

Review

Cis- and trans-splicing of group II introns in plant mitochondria

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Abstract

Group II-type introns in the mitochondrial genes of flowering plants belong to the ribozymic, mobile retroelement family, but not all exhibit conventional structural features and some follow unusual splicing pathways. Moreover, several introns have been disrupted by DNA rearrangements, so that separately-transcribed precursors undergo splicing *in trans*. RNA processing in plant mitochondria has the added complexity of C-to-U RNA editing which also sometimes occurs within core intron structures or at exon sites very close to introns. It appears that mitochondrial introns in flowering plants have followed quite different evolutionary pathways than other group II introns.

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1. Introduction

The mitochondrial genes of plants, protists and fungi contain varying numbers of introns, all of which have been categorized as members of the group I or group II families of mobile genetic elements (reviewed in Michel and Ferat, 1995; Bonen and Vogel, 2001; Lehmann and Schmidt, 2003; Lambowitz and Zimmerly, 2004). These two intron classes have distinctive structures, and some members encode their own protein machinery for splicing and/or mobility functions *in vivo*. In particular, group II introns spread by retrohoming and retrotransposition, processes which exploit intron-encoded reverse transcriptase/endonuclease activities. Over recent years, considerable insight has been gained about both group II intron self-splicing *in vitro* and mechanisms of intron mobility, with the genetic manipulation of those found in bacteria assisting in that undertaking (reviewed in Lambowitz and Zimmerly, 2004; Toro et al., 2007). In addition, the sequencing of mitochondrial genomes from fungi, protists, algae and land plants (which are particularly rich in group II introns), as

well as those of plastids, has enhanced the power of comparative analysis. In contrast, there have been only a few cases of introns identified in any mitochondrial genes in animals, and all from early diverging metazoan lineages such as corals, sponges and sea anemones (cf. Fukami et al., 2007). These are group I introns which appear to have arisen from fungal sources through recent horizontal transfer into specific homing sites. It is worth noting that some of the introns found in *Euglena* chloroplast genes were categorized as group III, but appear to be group II introns with extremely degenerate structure (reviewed in Bonen and Vogel, 2001; Lambowitz and Zimmerly, 2004). In addition, in both *Euglena* chloroplast and archaeobacteria, examples of “twintrons” that is, the nesting of one intron within another intron, have been identified (reviewed in Lambowitz and Zimmerly, 2004).

Over recent years, interest in mitochondrial introns and splicing has extended beyond solely understanding fundamental RNA level expression events, in part because of potential exploitation of their autocatalytic properties (e.g. in gene therapy, reviewed in Lambowitz and Zimmerly, 2004; Toro et al., 2007) and partly because they are providing new insights into the evolution of catalytic RNAs and the origin of introns. For example, the splicing mech-

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anism of group II introns (namely, two transesterification steps with the intron being released as a lariat) resembles that of nuclear spliceosomal introns and it has been proposed that these two classes of introns share a common evolutionary origin (reviewed in Jacquier, 1990; Valadkhan, 2005). Moreover, it has been suggested that spliceosomal introns may have originated by the introduction and spread of group II introns from the α -proteobacterial-type endosymbiont which gave rise to the mitochondrion during formation of the modern eukaryotic cell (cf. Cavalier-Smith, 1991; Martin and Koonin, 2006).

There are a number of recent reviews highlighting the features and behaviour of group I (cf. Haugen et al., 2005; Stahley and Strobel, 2006; Lang et al., 2007) and group II introns (cf. Lambowitz and Zimmerly, 2004; Pyle et al., 2007), so that this present account will focus specifically on the mitochondrial group II introns of flowering plants, their structural features, splicing and evolution. At the onset, it is worth noting that even though they possess some identifiable group II features, none of the mitochondrial introns in plants have been demonstrated to self-splice *in vitro*. A secondary structure model with the classical six helical domains (designated as dI–dVI extending from a central hub) is shown in Fig. 1 for intron 1 of the cytochrome oxidase subunit 2 (*cox2*) gene in plants. Sequence analysis has established however that many other flowering plant mitochondrial introns (see below) lack certain of the characteristic group II features found in bacteria, archaeal, plastids, fungal/protist/algal mitochondria, or even in non-vascular plants. Plant mitochondria are also distinctive in that the highly recombinogenic nature of their genomes and ability to readily accept foreign DNA sequences (reviewed in Knoop, 2004; Kubo and Mikami, 2007) can contribute to the disparate evolutionary directions taken by their introns.

