over. This is a strategy actually adopted by plants, although it looks like just a waste of energy.

The mysterious nature of RNA editing in plants raises the essential question of why plants prefer RNA editing to direct revision of the genome sequence. In contrast to the mammalian apoB system, partial editing is unlikely to increase the complexity of the proteome in plant organelles. To answer this fundamental question, I will revisit the model of the editing process in the light of recent breakthroughs in this field.

A *cis*-element required for editing site recognition

How is a specific C residue recognized for editing? In answering this question, the breakthrough was the establishment of a plastid transformation technique in tobacco. This technique was originally established in a unicellular green alga, *Chlamydomonas reinhardtii*, called green yeast [23] and significantly contributed to clarification of the function of plastid genes by reverse genetic strategies (reviewed in [24]). Application of this technique to research into RNA editing in plastids had to wait for the establishment of the technique in a higher plant, tobacco [25], since there are no RNA editing events in the plastids of *Chlamydomonas*.

In tobacco, functional *psbL* mRNA encoding a subunit of photosystem II is generated by editing an ACG codon to create an AUG translational initiation codon. This editing occurs in a chimeric RNA containing the N-terminal part of *psbL* fused with bacterial genes conferring resistance to antibiotics [26]. The plastid transformation technique was essential for the introduction of these foreign genes into tobacco chloroplasts. This system facilitated the introduction of the chimeric genes with *psbL* deletion derivatives to identify the RNA region required for RNA editing. This strategy revealed that a 22-nucleotide segment containing 16 nucleotides upstream and five nucleotides downstream of the editing site is sufficient to direct efficient editing [27]. A similar strategy on the *ndhB* editing sites, ndhB-6 and ndhB-7 (*ndhB* encoding a subunit of the chloroplast NAD(P)H dehydrogenase complex contains nine editing sites in tobacco) showed that the RNA segment from –12 to –2 is essential for editing [28]. In this manuscript, the editing sites are depicted based on the nomenclature proposed by Tsudzuki et al. [29], which allows comparison of sites between species.

In addition to the *in vivo* strategy using tobacco plastid transformation, the establishment of an *in vitro* editing system also significantly contributed to the identification of the *cis*-element required for RNA editing [30, 31]. RNA editing was successfully processed in extracts of tobacco chloroplasts, in a reaction in which the site-specific labeling of the RNA editing site facilitates detection of editing. The *in vitro* system identified the *cis*-elements which are required for RNA editing [30]. The result is consistent with that obtained by the *in vivo* system [27, 28].

The *cis*-sequences required for mitochondrial RNA editing were also determined using both *in vivo* and *in vitro* systems. Although mitochondrial transformation is not feasible in higher plant cells, foreign genes were successfully introduced into purified mitochondria, where the expressed RNA was processed precisely [32]. Using this system, both the 16 nucleotides upstream and 6 nucleotides downstream were shown to be essential for the editing of *coxII* mRNA encoding a subunit of cytochrome

c oxidase [33]. An *in vitro* system was also established using pea mitochondrial extract, clarifying two regions from –40 to –35, required for efficient editing, and from –15 to –5, essential for the reaction [34].
All the results suggest a particular feature of the *cis*-element required for RNA editing, which is common in both plastids and mitochondria. Generally, a fewer than 20-nucleotide upstream sequence, including a fewer than 10-nucleotide downstream sequence in some cases, is sufficient for RNA editing. It is conceivable that the *cis*-element is a binding site for a *trans*-acting factor, which would facilitate access of the editing enzyme to the editing site.

