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Intron RNA editing is essential for splicing in plant mitochondria

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ABSTRACT

Most plant mitochondria messenger RNAs (mRNAs) undergo editing through C-to-U conversions located mainly in exon sequences. However, some RNA editing events are found in non-coding regions at critical positions in the predicted secondary and tertiary structures of introns, suggesting that RNA editing could be important for splicing. Here, we studied the relationships between editing and splicing of the mRNA encoding the ribosomal protein S10 (*rps10*), which has a group II intron and five editing sites. Two of them, C2 and C3, predicted to stabilize the folded structure of the intron necessary for splicing, were studied by using *rps10* mutants introduced into isolated potato mitochondria by electroporation. While mutations of C2 involved in EBS2/IBS2 interactions did not affect splicing, probably by the presence of an alternative EBS2′ region in domain I of the intron, the edition of site C3 turned out to be critical for *rps10* mRNA splicing; only the edited (U) form of the transcript was processed. Interestingly, RNA editing was strongly reduced in transcripts from two different intronless genes, *rps10* from potato and *cox2* from wheat, suggesting that efficient RNA processing may require a close interaction of factors engaged in different maturation processes. This is the first report linking editing and splicing in conditions close to the *in vivo* situation.

INTRODUCTION

In plant mitochondria, transcripts undergo several post-transcriptional modifications, such as RNA editing, *cis*- and *trans*-splicing and trimming (1). RNA editing of organellar transcripts is found in all land plants with the exception of the basal liverwort *Marchantia polymorpha* (2). RNA editing occurs by base deamination of specific cytosines on the transcripts (3,4). In *Arabidopsis thaliana*, 456 editing events have been described in the different mitochondrial transcripts; most of the C-to-U conversions concern the coding region of messenger RNAs (mRNAs) and change the genomic encoded amino acid (5). Moreover, the number of editing sites can vary between different tissues or ecotypes (6). Changes in the coding region in plant mitochondrial transcripts increase the similarity of the encoded proteins with their counterparts from organisms which do not edit their transcripts. Thus, RNA editing constitutes an essential step to ensure the production of functional proteins and the proper functioning of mitochondria (7,8). However, a few editing events occur in non-coding regions, particularly in introns (9), but their role in mitochondria is not well understood. Recently, the study of *Vitis vinifera* mitochondrial transcriptome indicates that the extent of RNA editing in mitochondrial introns could be higher than anticipated in land plants (10).

Mitochondrial intervening sequences from higher plants belong to the group II introns family, with the exception of a group I intron found in some species, issued from horizontal transfer (11). Group II introns, either in *cis* or *trans* conformation (9), have a well-characterized secondary structure with particular regions which are essential to ensure a good secondary and tertiary conformation, as for example the intron binding site (IBS)/exon binding site (EBS) interaction (12). It is interesting to note that some editing sites are found in highly structured domains of introns or in the IBS domain of exon, very close to the intron. Consequently, it has been suggested that in some cases editing can be necessary for splicing, by allowing a canonical folding of the intron (13–16). It has also been proposed that RNA splicing could create the *cis*-elements necessary for the editing of some sites (17).

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To date, evidence for a possible link between splicing and editing is scarce, most arguments being issued from predictions of base pair interactions concerning the putative secondary structure of the intron. In wheat mitochondria, *nad1* and *nad5* present C-to-U transitions found in the domain 6 (D6) stem of the respective *trans*-introns (14). In both cases, RNA editing is postulated to facilitate the change of the secondary structure of D6 to a conformation apt to carry out the first transesterification step during splicing. While RNA editing in coding regions can reasonably be assumed as important for protein function, the inference on the consequence of RNA editing in non-coding regions is less evident necessitating additional experimental support. Börner *et al*. (13) suggested that editing was required for splicing using an *in vitro* system in which the stem of domain 6, either with the genomic C or the edited U, of intron *nad1-c/d* was grafted into an autocatalytic yeast intron and found that only the edited chimera was released as a lariat, while the precursor with the genomic encoded C remained inactive.

In this report, we addressed the question on the relationship between RNA editing and splicing using the ribosomal protein S10 gene (*rps10*) as a model. Previously we found that *rps10* constructs were correctly expressed, edited and spliced in electroporated potato mitochondria (18). *rps10* contains one intron and five C residues are involved in RNA editing; one of them is located in the intron. Based on the canonical structure of group II introns, two editing sites, one located at the IBS2 and another situated at the beginning of the intron, were proposed to be involved in the correct folding of the intron (16). Here, we tested this hypothesis by introducing the engineered *Solanum tuberosum rps10* gene and mutant derivatives into isolated cognate mitochondria by electroporation (18,19). Moreover, we examined the impact of the presence of the intron on the ability of the mRNA to be edited. To our knowledge, this is the first experimental exploration of the interaction between RNA editing and RNA splicing in plant mitochondria close to the *in vivo* situation.