2. Distribution and evolutionary history of group II introns in plant mitochondria

Introns have been identified in the mitochondrial genomes of all land plants examined to date, including both vascular and non-vascular plants. The mitochondrial genomes of the liverwort, *Marchantia polymorpha* (Oda et al., 1992) and the moss, *Physcomitrella patens* (Terasawa et al., 2007) contain 25 and 23 group II introns, respectively (as well as 7 and 2 group I ones). Among flowering plants, a total of 25 mitochondrial group II introns have been identified (Table 1), as well as one group I intron in the *cox1* gene in certain plants (Cho et al., 1998). The latter is believed to result from independent horizontal invasions into a specific *cox1* site at various times during evolution. In addition to having distinctive secondary structures, classical group II introns (particularly those in bacteria) possess an intron-encoded ORF showing homology to reverse transcriptase. In flowering plant mitochondria however, there is only one such ORF, namely *mat-r* in the fourth (and final) intron of *nad1*, and in *M. polymorpha*

and *P. patens* mitochondria there are nine and two RT-maturase intronic ORFs, respectively (Table 1).

Based on genomic data from a variety of land plants, it is apparent that the evolutionary history of group II introns is quite complex, and that they are being both gained and lost in a sporadic fashion over evolutionary time. Although the mitochondrial genomes of *Marchantia*, *Physcomitrella* and flowering plants each harbour about 25 group II introns, only eight are shared between *Physcomitrella* and flowering plants, three between *Physcomitrella* and *Marchantia*, and just one between *Marchantia* and flowering plants (namely *nad2* intron 3, which incidentally is absent from *Physcomitrella*). Similarly, studies in which individual introns were analyzed in a broad phylogenetic context have led to the conclusion that many independent intron gain and losses have occurred during land plant evolution (cf. Pruchner et al., 2002; Dombrowska and Qiu, 2004; Groth-Maloney et al., 2005). Even among flowering plants, intron content is seen to vary somewhat (cf. Gass et al., 1992; Kudla et al., 2002 and Fig. 2) and RNA editing data has provided compelling evidence that intron loss can occur through an RNA-mediated process whereby a spliced, edited cDNA copy serves as template with co-conversion of flanking exon sequences (Geiss et al., 1994). In keeping with this model, recent studies have shown the direct involvement of reverse transcriptase *in trans* on such a retro-deletion process in yeast mitochondria (Gargouri, 2005).

Mitochondrial intron length is also seen to vary (sometimes quite markedly) among homologues from different plants and this is typically due to insertion/deletions within dIV (Figs. 1 and 2). The *cox2* intron ranges from 462 nt in the “primitive” angiosperm *Acorus calamus* (sweet flag, Albertazzi et al., 1998) to 2660 nt in the gymnosperm *Ginkgo biloba* (Ahlert et al., 2006), and the latter contains remnants of a RT-maturase ORF within dIV, consistent with the view that as ribozymic group II introns degenerate they lose their intron-encoded splicing/mobility functions. The pattern of length variation among closely-related flowering plants within this *cox2* intron (Fig. 2, shaded blocks) suggests that a longer (ancestral) form has undergone several discrete deletions via recombination across short direct repeats during evolution. Interestingly, there are analogous examples of intron sequences contributing (through homologous recombination across repeats) to rearrangements correlated with cytoplasmic male sterility (e.g. *nad7* truncation in tobacco, Pla et al., 1995) and non-chromosomal stripe mutations (e.g. *nad4–nad7* fusion in maize; Marienfeld and Newton, 1994). The non-core regions of group II introns can also acquire extra sequences (cf. sugarbeet-specific insert in *cox2* intron, Fig. 2, dark grey block) and it has been observed that certain introns are more closely-related than are others (e.g. *nad1* intron 4 and *nad4* intron 1, Haouazine et al., 1993; *rps3* intron 1 and *nad5* intron 1, Laroche and Bousquet, 1999). This is suggestive of relatively recent spread, although gene conversion events might also contribute confounding effects. In this regard, the potential for hybrid introns being created from dis-

