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REVIEW

RNA Editing in Plant Organelles. Why Make It Easy?

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Abstract—Gene expression in plant organelles involves a number of distinct co- or posttranscriptional nucleic acid modifications: 5′ and 3′ RNA processing, *cis*- and *trans*-splicing, RNA stability, and RNA editing. All contribute to the steady-state RNA levels available for the translation of the reduced but essential organellar genetic information. Different from other maturation processes, RNA editing at the transcript level modifies the information encoded by organellar genes and is an essential step for the production of functional proteins. Editing changes are extensive in mitochondria from flowering plants with more than 400 cytidine-to-uridine changes that involve most transcripts, while in chloroplasts they are limited to some RNAs. An additional U-to-C RNA editing reaction is observed with the C-to-U transitions in fern and moss organelles. While RNA editing targets mostly concern coding regions, some events occur in untranslated regions. Whereas RNA editing is genetically and biochemically distinct from other RNA modification activities, evidence is growing for a tight connection between the different processing events. Although the understanding of this astonishing mechanism has increased since its discovery in 1989, some important questions remain unanswered. In this review we discuss the current knowledge on the different aspects of C-to-U, and to a lesser extent U-to-C, and look at RNA editing in plants with a particular emphasis on recent developments involving the role of pentatricopeptide repeat (PPR) proteins in this process.

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The discovery of RNA editing in plant mitochondria resulted from the comparison between genes and their corresponding cDNA sequences by three independent research teams [1-3]. Two years later, this process was described in chloroplasts [4]. In both organelles, some cytosines are changed into uracils on RNAs (Fig. 1; see color insert). The evidence that proteins are translated from edited RNAs came from direct sequencing of the mitochondrial encoded ATP9 [5]. While some RNAs are partially edited in the steady-state pool of transcripts in plant organelles [6, 7], the corresponding proteins derive from totally edited RNAs [8, 9]. RNA editing seems to be essential for the production of functional proteins, since it restores highly conserved amino acids [10]. Indeed, it has been shown that the mitochondrial accumulation of ATP9 proteins arising from unedited mRNAs (u-ATP9) results in a mitochondrial dysfunction leading to a male sterile phenotype in tobacco and *Arabidopsis* transgenic plants [11, 12]. This phenotype disappeared when the expression of the u-ATP9 protein was abolished [13, 14]. Similarly, the synthesis of an unedited *psbF* mRNA in chloroplasts resulted in a deficiency in photosystem II [15].

RNA editing has been found in all land plant lineages analyzed to date, except in marchantiid liverworts [16-18]. As some liverwort groups edit their organellar RNAs, the lack of editing in the subclass of marchantiid liverworts is probably a consequence of a secondary loss [19]. There is no evidence for RNA editing within green alga organelles, which are the closest relative of the land plants. Thus, it is probable that RNA editing is a derived trait appearing with the emergence of land plants.

RNA EDITING IN PLANT ORGANELLES

Typically, 300 to 500 C residues (henceforth referred to as editing sites) are edited in most plant mitochondria transcriptomes [20-24]. The number of editing sites can

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even exceed 1000 in the gymnosperm *Cycas taitungensis* [25] and reached 1782 in the fern *Isoetes engelmannii* [26]. Most of the editing sites are located in the first and second position of codons, changing the identity of the encoded amino acid [20, 27]. A consequence of the codon modifications produced by RNA editing is an increase in the hydrophobicity of the encoded proteins [20, 27]. Interestingly, in some cases, RNA editing can create start and stop codons, thus restoring an ORF absent from the gene [26]. In such cases, RNA editing directly affects the translation process.

A peculiar situation is the modification of the silent position of codons since these changes have no effect on the protein sequence. This is in agreement with the fact that the partially edited mRNAs found in plant organelles mainly concern the third position of the codon. Interestingly, it has been described that, in a single plant, the number of editing sites varies between different organs [28]. These differences are also essentially found in the silent position. The putative involvement of these residues in the regulation or other events during RNA maturation remains to be elucidated.