MATERIALS AND METHODS

All plasmids used in this study are based on the pRPS10St vector (18). The intronless construct, pRPS10Sti-, was constructed by creating two ApaI restriction sites, at the end of exon 1 and the beginning of exon 2 by site-directed mutagenesis with oligonucleotides I1 and I2. The mutant was then digested with ApaI endonuclease (Promega) and the fragment corresponding to the plasmid without the intronic sequence was recovered after separation on agarose gel electrophoresis and then circularized with T4 DNA ligase (Promega). The remaining ApaI site was eliminated by directed mutagenesis, using primer I3 to restore the wild-type sequence at this position. All mutants used here were obtained with the QuickChange® Site-Directed Mutagenesis kit (Stratagene) under the conditions indicated by the furnisher. Purification of restriction fragments and real-time polymerase chain reaction (RT–PCR) products was carried out with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Identical strategy was used to construct the wheat (*Triticum aestivum,* var. Ordeal) *pCOX2Tai-* vector, except the use of primers I4, I5 and I6.

Cytosines of sites C1 to C5 on the *rps10* transgene were changed into Ts (pre-edited) using primers C1 to C5. Mutants in EBS2 and EBS2′ regions were obtained using primers E2 and E2′. C3 was changed into A with primer A1 and the interacting residue (A) was changed into T with primer A2.

Oligonucleotides used for site-directed mutagenesis

Only sense primers are indicated. The residues changed in mutants are indicated by bold lowercase letters. The ApaI restriction site is underlined.

C1 ACCAAAAGTAA**t**GACCACCAAGA
C2 AGAAGTTCTTT**t**GGTTAAAACGCC
C3 GCCGTGCGACT**t**GGAGGACATAA
C4 CAAGACCCGTT**t**GGATAAGGGAA
C5 AGAGATTGCTT**t**GAAATAAGATC
E2 TAGACGCTTCGTAA**ttg**CGCACCGATCTACG
E2′ ATGATACAACAGAT**ttg**CTTAAGGAGTGGCG.
A1 GCCGTGCGACT**a**GGAGGACATAA
A2 GACCTGTGAAC**t**AGGTAAACCCA
I1 GGTTAAAACGCCGGGCCCGTGCGACTCGGA
I2 TCCCATCCCAATGGGCCCAGCGTATATTTG
I3 TTCGGTTAAAACGCCAGCGTATATTTGGAG
I4 AATGGTATCGGAGGGCCCGTGCGCCTCTTA
I5 CGTCGACCCAACGGGCCCCTTATGAGTATT
I6 AATGGTATCGGACTTATGAGTATT

Since the vectors used are based on p*RPS10*St *and pCOX2Ta*, all derivatives contain the inverted repeat from the wheat *cob* gene (*Ir-cob*) (accession no. AF337547). This sequence, combined with the 23 bp upstream insert, served to specifically amplify transgenic products with nested PCR as previously described (18). Primers 1a and 1b are common to all the vectors used in this work.

Primers used for RT–PCR analysis

1a GCGGTGCAGTCATACAGATCTGC
1b TCCCGCGGGAAGCGGAAAGC
2a GAGCAGAGCAGTCAAAGAATG
2b TATCCAGATTTGGTACCAAA
3a CCAAACCAAATGCATCAAAG
3b GGGAAAAACCCTAAATGTTG
2′a GAGCAGAGCTGAAAAAGATG
3′a TGAAAAAGATGGGAAATTCCA

Mitochondria purification

Solanum tuberosum tubers (var. Cherie) were bought at the local grocery store. Mitochondria were prepared essentially as described by Farré *et al*. (20). Two kilograms of tubers were homogenized in a buffer containing 0.4 M mannitol, 25 mM MOPS (pH 7.8), 1 mM EGTA, 8 mM cysteine and 1 mg/ml fatty-acid-free bovine serum

albumin (BSA). Homogenization was carried out in a Waring blender at full speed for 15 s. Homogenate was centrifuged in a Sorvall GSA rotor at 1500*g* for 10 min. The supernatant was then centrifuged in GSA rotor at 12 000*g* for 15 min. The mitochondrial pellet was resuspended in washing buffer containing 0.4 M mannitol, 5 mM MOPS (pH 7.8), 1 mM EGTA and 1 mg/ml fatty-acid-free BSA and centrifuged in a SS34 rotor at 1500*g* for 10 min. The supernatant was then centrifuged at 15 000 *g* for 15 min. All centrifugation steps were done at 4°C, and mitochondria were kept on ice during extraction and washing steps. The pellet was resuspended in washing buffer and mitochondria were purified by centrifugation on a sucrose gradient as previously described (20) and used immediately in electroporation experiments. Essentially the same procedure, with minor differences, was used to purify mitochondria from *Triticum aestivum* embryos (20).

Mitochondria electroporation

Electroporation was carried out with 1 mg of potato tuber mitochondrial proteins in 50 µl of 0.33 M sucrose and 2 µg of recombinant plasmid in the conditions previously described. (20). Electroporated mitochondria were incubated for 18 h at 25°C with shaking at 130 r.p.m. in a reaction mixture containing 0.33 M mannitol, 90 mM KCl, 10 mM $MgCl_2$, 12 mM Tricine (pH 7.2), 5 mM KH_2PO_4, 1.2 mM EGTA, 10 mM sodium succinate, 1 mM GTP, 2 mM ADP, 0.15 mM (each) CTP and UTP, 2 mM dithiothreitol and 1 mg/ml fatty-acid-free BSA. Mitochondria were recovered by centrifugation at 15 000*g* for 15 min at 4°C. RNA was purified with 800 µl Trizol® reagent (Invitrogen) according to the supplier's protocol.