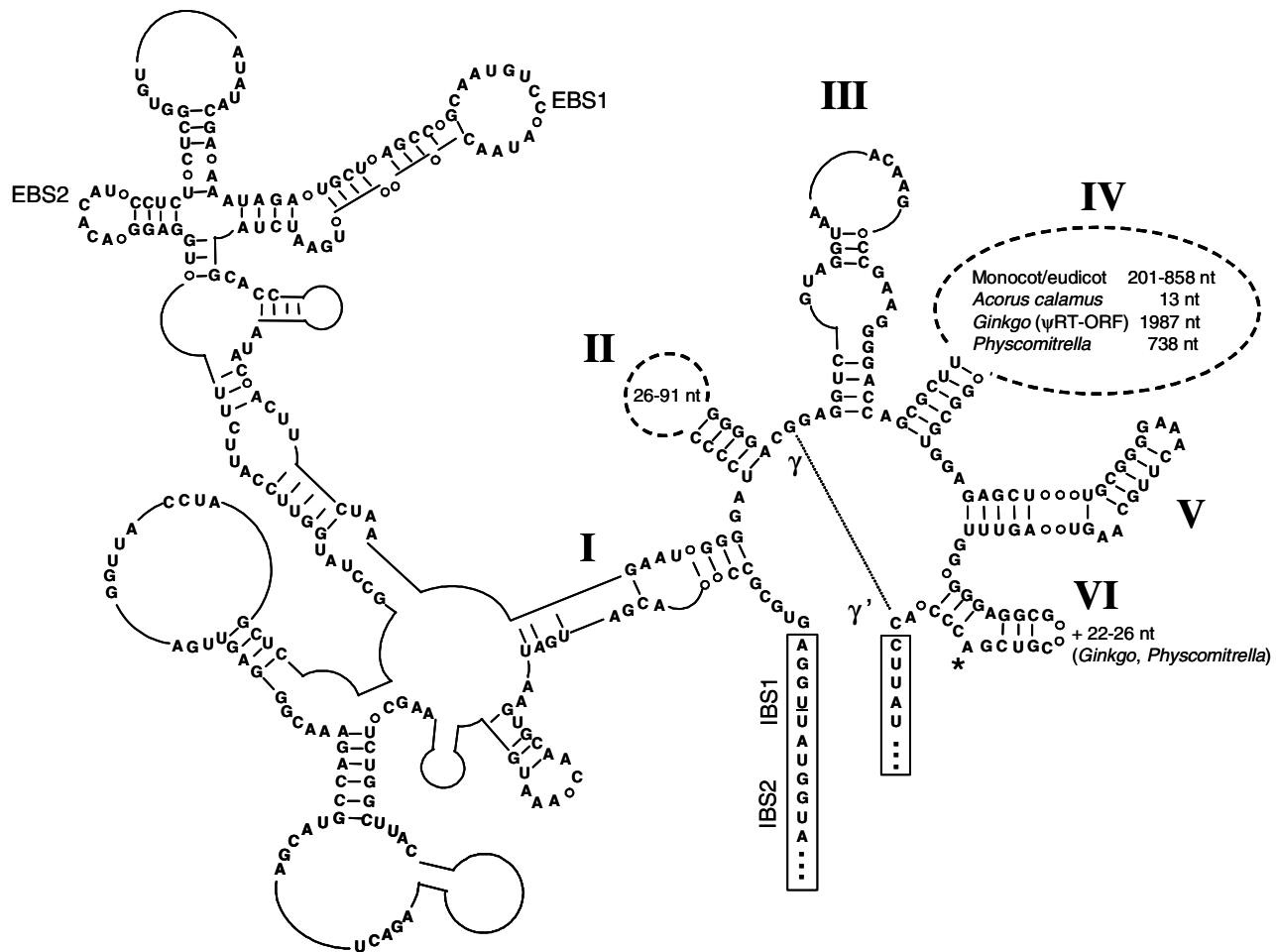


Fig. 1. Group II intron secondary structure model as illustrated by plant *cox2* intron 1. The six helical domains (I–VI) are shown extending from a central hub, according to the group IIA model of Michel and Ferat (1995). Nucleotide sequences are shown for core regions in which stretches of at least four nucleotides are identical among representative land plants, namely the four monocots wheat (AP008982), rice (BA000029), maize (AY506529) and sorghum (DQ984518); the four eudicots tobacco (BA000042), sugar beet (BA000009) carrot (X63625) and petunia (X17394), a gymnosperm, *Ginkgo biloba* (AJ874265), and a moss, *Physcomitrella patens* (NC_007945). Although the *cox2* intron of the “primitive” angiosperm *Acorus calamus* was omitted from this comparative analysis because of its exceptional divergence and short length (Albertazzi et al., 1998), the sizes of its dII and dIV loops have been included. The longest and shortest dII loops are for *Ginkgo biloba* and *Acorus calamus*, respectively. EBS (and IBS) indicate exon (and intron) binding sites, respectively; and γ (and γ') also illustrate one (of many) tertiary interactions (Bonen and Vogel, 2001). The dVI branchpoint adenosine is shown by an asterisk. Note that in flowering plants, the 34-nt domain V helix contains non-canonical U–C or U–U mismatches (represented by circles) unlike in non-vascular plants. The structural model shown is similar to that presented for *cox2* introns from petunia (Pruitt and Hanson, 1991) and *Triticum timopheevi* (Farré and Araya, 2002). Flanking exon sequences are boxed and a C-to-U edited exon site within IBS1 is underlined. Note that *trans*-splicing introns in flowering plant mitochondria are disrupted within dIV.

tantly-related homologues is worth considering, given that an extra copy of *nad1* intron 2 (and flanking exons) in the mitochondrial genome of the gymnosperm *Gnetum gnemonoides* appears to have been introduced from an angiosperm (asterid) source through horizontal transfer (Won and Renner, 2003). Remarkably, there is also evidence for widespread horizontal gene movement among plants, in some cases leading to the creation of functional, chimeric monocot/eudicot-type genes (Bergthorsson et al., 2003).