The general picture for chloroplast transcripts is similar, except that the number of editing events is drastically reduced to between 20 to 50 depending on the species [29-38]. As in mitochondria, editing sites are mainly located in the first and second codon position, silent positions remaining very rare [39]. Some ORF are restored by conversion at the RNA level of an ACG codon to AUG by editing [4, 37].

U-to-C TRANSITION CAN COEXIST WITH C-to-U EDITING

In addition to C-to-U editing, some plants possess a second RNA editing mechanism, U-to-C editing, totaling several hundreds of base changes on the transcriptome. This kind of event, described by some authors as "reverse editing", is particularly abundant in mosses of the *Anthoceros formosae* family [40, 41] and in ferns [26, 42]. A striking observation is that many U-to-C transitions change UAA, UAG, and UGA stop codons into CAA, CAG, and CGA coding for Gln and Arg, respectively (Fig. 1). Thus, U-to-C RNA editing corrects the reading frame of organellar-encoded pseudogenes and appears to be essential for the production of functional proteins [41]. While knowledge is still scarce concerning the mechanism of U-to-C transitions, it should be noted that this process is concomitant with C-to-U RNA editing and concerns both mitochondria and chloroplast transcripts. One important question is to know whether the two events are related, or if they occur independently. Analyses of editing intermediates from fern mRNAs suggest that the two events may be autonomous (D. Bégu, personal communication).

C-to-U RNA EDITING OCCURS BY A DEAMINATION MECHANISM

An essential step for the understanding of the mechanism of C-to-U RNA editing was the creation of an *in vitro* editing assay with mitochondrial extract [43]. Radioactive labeling of the C residues at either the α-5′-phosphate or the base with ^{3}H on the RNA substrate has shown that the phosphate-ribose backbone and the base are not eliminated [44-46]. A concomitant labeling on the base and the phosphate led to the final conclusion that C-to-U RNA editing was a deamination in plant mitochondria. Similar experiments have provided strong evidence that the same reaction occurs in chloroplasts [47]. In fern and mosses, where C-to-U and U-to-C editing coexist, it has been speculated that the latter is the reversal reaction of C-to-U conversion. If so, the presence of an enzyme able to perform both reactions, such as a transaminase, seems to be the most plausible explanation. However, the residues involved in the direct or reverse reactions are not the same. The only unambiguous way to solve this problem is to isolate the gene(s) fulfilling the catalytic function. The search for gene candidates using the conventional cytidine deaminase signature has not been conclusive [48]. Current studies aim at searching for deaminase activity associated with a particular set of genes coding for pentatricopeptide protein repeat (PPR) proteins [49].

RNA EDITING IN NON-CODING REGIONS

Although the vast majority of RNA editing sites is located in coding regions, some of them concern untranslated sequences. In *Arabidopsis thaliana*, eight out of 456 editing sites are located in introns [20]. However, a recent study by RNA deep-sequencing technology showed that the number of editing events in introns could be higher than previously thought [24]. If the editing events in non-coding regions are not in perfect line with the idea that RNA editing is essential for the production of functional proteins, it is obvious that the splicing reaction is required to produce translatable mRNAs. The vast majority of introns in plants are group II introns, which are characterized by a well-conserved secondary structure [50]. Interestingly, RNA editing sites in introns are located in highly structured domains that are important for the splicing reaction. This is particularly true when the editing event results in the correction of mismatches in double-stranded regions required for intron folding [26, 50, 51]. Thus, RNA editing in these domains may be postulated as a prerequisite for intron removal. This hypothesis, for which evidence was first indirect [51], was recently demonstrated by expressing mutant constructs into isolated mitochondria [52]. A similar situation seems to apply to group I introns in fern mitochondria, where

RNA editing restores structural motifs involved in the folding and catalytic reaction (D. Bégu, personal communication). In species that carry out both C-to-U and U-to-C RNA editing conversions, both events are also found in the intronic region [26].