RT–PCR

One microgram of nucleic acids obtained after Trizol® treatment was digested with 2 U of DNase I Amplification grade (Invitrogen) for 15 min at 25°C. cDNA synthesis was performed with 200 U of Superscript II RT (Invitrogen) using 100 ng of random primers hexamers as primers (Promega). The PCR amplifications were performed with primers 1a and 1b using Advantage 2 polymerase mix (Clontech) as follows: 95°C for 2 min, 20 cycles at 95°C for 30 s, 64°C for 1 min and 68°C for 2 min, and finally 68°C for 10 min. Primers 2a and 2b were used for nested PCR on 2 µl of PCR1, and primers 3a and 3b were used for nested PCR on 2 µl of PCR 2. Parameters were the same for these PCR, except for annealing temperature and cycling: 55°C and 20 cycles for PCR2, 52°C and 16 cycles for PCR 3. Primers 2′a and 3′a were used to analyze the wheat *cox2* transgene products.

Quantization of RNA splicing

RT–PCR products were separated on agarose gel containing SYBR®Safe DNA gel stain (Invitrogen) and run under standard electrophoresis conditions. The efficiency of splicing was determined from the fluorescence of SYBR Safe intercalated in DNA issued from precursor and mature molecules using a charge-coupled device (CCD) camera coupled to a PC computer. Images obtained were scanned using ImageJ64 software (http://rsb.info.nih.gov/ij/). The values expressed as a ratio of spliced/mature molecules were obtained after correction to an equivalent DNA size, assuming that the cyanine dye bound to double-stranded (ds)DNA with a stoichiometry of one dye molecule per 2 bp, characteristic of monointercalators (21,22).

Quantization of RNA editing

To determine the profile and the rate of C-to-U conversion in RT–PCR products, the precursor and mature PCR bands were excised from agarose gel after separation by electrophoresis and purified with the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The purified fragments were ligated into pGEM-T easy vector as described by the manufacturer (Promega). Cloned PCR products were sequenced with the BigDye® Terminator Cycle Sequencing Kit v 1.1 (Applied Biosystems). Sequences analyses were performed at the Genotyping and Sequencing Facility of Université Victor Segalen-Bordeaux 2.

RESULTS

The constructs, containing either *rps10* or *cox2* genes under the control of the potato or wheat *cox2* promoters, are depicted in Figure 1A. The *rps10* gene is formed by two exons of 307 and 110 bp, interrupted by an intron of 774 bp. The five cytosine residues, C1 to C5, that are changed into uracil in the transcript by editing are indicated (16). C1, C2, C4 and C5 are located in the translated region, whereas C3 is placed in the intron. The wheat *cox2* construct is formed by two exons of 388 and 392 bp, separated by a 1223-bp intron. The constructs were introduced into *S. tuberosum* or *T. aestivum* mitochondria by electroporation (20). The identity of precursor and spliced molecules produced by the transgenes was verified by sequencing mature and spliced RT–PCR products, after separation on agarose gel electrophoresis (see 'Materials and Methods' section). Similar results were observed for both wild-type constructs (Figure 1B, lanes a and c), confirming our previous data (18,19). As shown in Figure 1B, lanes b and d, the intronless *rps10* and *cox2* constructs were properly expressed in isolated mitochondria. To ensure that the analysis of intronless transcripts is not biased by vector DNA carryover in RNA preparations, RT–PCR experiments without reverse transcriptase were performed (Figure 1B). The editing efficiency of wild-type and mutant *rps10* transcripts was determined by sequence analysis of at least 16 individual RT–PCR clones from each electroporation experiments. For each construct, the results were validated by three independent electroporation tests.

The splicing efficiency is not affected in edited rps10 pre-mRNAs

The fate of transcripts from mutants on sites C2 and C3, and the C2/C3 double mutant were analyzed for intron removal. Residue C2 located in the IBS2 and residue C3