A *trans*-acting factor essential for RNA editing

The existence of a *trans*-acting factor was first suggested by an *in vivo* approach in tobacco plastids [26]. The expression of chimeric RNA containing the editing site of *psbL* in tobacco chloroplasts led to a significant decrease in the editing efficiency of the endogenous *psbL* RNA. This competitive effect of the transgene was specific to the *psbL* gene, with other endogenous sites being properly edited, indicating depletion of the *psbL*-specific *trans*-acting factor. This hypothesis, based on an *in vivo* analysis, was strongly supported by *in vitro* analysis [30]. The editing of *psbL* and *ndhB* mRNAs was arrested by adding an excess amount of RNAs, including *cis*-elements, as competitors. The effect of competitors is specific to the corresponding editing site, suggesting that the *trans*-acting factors are site-specific.
What is the *trans*-acting factor that recognizes a *cis*-element? The 'guide RNA theory' is based on a mechanism clarified in trypanosomes (reviewed in [2]). A pre-mRNA forms Watson-Crick base pairs and G/U base pairs with a guide RNA (gRNA), which specifies the sites of RNA editing. Endonucleotic cleavage of the pre-mRNA occurs upstream of the anchor duplex (8–10 bp) between the pre-mRNA and gRNA. Us are either added to or removed from the 3′ end of the upstream pre-mRNA based on the sequence information present in gRNA. Finally, the resulting upstream and downstream RNAs are ligated. In this process, the structural features of pre-mRNA and gRNA enable their recognition by an editosome, a piece of editing machinery.
To assess the involvement of *trans*-acting RNA in RNA editing in plastids, the chloroplast extract was pretreated with micrococcal nuclease [30]. However, the editing was still active, even after nuclease treatment. Furthermore, the attempt was unsuccessful in identifying which RNA components interact with *psbL* mRNA by cross-linking. Although the involvement of an RNA factor cannot be completely eliminated, there is no experimental evidence to support it so far.
In contrast, ultraviolet (UV)-cross linking in the *in vitro* editing system suggests the involvement of protein factors in RNA editing in the chloroplasts [30, 35]. Proteins with distinct molecular weights of 25, 56 and 70 kDa specifically bind the *cis*-elements required for editing in *psbL*, *psbE* and *petB*, respectively. These results suggest that the *trans*-acting factor is a protein rather than RNA in plastids. Consistent with this hypothesis, a gene encoding a PPR protein was recently identified as a *trans*-acting factor using a genetic approach [12].

PPR proteins: site-specific factors for organellar RNA maturation

PPR motif is a highly degenerate unit, consisting of 35 amino acids that usually appear as tandem repeats in a protein [36]. The most surprising feature of the family of proteins containing this motif is its extraordinarily large size, especially in higher plants. The *Arabidopsis* and rice (*Oryza sativa*) nuclear genomes, respectively, contain approximately 450 and 650 members of the PPR family, most of which are predicted to localize to plastids or mitochondria [13]. Although PPR proteins are widely distributed in eukaryotes but not in prokaryotes, the number of genes is very limited in non-plants. For example, human and *Saccharomyces cerevisiae* genomes encode only six and five putative PPR proteins, respectively [13].
The PPR family is divided into two subfamilies, the P and PLS subfamilies (fig. 1) [13]. The authentic PPR proteins are classified into the P subfamily, in which PPR repeats are relatively highly conserved. Except for the tandem array of PPR motifs and an N-terminal target signal to plastids or mitochondria, members of the P subfamily usually do not contain any other conserved motifs. In contrast, members of the PLS subfamily contain motifs related to a PPR motif, the PPR-like S (for short) and PPR-like L (for long), which are more variable in size and sequence than the PPR motif itself. This subfamily of PPR proteins was originally identified as the *Arabidopsis* plant combinatorial and molecular protein (AtPCMP) family [37]. In the PLS subfamily, the tandem arrays of PPR or PPR-like motifs are usually followed by several conserved motifs, E, E+ and DYW, arranged in this order. Based on the different appearance of the C-terminal motifs, the PLS subfamily is divided into four subgroups, PLS (without any C-terminal motif), E (with E), E+ (with E and E+) and DYW (with E, E+ and DYW) (fig. 1). Although members of the P subfamily are widely distributed in eukaryotes, the PLS subfamily is strictly restricted to plants [13].
The PPR motif is structurally similar to the TPR (tetratricopeptide repeat) motif consisting of 34 amino acids [36, 38]. The tandem arrays of TPR motifs are expected to function in protein-protein interactions [39]. However, genetic evidence suggests that PPR motifs may bind to