Base modifications by RNA editing are also observed in leaders or trailer sequences on the transcripts [20, 53]. Although the editing changes in these regions are thought to be involved in mRNA translation, no experimental evidence has yet been reported.

Some organellar tRNAs are edited. As in introns, RNA editing corrects mismatches in the stem-loop domains that allow the secondary structure folding [24, 54-56]. Although the RNA editing process does not seem to be tightly linked to tRNA aminoacylation, only the edited version of the transcript can undergo maturation *in vitro*, indicating a major role of tRNA editing in efficient precursor processing [54, 56-58]. Unsurprisingly, ferns edit their tRNAs with the two types of base conversion mechanisms [26].

Cis-RECOGNITION ELEMENTS

As plants possess hundreds of editing sites, the reaction needs to be very specific to ensure the production of functional proteins. One major question was to understand the *cis* elements defining the C residue to be edited. Indeed, an examination of the neighboring region of the target Cs does not show any consensus element, either in sequence or secondary structure. In both organelles, bioinformatic analyses have suggested that the information for editing site recognition is contained in the proximity of the C residue, with a bias for a pyrimidine in −1 and a purine in +1 position [20, 59, 60].

The production of transplastomic plants has facilitated the search for editing *cis* elements in chloroplast RNAs. The use of transgenes bearing nucleotide deletions has shown that a short sequence encompassing the target C residue is essential to the reaction [61-64]. The *cis* elements contain 22 nucleotides, 16 upstream from the editing site and 5 downstream, the upstream region being more important [62, 64]. Site-directed mutagenesis on individual nucleotides has shown that the residues immediately adjacent to the editing site have an important role in target recognition [61, 62, 64, 65]. These results were later confirmed by *in vitro* assays [32, 66-68]. Moreover, mutagenesis analyses revealed short upstream sequences essential for the reaction conserved in different plants [69, 70].

The first information on the mitochondrial *cis* elements was obtained by analyzing the editing of natural chimera. It was reported that changes upstream from the editing site could abolish editing, whereas changes downstream had slighter effects [71, 72]. The lack of a transformation system for mitochondrial genomes has led to the creation of an alternative *in organello* strategy [73]. It is a powerful tool to study RNA editing and other maturation processes such as splicing [74]. The introduction of foreign DNA into mitochondria isolated from wheat, potato, maize, sorghum, and cauliflower has been reported [75-77]. With this strategy, it was demonstrated that mitochondrial *cis* elements reside in the region −16 to +6 encompassing the editing site [78, 79]. Single mutants along this sequence revealed a different role played by individual residues [78]. The extent of the recognition region was confirmed by *in vitro* experiments using pea and cauliflower mitochondrial extracts [80, 81]. In most cases, the upstream sequence was found essential for the editing reaction, the downstream nucleotides playing a role in the efficiency but not in the reaction itself [80-82]. However, spacing up- and downstream regions by more than two nucleotides results in complete loss of the editing ability [78, 79].

Distal elements have been revealed by deletion experiments upstream of an editing site, which resulted in a concomitant decrease in RNA editing of a second site 50-70 nucleotides downstream [83]. The effect of modifications in remote regions from the −16/+6 *cis*-acting elements on RNA editing has been confirmed by both *in vitro* and *in organello* approaches, but the mechanisms responsible for these effects are still a matter of debate [83, 84]. A major concern with the *in vitro* test is the low efficiency of C-to-U conversion. The use of an RNA substrate with duplicated *cis* elements greatly increased the reaction efficiency, thereby confirming the role of this sequence in the editing site recognition [85].