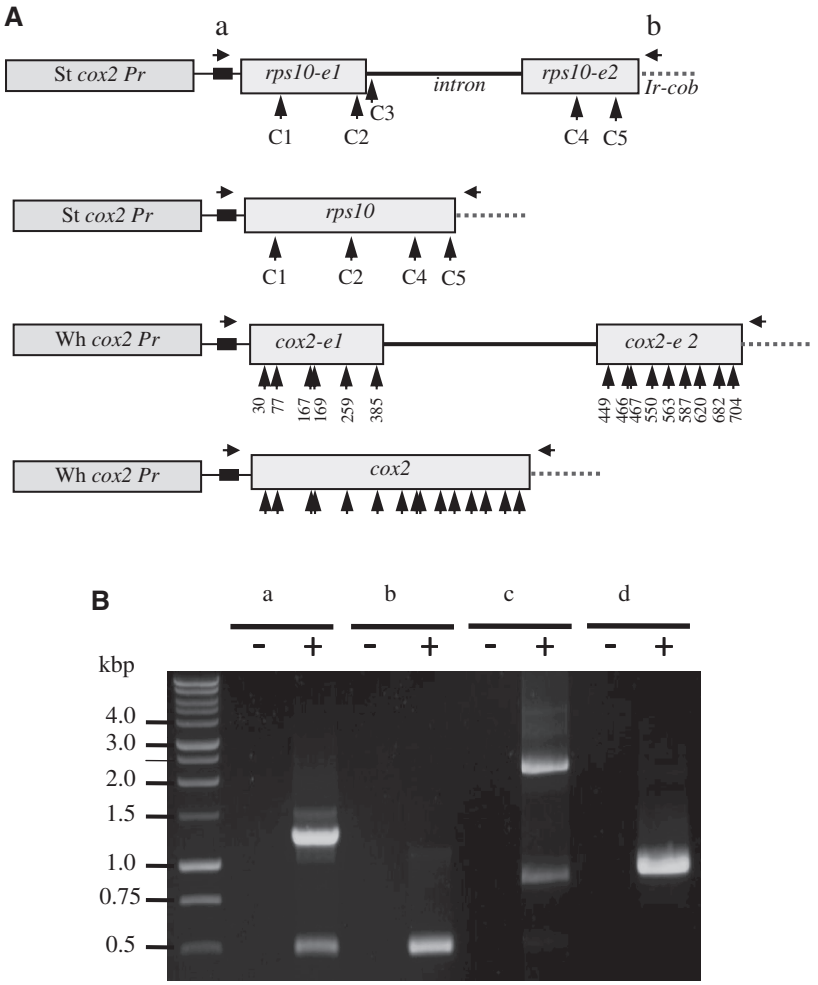

Figure 1. (**A**) Scheme of the constructs used. *rps10* and *cox2* are dependent of *cox2* promoters from potato or wheat, depending the source of mitochondria. St, *Solanum tuberosum*; Wh, wheat; Pr, promoter. C residues involved in RNA editing are indicated by vertical arrows. Black boxes represent a 23-bp insertion used for specific PCR amplification present in all constructs. Dot lines represent the inverted repeat region from the wheat *cob* gene (*Ir-cob*). *e1* and *e2* indicate the exons 1 and 2 from both genes. Horizontals arrows signal the position of primers a and b used for specific RT–PCR amplification of both transcripts. For details on *cox2* mRNA editing sites see Farré *et al*. (19). (**B**) Agarose gel electrophoresis of RT–PCR amplification products revealed by fluorescence of SYBR®Safe DNA gel stain. (a) Wild-type *rps10*; (b) intronless *rps10*; (c) wild-type *cox2*; (d) intronless *cox2*. The RT–PCR products of precursor (1281 bp and 2076 bp) and mature (417 bp and 882 bp) transcripts from *rps10* and *cox2* respectively were confirmed by sequence analysis. Symbols (–) and (+) indicate either the absence or the presence of reverse transcriptase in the cDNA reaction previous to PCR amplification.

located in the domain 1 of the intron are putatively involved in the splicing reaction. The mutations consist in introducing a T residue instead of C on the gene (pre-edited). The efficiency of splicing was evaluated by scanning the fluorescence of SYBR®Safe staining and normalized according to the size of the precursor and mature products. Under the test conditions used, ~50% of the *rps10* transcripts were spliced (Figure 2A), a value consistently found in different electroporation experiments. When the transcripts contained a T residue at either individual C2 and C3, or when both mutations were present in the same transcript, no significant changes in splicing efficiency were found (Figure 2A). We extended these observations to a full edited transcript by changing the five editing sites into Ts. The edited version of *rps10* mRNA was correctly spliced, with the same efficiency than the wild-type mRNA (Figure 2B).

Excision of the *rps10* intron involves an alternative EBS2 region

The domain I (D1) of *rps10* intron presents a region of five nucleotides, EBS2, complementary to the exonic sequence IBS2. The editing status of C2 determines whether either a G-C or a G-U base pair occurs during the EBS2/IBS2 interaction characteristic of the canonical tertiary structure of group II introns (12). A second potential EBS2 region, called EBS2′, can be detected 65 nt upstream of EBS2 (Figure 3A). The IBS2–EBS2′ interaction should be unaffected by RNA editing since the C2 residue is not directly involved in the putative IBS2–EBS2′ base pairing (Figure 3B). To assess the role of EBS2′ in the splicing reaction, mutants impairing the IBS2/EBS2 and the potential IBS2/EBS2′ interactions or both were constructed (Figure 4A). EBS2 mutant showed only 30% of

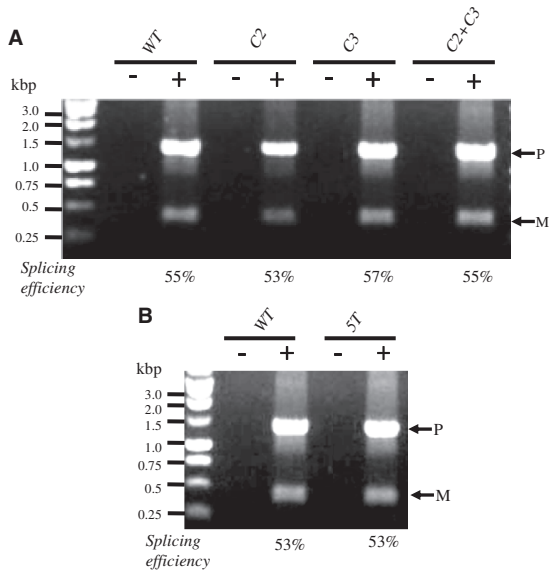

Figure 2. Splicing of C2 and C3 *rps10* mutant transcripts in electroporated potato mitochondria. (**A**) Mutants C2 and C3 were obtained by changing the respective cytosine by a T residue; C2+C3 represents a double mutant. (**B**) 5T corresponds to a fully edited construct with all five editing sites changed into Ts. The pRPS10St wild-type construct and the respective mutants were introduced into potato mitochondria and analyzed as described in Figure 1. WT, wild-type control construct; P, precursor mRNA; M, spliced form. Symbols (–) and (+) indicate either the absence or the presence of reverse transcriptase in the cDNA reaction previous to PCR amplification.