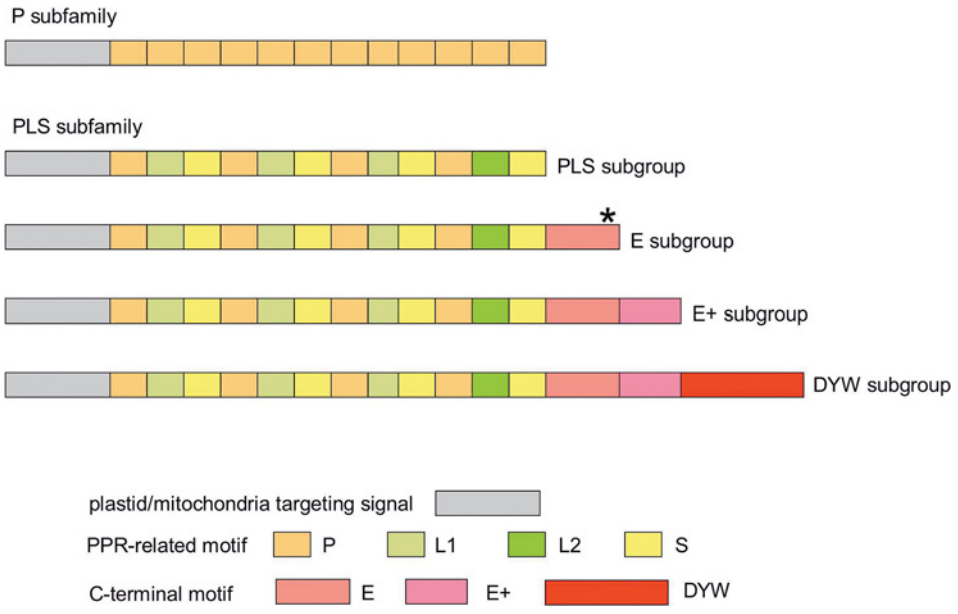

Figure 1. Structure of PPR proteins. Typical structures of proteins of each subfamily and subgroup. The classification of proteins and nomenclature of motifs are based on Lurin et al. [13]. The number of motifs and often, also, the order of P, L1, L2 and S motifs in the PLS subfamily are variable in individual members. An asterisk represents the 15-amino-acid motif conserved in some members, including CRR4 [12]. This figure was modified from [13] with permission of the authors

specific RNA sequences (table 1), as do some TPR motifs [40, 41]. The function of the plant PPR protein was first experimentally identified in a maize mutant, *crp1* (*chloroplast RNA processing*), defective in photosynthetic electron transport [42]. CRP1 is a member of the P subfamily and is required for the translation of plastid-encoded genes *petA* and *psaC*, and also for generation of a monocistronic *petD* RNA [43, 44]. While *pet* genes encode subunits of the cytochrome b_6f complex, *psaC* encodes a subunit of photosystem I. This discovery was followed by characterization of two members of the P subfamily, HCF152 and PGR3, both of which were identified by genetic strategies in *Arabidopsis*. HCF152 is involved in the splicing of *petB* RNA and the stabilization of the spliced product, and also in the intergenic RNA cleavage between *psbH* encoding a subunit of photosystem II and *petB* in the plastid [45, 46]. In contrast, PGR3 is involved in stabilization of *petL* operon RNA, and also in translation of *petL* and one of the *ndh* genes in plastids [47]. Taken together with earlier discoveries in the mitochondria of yeast [48] and *Neurospora* [49], all the results are consistent with the idea that PPR proteins are involved in RNA maturation steps in organelles.

Identification of an RNA editing mutant in *Arabidopsis*

PPR proteins are involved in organellar RNA maturation and form a family that is large enough to maintain all the editing events in plants. Thus, it was plausible to suppose that a PPR protein is a *trans*-acting factor essential for RNA editing in plants [13]. The expected breakthrough was the identification of a mutant impaired in RNA editing due to a defect in a PPR protein. The mutants were isolated by genetic screening focusing on a defect in photosynthetic electron transport.