Trans-ACTING FACTORS

Different from the trypanosome editing model, in plant organelles a particular class of proteins has been proposed to play the role of *trans*-recognition elements. Albino mutants containing chloroplasts unable to perform protein synthesis are able to sustain the editing reaction, demonstrating that *trans*-acting factors are not encoded by the chloroplast genome [86]. Similar results have been obtained for mitochondria using *in organello* editing assays, since the inhibition of organellar protein synthesis did not abolish the reaction [87]. This indicates that the factors involved in the recognition of *cis* elements are nucleus-encoded. The existence of *trans*-acting factors was the initial explanation for the editing inhibition of an endogenous site in transgenic plants expressing a competitor sequence [63]. Using chloroplast extracts in *in vitro* competition experiments, it was suggested that some *trans* elements could recognize more than one *cis* element [88, 89].

The binding of *trans*-acting factors to the target sequence was demonstrated by UV cross-linking experiments using tobacco chloroplast extracts [47, 66, 90].

Moreover, a strong correlation was observed between the editing efficiency and the binding capacity of one of the proteins, suggesting a direct link between binding and the editing reaction [68]. From competition experiments, it was inferred that a single *trans*-acting factor could bind to two different editing sites [90]. However, *in vitro* experiments have not yet allowed the identification of the cross-linking proteins.

Immunodepletion of CP31, a protein containing two RNA recognition motifs, has shown that it is involved in RNA editing in tobacco [47]. These results were later confirmed by *Arabidopsis* mutant analyses. However, it is difficult to conclude in a direct role in RNA editing reaction as the accumulation of most of the mRNA is lowered in these mutants [91].

The direct identification of *trans*-acting proteins was achieved by screening nuclear mutants of the model plant *Arabidopsis thaliana* based on the analysis of a failure of the cyclic electron transport in the chloroplast NDH complex. The first gene identified was *crr4*. The product of this gene is essential for one RNA editing site on the *ndhD* transcript (*ndhD-1*), which creates the AUG initiation codon [92]. Following this discovery, several proteins were identified by analyses of *Arabidopsis* nuclear mutants [93-101]. Another *trans* factor was identified with a different strategy by using a comparative genomics approach [102].

The identification of mitochondrial factors is hampered by the difficulty to isolate mutants. The observation that amino acid sequence of *trans*-acting factors influences editing efficiency [103] leads to an alternative strategy to identify the genes involved in RNA editing. It is based on the idea that differences in editing efficiency for a particular site between different plant ecotypes reflect modifications on the *trans*-acting genes [28, 104]. Reciprocal crosses of ecotypic RNA editing variants can be used to identify the locus responsible for the editing variations. With this strategy, the first *trans*-acting factor associated with mitochondrial editing events, MEF1, was identified [105]. The use of a high throughput method to screen for editing variations [106] facilitated the identification of other mutants [107]. Several other proteins have now been identified in different organisms [107-115]. The proteins involved in RNA editing described so far are indicated in table.

PENTATRICOPEPTIDE REPEAT PROTEINS AS RNA EDITING *trans*-ACTING FACTORS

An important observation is the fact that all the *trans* factors identified so far belong to the pentatricopeptide protein repeat (PPR) family (table). These proteins are characterized by the repetition of a 35-amino acid motif (P motif) [116-118]. Most *PPR* genes code for proteins possessing motifs defined as putative organelle-addressing sequences, suggesting that they may play an important role in mitochondria and chloroplasts [117, 118]. It has been shown that some of them are able to bind RNA in a sequence-specific manner and that they are involved in different stages of organellar genetic expression [117, 119]. Although present in all eukaryotes, this family has greatly expanded in plants, which raises the question of its involvement in plant-specific processes.

Interestingly, a subfamily of PPR proteins containing longer (L) and shorter (S) motifs in addition to the P (called PLS subfamily) is specific to land plants. In addition to the PLS domain responsible for the interaction with RNA, they contain C-terminal extensions called E and DYW domain that potentially bear catalytic activities [117, 119]. In fact, the DYW domain contains conserved amino acids equivalent to the one found in the active site of cytidine deaminase [49]. Moreover, there is a striking correlation between the occurrence of RNA editing and the presence of *PPR-DYW* genes in land plants [49, 120]. Based on these observations, it was proposed that the PPR-DYW proteins could act as the catalytic factor for C-to-U conversion [49] (Fig. 2a). However, no deaminase activity has been detected *in vitro* for PPR-DYW proteins. Additionally, it was demonstrated that the DYW domain is not required for the editing reaction *in vivo* [96, 97].