3. *Trans*-splicing introns in plant mitochondria

One of the most striking examples of the impact of the dynamic nature of plant mitochondrial genomes is the cre-

ation of *trans*-split genes, that is genes in which rearrangements have occurred within group II introns so that exons (and flanking half-introns) are dispersed around the genome, independently transcribed and the mRNA is generated through splicing *in trans* by re-assembling proper intron folding (reviewed in Bonen, 1993). There are six such documented cases in flowering plant mitochondria, involving the *nad1*, *nad2* and *nad5* genes (Table 1), with disruptions having occurred within dIV in each case. As yet it is unknown how the appropriate intron halves correctly recognize each other, and whether it is mediated by RNA chaperones as well as base-pairing within the dIV helical stalk (and other higher-order RNA interactions). In *Oenothera*, *nad5* intron 3 has been further fractured into three

Table 1
Distribution of group II introns in mitochondrial genes of selected land plants

		<i>Arabidopsis</i>	Rice	<i>Physcomitrella</i>	<i>Marchantia</i>
<i>nad1</i>	i1	+ (<i>trans</i>)	+ (<i>trans</i>)		
	i2	+	+		
	i3	+ (<i>trans</i>)	+ (<i>trans</i>)		
	i4	+ (ORF)	+ (<i>trans</i> , ORF)	+	
<i>nad2</i>	i1	+	+	1	
	i2	+	+	+	
	i3	+ (<i>trans</i>)	+ (<i>trans</i>)		+
	i4	+	+		
<i>nad3</i>					1
<i>nad4</i>	i1	+	+	+	
	i2*	+	+		
	i3*	+	+		
<i>nad4L</i>				+	1
					+
<i>nad5</i>	i1	+	+	+	
	i2	+ (<i>trans</i>)	+ (<i>trans</i>)	+ (ORF)	
	i3	+ (<i>trans</i>)	+ (<i>trans</i>)		
	i4	+	+		
<i>nad7</i>	i1	+	+	+	
	i2	+	+	+	
	i3	+	+		
	i4*	+	+		
<i>nad9</i>				1	
<i>cox1</i>				+	+ (ORF)
				+ (ORF)	+ (ORF)
<i>cox2</i>	i1*		+	1	1 (ORF)
	i2*	+		+	
<i>cox3</i>				1	2 (1 ORF)
<i>cob</i>				1	2
<i>atp1</i>				1	3 (1 ORF)
<i>atp6</i>				1	2 (2 ORFs)
<i>atp9</i>				3	
<i>sdh3</i>				1	1 (ORF)
<i>cemFc</i>	i1	+	+		
<i>rpl2</i>	i1	+	+		
					1
<i>rps3</i>	i1*	+	+		
<i>rps10</i>	i1*				
<i>rps14</i>					1
<i>trnS</i>					1
18S rRNA					1 (ORF)
26S rRNA					1