The light reactions of photosynthesis are a process for converting the light energy of the sun into chemical energy in the form of NADPH and ATP, and consist of two types of electron transport, the linear and cyclic around photosystem I (PSI), which occur in the chloroplast (reviewed in [50]). In higher plants, the PSI cyclic electron transport consists of PGR5- and NDH [NAD(P)H dehydrogenase]-dependent pathways. The PGR5-dependent pathway was identified by characterization of an *Arabidopsis* mutant, *pgr5* (*proton gradient regulation*) defective in response to excessive light intensity [51]. On the other hand, the NDH-dependent pathway was clarified by reverse genetics using plastid transformation in tobacco [52, 53]. The eleven genes, *ndhA–ndhK*, encoding subunits of the NDH complex are present in the plastid genome in higher plants [54]. To clarify the physiological function of PSI cyclic electron transport, it was essential to characterize the double mutant completely defective in PSI cyclic electron transport, hopefully in *Arabidopsis*, in which the further genetic approach was possible [55]. Since plastid transformation was not feasible in *Arabidopsis*, our strategy involved the screening of nuclear mutants defective in NDH activity [56].

Table 1. List of PPR characterized proteins.

	Localization	Subclass	Function	Target RNA[1]	References
Maize					
CRP1	plastids	P	translation RNA cleavage	*petA*, *psaC* *petB*/*petD*	42, 43, 44
PPR2	plastids	P	plastid ribosome accumulation	ND[2]	86
Arabidopsis					
HCF152	plastids	P	RNA cleavage splicing	*psbH*/*petB* *petB*	45, 46
PGR3	plastids	P	RNA stabilization translation	*petL*, *ndhX*[3]	47
CRR2	plastids	DYW	RNA cleavage	*rps7*/*ndhB*	56
CRR4	plastids	E+	RNA editing	*ndhD*	12
EMB175	plastids	P	embryogenesis	ND	87
Rice					
OsPPR1	plastids	P	chloroplast biogenesis	ND	88
Rf-1	mitochondria	P	fertility restoration	*atp6*/*orf79*	78, 80, 81, 89
Petunia					
Rf	mitochondria	P	fertility restoration	*pcf*	75
Radish					
Rfk1	mitochondria	P	fertility restoration	*orf125*	77
Rfo	mitochondria	P	fertility restoration	*orf138*	76, 79
Yeast					
PET309	mitochondria	P	transcription/RNA stabilization, translation	COX1	48
Neurospora					
cya-5	mitochondria	P	translation	COX1	49

[1] Including suggestions based on the mutant phenotypes.
[2] Not determined.
[3] One of the 11 *ndh* genes.
Many other PPR proteins were partially characterized in [13, 87].

Our mutant screening was based on the technique of chlorophyll fluorescence imaging, which makes even subtle changes in photosynthetic electron transport visible under a CCD (charge-coupled device) camera [56–58]. To select mutants specifically impaired in NDH activity, which are referred to as *crr* mutants (*chlororespiratory reduction*), we focused on the change in chlorophyll fluorescence that was detected in the tobacco knockout lines of chloroplast *ndh* genes [52, 53] (fig. 2). Consequently, our targets were genes encoding nuclear-encoded subunits of the NDH complex. Although 11 plastid-encoded [54] and 3 nuclear-encoded subunits [59] have been identified so far, they are not sufficient to account for the activity of the NDH complex (reviewed in [60]). Several candidates, including CRR7, for the additional subunits have been identified and characterized [61]. Since the 11 subunits of the NDH complex are encoded by the plastid genome, we also expected to be able to identify nuclear genes related to the expression of plastid *ndh* genes, which might include genes related to RNA editing. By focusing on NDH activity, we were able to pinpoint candidates for RNA editing mutants from the bulk of mutants that simply showed faulty photosynthetic electron transport due to a variety of defects.