All but one PPR proteins involved in RNA editing identified so far contain the E domain in their C-terminal

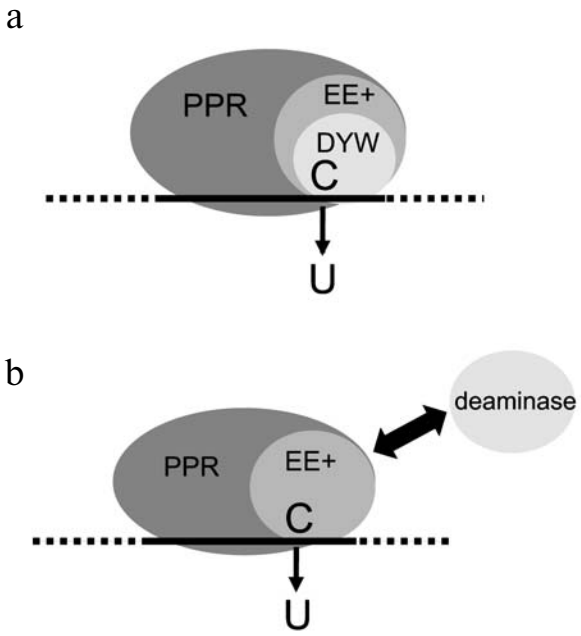

Fig. 2. Putative function of PPR proteins in plant organellar RNA editing. a) PPR-DYW protein may function in the recognition of the target RNA, and the DYW domain catalyzes the deamination of the C residue [49]. b) The PPR recognize the target RNA, and the E domain participates in recruiting the catalytic factor [121]. Adapted from Okuda et al. [122].

PPR protein genes involved in organellar RNA editing

Protein	Organism	Organelle	PPR family	Target mRNA	Reference
CRR4	Arabidopsis	chloroplast	E	*ndhD*	[92]
CRR21	–"–	–"–	E	*ndhD*	[98]
CRR22	–"–	–"–	DYW	*ndhB, ndhD, rpoB*	[96]
CRR28	–"–	–"–	DYW	*ndhB, ndhD*	[96]
CLB19	–"–	–"–	E	*rpoA, clpP*	[94]
LPA66	–"–	–"–	DYW	*psbF*	[93]
RARE1	–"–	–"–	DYW	*accD*	[102]
YS1	–"–	–"–	DYW	*rpoB*	[101]
ECB2/VAC1	–"–	–"–	DYW	*accD, ndhF*	[99, 100]
OTP80	–"–	–"–	E	*rpl23*	[95]
OTP81	–"–	–"–	DYW	*rps12*	[95]
OTP82	–"–	–"–	DYW	*ndhG, ndhB*	[97]
OTP84	–"–	–"–	DYW	*ndhF, psbZ, ndhB*	[95]
OTP85	–"–	–"–	DYW	*ndhD*	[95]
OTP86	–"–	–"–	DYW	*rps14*	[95]
MEF1	–"–	mitochondrion	DYW	*rps4, nad7, nad2*	[105]
MEF9	–"–	–"–	E	*nad7*	[113]
MEF11/LOI1	–"–	–"–	DYW	*cox3, nad4, ccb203*	[107, 124]
MEF8	–"–	–"–	DYW	unknown	[113]
MEF18	–"–	–"–	E	*nad4*	[113]
MEF19	–"–	–"–	E	*ccb206*	[113]
MEF20	–"–	–"–	E	*rps4*	[113]
MEF21	–"–	–"–	E	*cox3*	[113]
MEF22	–"–	–"–	DYW	*nad3*	[113]
SLO1	–"–	–"–	E	*nad4, nad9*	[112]
PPR596	–"–	–"–	P	*rps3*	[109]
REME1	–"–	–"–	DYW	*nad2, orfX*	[108]
OGR1	*Oryza sativa*	–"–	DYW	*cox2, cox3, ccmC, nad2, nad4*	[110]
PpPPR_71	Physcomitrella	–"–	DYW	*ccmFc*	[115]
PpPPR_56	–"–	–"–	DYW	*nad3, nad4*	[111]
PpPPR_77	–"–	–"–	DYW	*cox2, cox3*	[111]
PpPPR_91	–"–	–"–	DYW	*nad5*	[111]