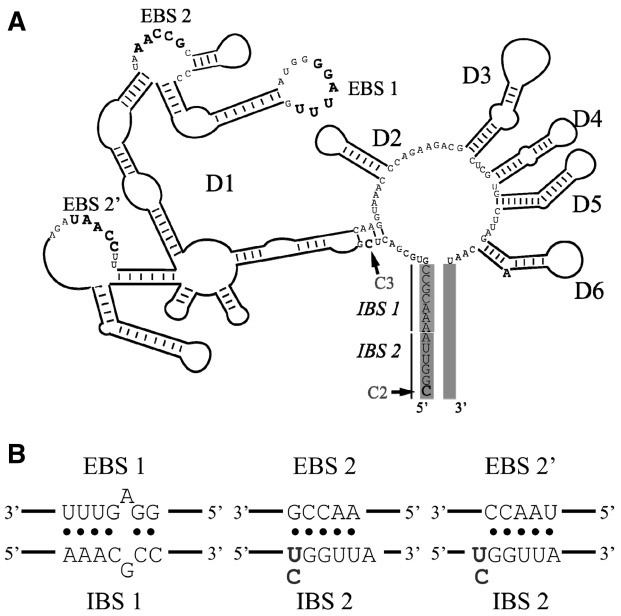

Figure 3. Schematic representation of the secondary structure of the *rps10* intron (**A**) and of the EBS/IBS interactions (**B**). Nucleotide sequence of EBS1, EBS2 and putative EBS2′ regions are indicated in bold. Exon 1 sequences forming the IBS1 and IBS2 regions are indicated by gray boxes. Editing sites C2 and C3 are signaled by arrows. The implication of C2 editing site (red) in different IBS/EBS interactions is indicated.

spliced products compared to 54% in the wild-type construct; no significant difference with wild type *rps10* was found for EBS2′ mutants (Figure 4B). However, when both EBS2 and EBS2′ mutations were combined, the splicing was completely abolished (Figure 4B, lane E2 + E2′). To verify the effect of the C-to-U change at editing site C2 (IBS2) in the EBS2′ mutant, the corresponding C residue was pre-edited (C2T). No difference in splicing efficiency was observed compared to unmodified C2 control mutant (Figure 4B). These results indicate that the editing of C2 is not required for *rps10* mRNA splicing.

The editing site C3 is essential for splicing

C3, present on the intronic region, is predicted to participate in the correct stem formation at the base of domain I (D1) of the *rps10* intron (16) (Figure 3A). C-to-U change by RNA editing of C3 residue corrects the A:C mispair to a canonical A:U base pair and consequently generates a more stable stem at the beginning of D1. To check the hypothesis that editing of C3 facilitates the excision of the intron, this residue was changed into an A (C3A). Transcripts from this mutant were unable to undergo splicing in electroporated potato mitochondria (Figure 5). To validate this observation, the A residue, supposed to interact with the edited C3 residue in the D1 stem, was changed into a T (A3T). Once again, splicing of *rps10* mutant was not detected. Interestingly, a normal level of splicing was restored when the correct

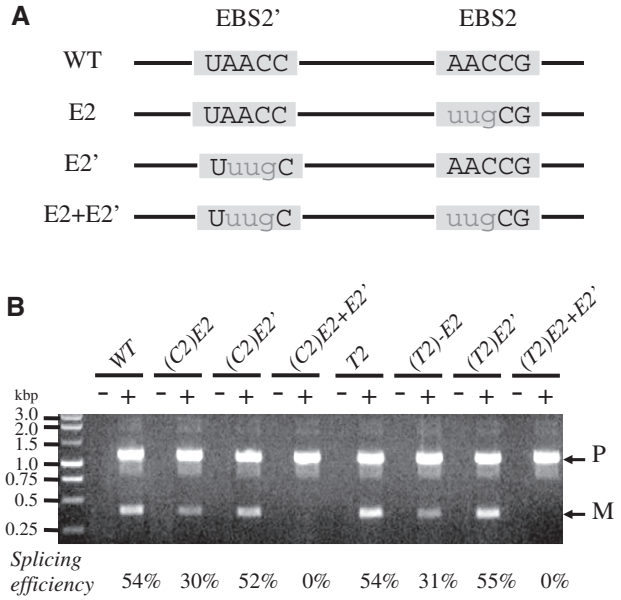

Figure 4. Expression of mutants with impaired EBS2/IBS2 interactions. (**A**) Scheme of the mutants EBS2 (E2), EBS2′ (E2′) and EBS2 combined with EBS2′ (E2 + E2′) in domain I of the *rps10* intron. Modified residues are indicated by lowercase letters (**B**) Analysis of the splicing efficiency of *rps10* and the different EBS2 and EBS2′ mutants indicated in (A). Symbols (–) and (+) indicate the absence or the presence of reverse transcriptase in the cDNA reaction. (C2) signals the construct with a cytosine residue at site C2 and (T2) the mutants with this residue in the pre-edited form. WT, wild type control construct.