An *Arabidopsis* mutant, *crr4*, was identified based on its defective NDH activity [12]. The *CRR4* gene encodes a member of the PLS subfamily, suggesting that *crr4* is defective in the maturation of a plastid *ndh* gene. However,

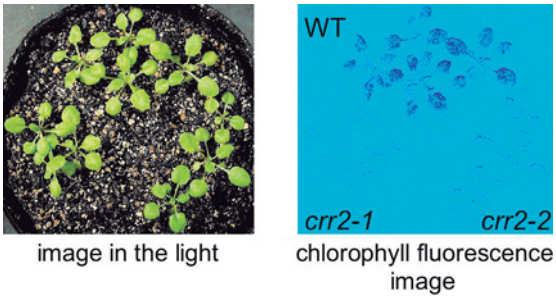

Figure 2. Visualization of NDH activity using chlorophyll fluorescence imaging. Chlorophyll fluorescence reflects the status of photosynthetic electron transport. By monitoring a transient increase in the fluorescence level after illumination, NDH activity can be detected under a CCD camera. The image appears in the wild type (WT), while it is not present in alleles of *crr2*, which is defective in NDH activity. This figure was modified from [56]

Northern analysis failed to detect any alteration of RNAs from 11 *ndh* genes in their size or abundance in *crr4*. We therefore analyzed the efficiency of RNA editing of plastid *ndh* genes, resulting in the identification of a specific defect in RNA editing of *ndhD*. In *Arabidopsis*, the translational initiation codon of *ndhD* encoding a subunit of the NDH complex is encoded by ACG, which is converted to AUG by RNA editing (the ndhD-1 site). The other editing sites were properly processed, as in the wild type, indicating that *crr4* is specifically defective in the RNA editing of ndhD-1.

The machinery of RNA editing in plants

The machinery of the RNA editing process is largely unknown in plants, except for the involvement of PPR protein. Using an *in vitro* editing system, mitochondrial editing was shown to be a process of deamination of the specific C, rather than the substitution of a nucleotide or transglycosylation [62]. A similar story is true for the plastids [30]. These results imply that C deaminase is also involved in RNA editing in plants, as well as APOBEC-1 in the mammalian system [7].

CRR4 was identified as a factor required for RNA editing in the plastids. CRR4 belongs to the E+ subgroup, which is lacking a well-conserved C-terminal DYW motif (fig. 1). CRR4 is structurally similar to CRR2, which was also identified by our screening of *Arabidopsis* mutants defective in NDH activity [56]. CRR2 is involved in RNA cleavage between *rps7* and *ndhB*, which may be essential for *ndhB* translation. CRR2 is a member of the DYW subgroup (fig. 1). This means that CRR2 contains every motif present in CRR4, except for a short motif consisting of 15 amino acids. This short motif is related to the E+ and E motifs, but is well conserved in some members, including CRR4. Despite their structural similarity, CRR2 and CRR4 are involved in different processes of RNA maturation, RNA cleavage and RNA editing. These results show that it is unlikely that CRR4 contains a domain responsible for the activity of C deaminase, although it is still possible that the DYW domain of CRR2 is essential for RNA cleavage. In our model, the PPR protein acts to recognize the target RNA for maturation and recruits the general machinery of RNA maturation, such as C deaminase or endonuclease, to the target site (fig. 3).

In contrast to our multi-component model of the RNA editing machinery, the results of biochemical analysis using *in vitro* editing system favor a single-subunit model [31]. UV cross-linking suggests that an unidentified protein, which may be C deaminase or a *trans*-acting factor, interacts with both the *cis*-element and the editing site. It is possible that the interaction among the PPR protein, C deaminase and the target RNA is transient, and that the PPR protein may be released from the target site after recruiting C deaminase.