part. Complementation experiments have shown that this domain is essential for RNA editing and that it can be exchanged between different PPR proteins [96, 98]. It has been proposed that the E domain participates in recruiting the catalytic factor, either a PPR-DYW or another accompanying factor [121] (Fig. 2b).

RECOGNITION OF THE RNA EDITING SITE

An unresolved question is how PPR proteins recognize the *cis*-acting elements. It has been reported that a single *trans*-recognition element can interact with different *cis* elements. However, such an event has been experimentally demonstrated only for an unidentified tobacco protein [90]. Until now, the specific binding of two *trans*-acting PPR factors to the respective target sequences has been reported for mitochondria [115] and chloroplast [121]. In both cases the proteins recognized a single *cis* element.

While a growing number of PPR protein genes have been shown to be related with the editing reaction, the mechanism allowing the recognition of different *cis* elements by a single protein is still unknown. It has been

hypothesized that not all the nucleotides belonging to the *cis* elements play equivalent roles in the recognition of the C residue. Site-directed mutagenesis has confirmed this idea by showing that the nucleotides immediately adjacent to the target C play a major role [61, 62, 64, 68, 78-80]. Moreover, bioinformatic analyses on chloroplast RNA editing have suggested that the specificity of recognition by *trans*-acting factors could be due to the ability to discriminate between purines and pyrimidines, and in some cases to choose one of the four nucleotides [95]. This mode of interaction is predictive and needs to be validated.

Understanding the interaction between a PPR protein factor and its multiple target sequences needs better knowledge of both *cis* and *trans* elements, by carefully deciphering and validating their roles in the editing event. Indeed, growing evidence suggests that the different maturation processes inside plant organelles are tightly connected [52, 76, 122]. As some PPR proteins may influence RNA editing without playing a direct role in it, caution is required when discriminating between a direct contribution and a side effect in the process [108, 109]. An example of this concerns AtECB2 and RARE1 PPR protein which are defined as *trans*-acting factors for one editing site on chloroplast *accD* mRNA. Mutants of these proteins induce different phenotypes, whereas the editing defect is the same [100, 102]. It may be that, at least in one case, the lack of editing is due to a side effect of the mutation. Another discrepancy results from the analysis of *otp85* mutants. It has been described as a gene encoding for a RNA editing chloroplast *trans*-acting factor with no visible phenotype [95], whereas a severe phenotype and mitochondrial location were observed by other authors [123]. Thus, clear understanding of how a PPR protein recognizes different specific RNAs first requires thorough validation of the partners involved, as the editing response observed in mutants might reflect complex interactions between several different proteins.

Since its discovery in 1989, knowledge of C-to-U RNA editing has greatly expanded. In addition, U-to-C RNA editing found in certain mosses and ferns act in concert with the C-to-U editing, but their mechanism is still poorly understood. A combination of *in vivo*, *in vitro*, and *in organello* experiments has shown that the specificity of recognition resides in a short region fewer than 30 nucleotides encompassing the RNA editing site. These *cis* elements are recognized by a large number of proteins belonging to the PPR family. The picture emerging from the different studies is that the RNA editing process seems to be more complex than previously thought. To date, three major problems remain unsolved. First, to identify the enzyme responsible for C deamination. Second, while it is clear that PPR proteins take part in RNA editing, how they recognize their RNA target is still unclear. Third, since different mechanisms of RNA processing may influence each other, the precise role of protein factors in organellar gene expression processes remains to be defined.

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