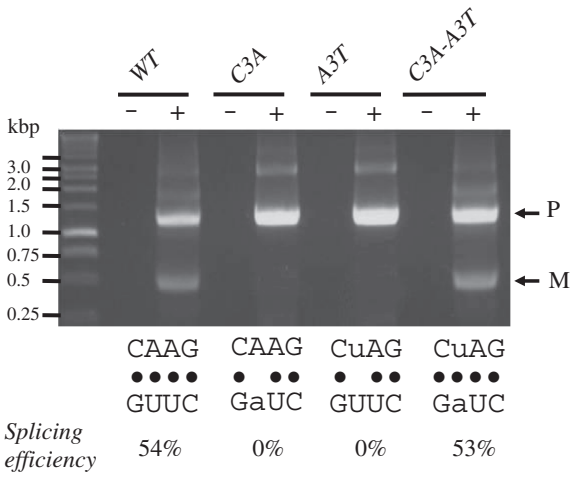

Figure 5. Splicing of *rps10* C3 mRNA mutants. Agarose gel electrophoresis of RT–PCR products of wild type (WT) *rps10* and their C3 mutants. In the C3A mutant, the C3 residue was changed into A. In the A3T mutant, the A residue interacting with C3 was replaced by a T residue. The C3A-A3T double mutant combines both mutations and ensures a U:A base pairing in the D1 stem. The base pairing of the four nucleotides at the base of D1 in the *rps10* intron in the WT and the mutants are shown. The splicing efficiency was determined as indicated in 'Materials and Methods' section. WT, wild-type control construct; P, precursor mRNA; M, mature spliced form. Base changes are indicated in lowercase letters.

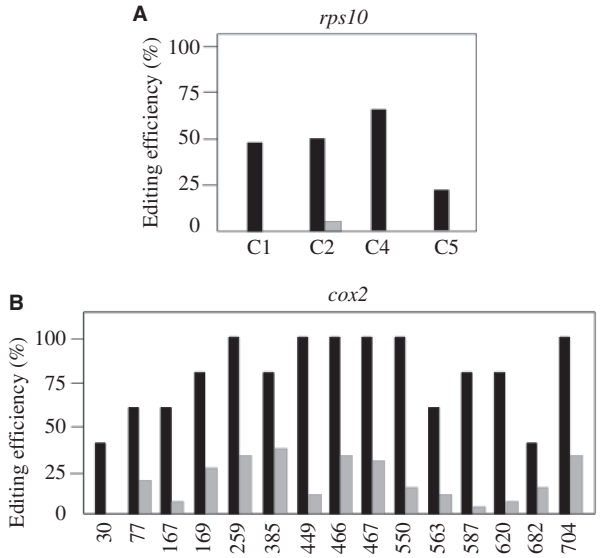

Figure 6. Editing of the intronless potato *rps10* (**A**) and wheat *cox 2* (**B**) mRNAs in cognate mitochondria. Editing status of residues involved in editing was assessed by sequencing of at least 16 clones for each experiment. The results represented are the average of three independent experiments for each construct. Black bars represent the editing status of mature transcripts from wild-type constructs. Gray bars represent the editing status of the transcripts from intronless genes.

base pairing was allowed by combining both mutations in the construct (Figure 5, lane C3A-A3T).

The efficiency of editing is reduced in intronless transcripts

To explore the effect of the splicing process on RNA editing events, constructs containing *rps10* and *cox2* genes devoid of their introns (Figure 1) were used in electroporation experiments. Spliced transcripts from wild-type *rps10* vector presented one or several editing residues unchanged (Figure 6A), henceforth designated as partially edited transcripts. While C4 was found edited in 65% of the mature transcripts, C1 and C2 were found edited only in 50% of mature transcripts and C5 was edited in 25% of spliced mRNAs. Strikingly, the intronless *rps10* transcript presented a dramatic decrease in the yield of RNA editing on the different sites (Figure 6A). Editing of sites C1, C4 and C5 could not be detected in the clones sequenced, whereas only two out of 16 transcripts presented the C2 residue edited (Figure 6A). To verify whether this situation is particular to *rps10* mRNA or a more general phenomenon, we use the intronless wheat *cox2* expressed in cognate mitochondria (23). Sequence analysis of 16 individual cDNA clones showed a dramatic reduction in the efficiency of the C-to-U conversion for all 15 editing sites (Figure 6B). Similar results were obtained in three separate electroporation experiments for each construct.

DISCUSSION

In plant mitochondria, almost all mRNAs undergo RNA editing and some of them are also subject to splicing (5,9,24). *Arabidopsis* mitochondrial transcriptome presents eight transcripts needing both editing and splicing. Most RNA editing events concern the production of functional proteins since they affect coding regions. In fact, they occur mainly at significant positions, i.e. the first and second residue of the codon (5,25). However, several reports describe C-to-U editing occurring in intronic regions and potentially affecting splicing. These events concern strategic positions in the putative secondary structures or tertiary interactions of introns.