Data are shown for the eudicot *Arabidopsis thaliana* (Y08501), the monocot rice (BA000029), the moss *Physcomitrella patens* (NC_007945) and the liverwort *Marchantia polymorpha* (NC_001660). For simplicity, the intron nomenclature is based on the flowering plant mitochondrial literature, rather than the more generalized land plant one introduced by Dombrowska and Qiu (2004). Introns shared by two (or more) lineages are shown by (+) symbols, and additional group II introns present in *Physcomitrella patens* or *Marchantia polymorpha* are simply indicated by numbers. Introns with an RT-maturase ORF are indicated by (ORF). *Trans*-splicing introns in flowering plants are shown by (*trans*), whereas all group II introns in *Physcomitrella patens* and *Marchantia polymorpha* mitochondria are of the *cis*-splicing type. Asterisks indicate introns present in only certain flowering plants (cf. presence of *rps10* intron in plants such as tobacco (BA000042) and potato (Zanlungo et al., 1995), but whole gene is absent in plants such as *Arabidopsis* and rice). Note that an additional *rps3* intron has been identified in the gymnosperm *Cycas* (Regina et al., 2005) and that *nad7* is a pseudogene in *Marchantia polymorpha* mitochondria. The *Physcomitrella patens* intron data was taken from the databank genome sequence (NC_007945) which differs slightly from Terasawa et al. (2007), for example in the number and positions of *cox2* introns.

segments, with an additional break within domain I (Knoop et al., 1997). Only a few other cases of naturally-occurring *trans*-split group II introns have been identified

as yet: two mitochondrial *nad3* introns in the green alga *Mesostigma viridae* (Turmel et al., 2002), one *rps12* intron in land plant chloroplast, and two *psaA* *trans*-introns in