Provided that the PPR protein does not contain any domains responsible for C deaminase activity, it is necessary to hypothesize that a second factor is involved in this activity. *in vitro* editing in *Arabidopsis* chloroplasts is sensitive to a zinc chelator, suggesting the involvement of a zinc-dependent C deaminase in the same way as mammalian APOBEC [63]. The *Arabidopsis* genome encodes several proteins containing a zinc-dependent deaminase domain [64]. However, reverse genetic strategies have so far failed to find an enzyme essential for RNA editing in the plastid [unpublished results]. In contrast, *in vitro* editing in pea mitochondria is insensitive to a zinc chelator [65]. The editing enzyme may be different between mitochondria and plastids especially in higher plants. This idea is consistent with the fact that C deaminase cannot

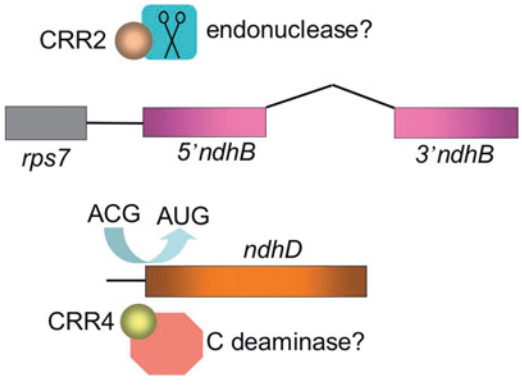

Figure 3. Function of PPR proteins CRR2 and CRR4. CRR2 is essential for intergenic RNA cleavage between *rps7* and *ndhB* [56]. On the other hand, CRR4 is essential for RNA editing of the ndhD-1 site [12]. CRR2 and CRR4 may function in the recognition of the target RNA and recruit an endonuclease and a putative editing enzyme (C deaminase), respectively, to the processing sites

catalyze the reverse reaction of U to C, which often occurs in mitochondria [65].

How many *cis*-elements does a PPR protein recognize?

In *Arabidopsis*, 441 sites are edited in mitochondria, [66], while 28 sites are edited in plastids [67]. If a specific *trans*-acting factor exists for each individual editing site as suggested [26, 30], the nuclear genome would need to encode 469 genes to maintain all the RNA editing processes in *Arabidopsis* organelles. The *Arabidopsis* nuclear genome encodes approximately 450 PPR proteins, roughly enough to cover all of them. However, PPR proteins are involved not only in RNA editing but also in other RNA maturation steps, including RNA stabilization, RNA cleavage, splicing and translation (table 1). Are 450 PPR proteins enough for all the RNA maturation processes?

An alternative idea is that a single PPR protein is involved in RNA editing at multiple sites. This idea was proposed based on the effect of high-level expression of the sequences carrying the rpoB-2 and ndhF-2 editing sites on all the editing events in the tobacco chloroplast [68]. The editing efficiencies were reduced, both in the corresponding endogenous genes and in several other genes. Interestingly, weak conservation of nucleotides was detected in the 5′ sequences of the editing sites among genes which experienced a cross-competitive effect. This result suggests that the plastid *cis*-elements and also some mitochondrial *cis*-elements can be classified into several groups which would be recognized by a single *trans*-acting factor.

However, this model is not supported by the *crr4* phenotype. The efficiency of ndhD-1 editing, which requires a CRR4 function in *Arabidopsis*, was reduced by overexpression of the ndhF-2 editing site, suggesting that editing of ndhD-1 and ndhF-2 requires the same *trans*-acting factor [68]. However, the *crr4* phenotype is specific to the ndhD-1 site, and the editing of ndhF-2 was not affected in *crr4* [12]. It is possible that high-level accumulation of RNA containing the ndhF-2 site may have caused an artificial interaction of CRR4 with the ndhF-2 cis-element. It is also possible that a *trans*-element other than CRR4 is required for RNA editing of both ndhD-1 and ndhF-2, which is rate-limiting when the ndhF-2 site is overexpressed.

However, biochemical and genetic evidence supports the idea that a PPR protein is involved in multiple editing events. CRP1 binds 5′ untranslated regions of both *petA* and *psaC*, where consensus sequences of 7 and 11 nucleotides separated by 51 nucleotides have been discovered [44]. Genetic analysis also suggests that PGR3 interacts with multiple targets and is involved in the different steps of RNA maturation [47]. HCF157 is also involved in multiple events, intergenic RNA cleavage and splicing of the same target RNA [45]. These results suggest the possibility that a single PPR protein is involved in RNA editing of multiple sites and even shares the function with other RNA maturation processes, such as RNA cleavage.