Based on these observations, it has been proposed that RNA editing might be required for RNA splicing (5,16,26–29). For example, C-to-U editing in the domain 6 (D6) of *nad1* and *nad5 trans*-introns from wheat mitochondria is needed to obtain the canonical D6 stem–loop structure required for the first transesterification step during splicing (14). Similarly in *Arabidopsis*, different editing events were predicted to improve the secondary structure of the *nad5* a/b and *nad 7* b/c introns (5). In *Oenothera*, editing occurs in the basal stem of the domain 1 (D1) of the second intron of the *nad2* gene, at an analogous position to the *rps10* gene described in this study (26). However, the study of the editing status of these particular editing sites in precursor molecules is not informative enough to conclude on a direct role of editing in the splicing process. Previously, one indirect evidence of the implication of RNA editing in the splicing process has been reported by Börner *et al.* (13). In this work, the edited or the unedited version of *nad1* c/d intron D6 from *Oenothera* mitochondria was grafted into a yeast self-splicing intron. Only the chimera containing the edited form of D6 was able to undergo self-splicing.

Although both editing and splicing events are essential for the production of functional proteins, no clear understanding of how transcript maturation proceeds is available. In this work, we studied the eventual link between RNA editing and splicing using an *in organello* model to introduce engineered DNA constructs into mitochondria (20). Using this approach, we have determined the *cis*-acting regions required for RNA editing in plant mitochondria (19,30), and the functional elements necessary for splicing of the *cox2* intron (23). Previously, we observed that when constructs containing the *S. tuberosum rps10* gene, under the control of *cox2* promoter, were electroporated into potato tuber mitochondria, the transgene was correctly transcribed, spliced and edited (18). Sequence analysis of spliced mRNAs showed the presence of partially edited molecules, indicating that full RNA editing is not a prerequisite for splicing. Of particular interest is the site C2 which was found edited in only 50% of mature transcripts (Figure 6A). This result is in agreement with observations made with *cox2* constructs in wheat mitochondria (23).

Among the five cytosine residues edited in *rps10* mRNA, two of them, C2 and C3 have been proposed to participate in secondary and tertiary interactions characteristic of group II introns (16,31). C2 participates in stabilizing the tertiary structure of the intron, linking the IBS2 region of exon 1 with EBS2 sequence through Watson–Crick interactions. C3 is found mispaired with an A residue in the stem connecting D1 with the central wheel (Figure 3A). The unedited C2 can interact with the G residue of the 3′ end of EBS2. When edited, C2 can form a G:U wobble base pair (Figure 3B), a type of interaction often observed in RNA secondary structure (32). Mutant transcripts in which the C2 residue was replaced by U gave a similar result compared to the wild type (Figure 2A). In addition, when a completely edited transgene was used to electroporate mitochondria, no difference in splicing efficiency was found (Figure 2B). These results indicate that editing in the *rps10* coding region is not involved in splicing, and explain why partially edited transcripts can be found amid spliced molecules.

An alternative IBS2–EBS2′ interaction act in concert with the canonical IBS2–EBS2

In previous work, we found that EBS2 region was not acting alone in the splicing reaction, but that a second upstream region, designated as EBS2′, participates in the intron removal in wheat mitochondrial *cox2* mRNA (23). Bioinformatic analysis revealed that a potential EBS2′ sequence also exists in the D1 domain of the *rps10* intron (Figure 3A). It should be noted that the C2 editing site is excluded from the putative IBS2:EBS2′ interaction (Figure 3B). To verify the function of EBS regions, three internal residues were changed in EBS2 (Figure 4A). A mutant which cannot sustain base pairing with IBS2 did not abolish, but reduced significantly the splicing efficiency, compared to the wild-type transcript (Figure 4B). Oppositely, when the EBS2′ region was mutated, no changes in splicing efficiency were observed. Only when both EBS2 and EBS2′ mutations were introduced in the same *rps10* construct, splicing was completely inhibited (Figure 4B). These results suggest that while EBS2 and EBS2′ do not play a crucial role individually, they probably act concertedly to facilitate the splicing reaction. However, we cannot exclude the possibility that EBS2′ plays other role in the complex interactions responsible for the three-dimensional folding of group II introns (33). This observation, which confirms previous results with *cox2* in a different plant model (23), clearly suggests a general role for the EBS2′ motif in intron excision of group II introns from plant mitochondria.

Since modifications in the vicinity of the target C residue may affect the editing reaction (30), the same experiments were performed with constructs having C2 replaced by a T residue. Identical results were obtained with the edited (T2) version of the transgene (Figure 4B). These results confirm that editing of C2 is not connected with the splicing reaction, and is consistent with the fact that unedited intermediates can be found in spliced mRNAs (Figure 6A). It was proposed that exon fusion after splicing generated the *cis*-recognition elements for editing (17). This is not the case for *rps10* since C2 can be found edited in precursor mRNA (16). The ability of RNA to sustain a stable G-U wobble base pair and the presence of a second EBS2′ region, which exclude the C2 residue in the IBS2-EBS2′ interaction, may explain why editing of the C2 residue is not a prerequisite for splicing. Most probably, the C-to-U transition of site C2 is connected with translation since C2 editing induces an important change in the encoded amino acid. In fact, the genomic encoded R (CGG) residue is changed into W (UGG) (16). Although we confirmed the role of an optional EBS2′ region, the effect of mutations in EBS sequences from *S. tuberosum* mitochondrial *rps10* was less drastic than those observed for *T. aestivum cox2* (23). Further work will be required to understand the actual role of these domains during the splicing process.