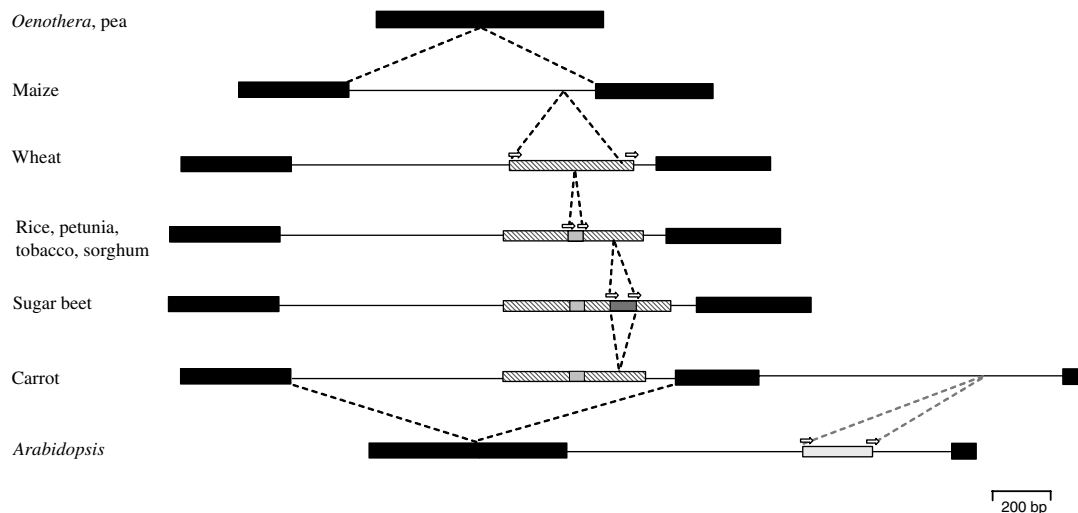


Fig. 2. Schematic of the mitochondrial *cox2* gene illustrating variation in intron content and length among selected flowering plants. Exons are shown by black blocks and introns by lines or by hatched, light grey and dark grey blocks within dIV to depict lineage-specific insertion/deletions. Note that carrot, sugar beet, petunia and tobacco have several additional short insertions relative to rice and sorghum. Arrows show positions of short direct repeats. Accession numbers are as given in Fig. 1 and *Arabidopsis* (Y08501), *Oenothera* (X00212), pea (X02433), petunia (X17395), sorghum (DQ984518), sugar beet (BA000009) and carrot (X63625).

Chlamydomonas reinhardtii chloroplast (cf. Perron et al., 2004). One of the latter is a tripartite intron, with a short separate *tscA* RNA comprising part of domains I–IV. Recently, an elegant genetic assay to generate and monitor *trans*-splicing behavior *in vivo* in bacteria has been developed and it should provide valuable new insights (Belhoucine et al., 2007).

To estimate the timing of conversion of *cis*-splicing introns to *trans*-splicing forms during evolution, homologous genes from divergent land plants (such as ferns, hornworts and mosses) have been examined. The disruption of *nad1* intron 1 appears to be the earliest such event, occurring in the ancestor of seed plants and ferns (Malek and Knoop, 1998), whereas on the other extreme, fission of *nad1* intron 4 has happened recently and multiple times within the flowering plant lineage (Qiu and Palmer, 2004). The break within this intron has sometimes occurred upstream of the *mat-r* ORF and in other cases downstream of it. For example in cereals, the former type of *trans*-configuration appears to have been generated by homologous recombination across a short purine-rich stretch located upstream of *mat-r* (Chapdelaine and Bonen, 1991).

The fragmented and dispersed nature of plant mitochondrial coding segments (and flanking intron pieces) resulting from mitochondrial DNA rearrangements raises questions about their expression. Some appear to recruit new promoters from spacer regions for transcription, and in other cases they hitchhike along with pre-existing transcriptional units. Co-transcripts sometimes even contain exons from different genes, an example being *nad1* exon 5 and *nad5* exon 3, which are just 372 bp apart in the wheat genome (Pereira de Souza et al., 1991; Farré and Araya, 1999). The *nad5* gene has five exons, with the third exon (which is only 22 nt long) being flanked by *trans*-splicing

introns, and maturation of its mRNA is particularly interesting. Recently detailed analysis of *nad5* splicing pathways in several plants has revealed the presence of incorrectly spliced products that appear to result from the activation of cryptic 5' splice sites within exon 2 due to mis-folding of precursors (G.G. Brown, personal communication). This suggests that for functional *nad5* mRNA to be produced a particular order of intron excision is needed.

4. Intron structure and splicing in flowering plant mitochondria

Because correct intron folding is crucial for ribozymic group II splicing, it might be expected that core structures of plant mitochondrial introns would similarly be under strong functional constraint. In particular, high conservation might be predicted near the 3' end of introns, since dV is part of the catalytic core and dVI provides the branchpoint adenosine for the first transesterification step. Although this is the case for some introns, it is not seen for all (Bonen and Vogel, 2001; discussed in Hausner et al., 2005). For example, the *trans*-splicing *nad1* intron 1 has a poorly structured dVI without a bulged nucleophilic adenosine, an aberrantly large dV loop, and varies in sequence and editing status among closely-related plants (Carrillo et al., 2001). A second example is *nad1* intron 2 which has a very short, strongly base-paired dVI helix (Li-Pook-Than and Bonen, 2006) and a third unusual one, namely *nad4* intron 2, is depicted in Fig. 3a. It lacks a bulged adenosine at the expected position (7–8 nucleotides from the 3' end of the intron) and varies in sequence, length and predicted dVI structure among plants. Interestingly, in the green alga *Chara vulgaris* (which represents the lineage believed to be most closely-related to land plants, Turmel