Interestingly, transcripts of the most NDH subunit genes are inefficiently edited in the roots, where the NDH complex does not accumulate [69]. It is plausible that a common *trans*-acting factor regulates several RNA editing events essential for the functioning of the NDH complex. However, it is apparent that this *trans*-acting factor is not CRR4, since the *crr4* phenotype is specific to the ndhD-1 site. Overexpression of *CRR4* does not promote the editing of ndhD-1 in roots [12]. Furthermore, endogenous *CRR4* is expressed in roots, where ndhD-1 is not edited. All these results suggest that an unknown factor other than CRR4 limits the RNA editing of ndhD-1 in roots. It is possible that this factor is common to more than one RNA editing event related to expression of the functional NDH complex.

The factor required for the editing of multiple editing sites was also suggested in mitochondria. Cytoplasmic male sterility (CMS) is a maternally inherited trait, in which plants cannot produce functional pollen. In *Sorghum bicolor*, CMS is closely related to the reduced extent of RNA editing in *atp6* transcripts [70]. The *Sorghum atp6* gene contains 19 RNA editing sites, which are inefficiently edited in CMS plants. However, under the specific nuclear background in which fertility is restored, the editing efficiency of all the sites increases to the level of the fertile cytoplasm. Interestingly, this restoration was suggested to require a factor which is involved in the multiple editing events present in the same transcript [71]. It is unclear on the relationship of this putative transcript-specific factor and the editing site-specific factor.

The physiological function of RNA editing

What is the physiological function of RNA editing in plant organelles? Although RNA editing is essential to express functional proteins, almost all the editing sites can probably be revised at the genomic DNA level. Is RNA editing just a habit of plant organelles that lacks any physiological function? Before heading towards this discouraging conclusion, let us try to find an example in which RNA editing may be beneficial for plants.

The initiation codon of *ndhD* is encoded by ACG in the plastid genome, and is modified to AUG by RNA editing. In *crr4*, which is defective in this RNA editing, accumulation of the NDH complex is severely impaired, indicating that RNA editing is essential to expression of *ndhD*.

Thus, editing ndhD-1 may regulate the efficiency of the *ndhD* translation. This idea is supported by the fact that the efficiency of this RNA editing is developmentally regulated [72]. The ndhD-1 site is partially edited, even in leaves, where the NDH complex is active, whereas it is not edited in roots, where the NDH complex is absent.

How is the efficiency of ndhD-1 regulated? A possible idea is that RNA editing is limited by the availability of the *trans*-acting factor CRR4. However, overexpression of *CRR4* under the control of 35S promoter of cauliflower mosaic virus did not increase the efficiency of ndhD-1 editing in either *crr4* or the wild type [12]. The simplest conclusion is that a factor other than CRR4 limits the efficiency of ndhD-1 editing. It is unlikely that a common factor for all the editing events, such as a putative C deaminase, would limit the efficiency. This means that multiple specific factors are required for a single editing event. If this really is the case for all editing sites, double the number of specific factors would be required. It is also possible that CRR4 is modified to facilitate target binding.

Even though the editing of ndhD-1 is beneficial for plants in that they can regulate the translation of *ndhD*, *Arabidopsis ndhD* has an additional four editing sites, which always need to be edited in RNA containing a translational initiation codon to prevent expression of mutant versions of NdhD. Why must these editing sites be regulated independent of ndhD-1 editing? Furthermore, plants have a mechanism for regulating *ndhD* expression via alternative RNA cleavage between *psaC* and *ndhD* [73]. How does the regulation of the ndhD-1 editing efficiency contribute physiologically *in vivo*? At least in the monocots wheat, rice and maize, the *ndhD* translational codon is encoded by ATG in the genome [29], suggesting that translational regulation via the RNA editing is not essential.