Editing of C3 is a prerequisite for splicing of *rps10* mRNA

The basal stem of intron domain I is crucial for the excision of the first intron of *cob* mRNA from yeast mitochondria; a single mutation impairing this region abolished the splicing reaction *in vivo* and *in vitro* (34, 35). Interestingly, the C3 residue in the *rps10* intron is found in an equivalent position to those described for the yeast intron (34). In the canonical secondary structure of the *rps10* intron, C3 is opposed to an A residue (Figure 3A). Editing of the C3 residue should restore the A:U base pairing allowing the formation of a more stable stem and facilitate the splicing reaction. This hypothesis was verified by changing C3 by an A, or by changing the opposite A residue by a T. Both mutants, unable to ensure base pairing, showed a complete inhibition of splicing (Figure 5). However, by combining both mutations, which restores the base pair ability at this position, a complete recovery of the splicing process was observed (Figure 5). From these results, we conclude that the structure of the basal stem of the domain I is essential for intron splicing and that RNA editing of C3 residue is

crucial to ensure the correct secondary structure required for mRNA splicing. This mechanism may also operate for other genes in different plant models since a similar editing site has been described in a *trans*-splicing intervening sequence (26). To our knowledge, this is the first experimental evidence that RNA editing of a C residue is mandatory for the splicing of a mitochondrial mRNA precursor in conditions closer to the *in vivo* situation.

Post-transcriptional processing events may be coupled in plant mitochondria

Taken together, these observations indicate that RNA editing constitutes a general correction mechanism devoted to the proper functioning of different steps of gene expression in plant mitochondria. If this is true, effects on RNA editing would be expected when other RNA processing events are modified. In previous work, we found that a potato *rps10* mRNA expressed in wheat mitochondria remained unspliced and concomitantly, an editing site from wheat grafted in the chimeric transgene was not recognized by the cognate organelle (18). We speculated that this behavior could reflect a complex system conveying the primary transcript to processing, i.e. that RNA editing occurs mainly when transcripts are engaged in post-transcriptional processing. To get further insight about this issue, we analyzed the fate of editing sites in an intronless *rps10* construct, which obviously does not require the splicing step. Surprisingly, a significant reduction in editing efficiency was observed for all four editing sites (Figure 6A), suggesting that in some way the different maturation processes are potentiating each other.

The inhibition of RNA editing in transcripts devoid of intron seems to be a more general phenomenon, since a similar situation was observed when an intronless *cox2* construct was expressed in wheat mitochondria. The *cox2* mRNA undergoes 15 C-to-U editing changes in the translated region when the transgene is expressed in isolated mitochondria. The editing efficiency for individual sites is in the range between 40% and 100%. However, the intronless *cox2* mRNA was severely affected in editing efficiency for all the editing sites (Figure 6B).

The fact that *cis*-recognition elements for RNA editing reside in the vicinity of the target C (19), and the observation that heterologous editing sites can be recognized when grafted in chimeric genes outside their natural context (18,36), suggest that this situation was probably not connected with structural modifications generated by the lack of the intron. It should also be noted that some sites are found edited in precursors (16) and, conversely, partially edited and unedited mature transcripts can be found in spliced mRNAs. We interpret these results as a cooperation of both, splicing and editing mechanisms. Obviously, this situation does not apply to transcripts from genes naturally devoid of introns; in that case, RNA editing proceeds efficiently. However, other maturation processes such as RNA trimming seem also to affect RNA editing of mRNAs and tRNAs (37,38). The possibility to introduce *in vitro* transcribed RNA into mitochondria (39) may help to answer some of the questions raised in this study, under conditions independent of the gene expression pathway.

Interestingly, the lack of maturation (trimming) of *atp6* mRNAs, in a *Rf-1* rice mutant with a cytoplasmic male sterile (CMS) phenotype, correlates with the presence of unedited and partially edited RNAs, indicating that the presence of the *Rf-1* gene influences the RNA maturation and editing of the *B-atp6* RNA (37). Nuclear suppressors of the sterile phenotype, *Rf-1a* and *Rf-1b*, have been identified as members of the Pentatricopeptide Repeat Protein (PPR) family (40). This type of proteins has been implicated in several steps of gene expression in plant organelles (41,42). The PPR protein OTP43 is important for *trans*-splicing of the first *nad1* intron in *Arabidopsis* mitochondria (43). Moreover, some proteins belonging to a particular PPR subfamily have been identified as potential *trans*-acting factors for mitochondrial RNA editing (44–50). According to the picture emerging from the present study, one can imagine that some protein factors, for example PPR proteins, act as chaperones ensuring the engagement of transcripts into the maturation process. Thus, the intron removal of *rps10*, a naturally interrupted gene, may result in impaired recognition of the transcript by processing proteins, leading to a global maturation defect, and therefore alter the editing process.

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