Evolution of PPR protein and RNA editing

The evolutionary process of RNA editing was proposed in an early review [74]. The story is still plausible after the discovery of *trans*-acting factor of PPR protein. Initially, the ancestral editing activity would allow the mutation by which T is replaced with C in organellar genomes. Accumulation of mutations would require the conservation of RNA editing as an essential function. As evolution progressed, PPR proteins may have allowed the number of editing sites to be increased. By multiplying the family members with variations, which recruit the editing machinery to the editing site, plants may have easily managed the newly occurring mutations. It is also possible that a mutation in an existing PPR protein enables it to recognize an additional target. If this is the case, it is not surprising that a single PPR protein is involved in both RNA editing and other RNA maturation processes.

Several examples of evolutionary processes in which PPR proteins allowed plants with mutations in the mitochondrial genome to survive have recently been shown [75–81]. Unusual genes consisting of chimeric fusions of mitochondrial genes often cause a maternally inherited trait in which plants cannot produce functional pollen (CMS). Dominant nuclear genes, restore fertility (*Rf*), suppress the CMS phenotype. The mitochondrial genome consists of hundreds of copies per cell and undergoes a wide range of recombination events through repeats. Chimeric genes associated with CMS are often generated by recombinations such as these, which have to been accompanied by co-evolution of the *Rf* gene to sustain fertility. The levels of RNA editing in mitochondria were also suggested to be involved in CMS [70, 71, 82, 83]. Interestingly, some *Rf* loci contain several copies of PPR genes, strongly suggesting that expression of CMS genes is suppressed via RNA processing related to the function of PPR proteins which may have been generated by gene duplication events that occurred recently. These results suggest a strategy of the plant in which a new function of the *Rf* gene is acquired via the duplication and mutation of a PPR gene.

If RNA editing sites really have a history of co-evolution with PPR proteins, we may find a phylogenetic correlation between RNA editing and PPR proteins. The topic was extensively discussed in a recent report [13]. Conversion of a specific C to U is unique to land plants [84]. In contrast, PPR proteins are widely distributed in eukaryotes, consistent with the fact that a PPR protein is a factor involved in general RNA maturation steps. However, the drastic expansion of the PPR family and the appearance of the PLS subfamily correlate with C-to-U RNA editing in organelles. At present, only two members of the PLS subfamily were characterized at the level of their function, and only CRR4 is clarified to be related to RNA editing [12, 56]. It is essential to accumulate information, especially on PPR proteins related to RNA editing processes.

Concluding remark

As in the aforementioned example of evolution of *Rf* genes, the PPR family may have expanded to suppress defects in the mitochondrial genome. The same story may be true for the evolution of RNA editing sites, both in mitochondria and plastids. The nuclear genome may have utilized PPR proteins to manage growing numbers of RNA editing events in the mitochondria. Once the mitochondria acquired the system, it was probably a simple step to transfer the system to the plastids by modifying the machinery to target both organelles. A similar evolutionary strategy is observed in a nuclear-encoded RNA polymerase in plastids, which was acquired via the dual

targeting of RNA polymerase originally derived from the mitochondria [85].

Is RNA editing in plant organelles the result of a failure in training of the mitochondrial genome by the nuclear genome during evolution? Although RNA editing does not seem to be essential in present-day plants, it may have been beneficial to plants in the past, during evolution. To investigate this possibility, it is necessary to accumulate more information on the relationship between PPR protein and the editing event.

One essential question concerning plant RNA editing is whether the editing mechanism is single or not. Although the PPR protein is involved in the editing of ndhD-1 in plastids, the guide RNA hypothesis has still not been completely eliminated, especially in complex editing events in mitochondria. It is also not known whether the enzyme catalyzing nucleotide modification is single or not. If it is true that C deaminase is involved in RNA editing, as in mammals, how can reverse editing of U to C be explained in plants? The presence of reverse editing implies that unknown enzyme activity other than C deaminase is involved in RNA editing, or that it has multiple mechanisms.

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