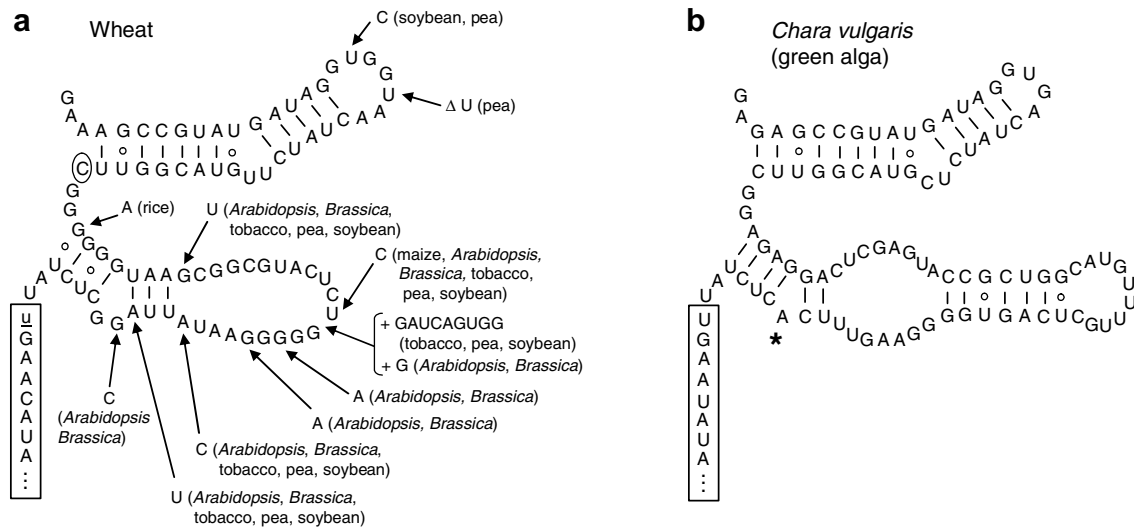


Fig. 3. Secondary structure models of the domain V/VI region for *nad4* intron 2 in (a) wheat and (b) its homologue in the green alga *Chara vulgaris*. (a) Nucleotide differences relative to wheat (AP008982) are indicated by arrows for rice (BA000029), maize (AY506529), *Arabidopsis thaliana* (Y08501), *Brassica napus* (AP006444), tobacco (BA000042), and for pea and soybean (unpublished data, C. Carrillo, N. Niknejad and L. Bonen). The circled C is an A–C mispair editing candidate which remains unedited in wheat (C.C. N.N. and L.B. unpublished data). (b) The dVI branchpoint adenosine in *Chara vulgaris* (NC_005255) is shown by an asterisk. The C-to-U edit seen at position +1 of the downstream exon in flowering plants (underlined u within box in a) corresponds to a genomically-encoded T in *Chara vulgaris*. Note that dVI of *nad4* intron 2 is markedly more divergent than that of *cox2* (which has an identical dVI among all eight flowering plants shown in Fig. 1).

et al., 2003) the homologous *nad4* intron conforms to classical group II features (Fig. 3b).

The core helices of mitochondrial introns in flowering plants sometimes contain non-canonical base-pairs, and those that are A–C can be corrected to A–U pairs by RNA editing (cf. Wissinger et al., 1991; Lippok et al., 1994; Zanlungo et al., 1995; Carrillo and Bonen, 1997; Farré and Araya, 1999). Curiously though, editing is not always observed at predicted positions (cf. Carrillo et al., 2001 and Fig. 3, circled) and similar observations have been made for barley chloroplast group II introns (Vogel and Börner, 2002). On the other hand, for a chimeric yeast mitochondrial (ribozymic) intron containing dVI of *nad1* intron 3 from *Oenothera* mitochondria, self-splicing *in vitro* was observed only in an edited (A–U basepair) form (Börner et al., 1995). In addition to intron editing, some exon editing sites are located very close to intron junctions, and this raises the question as to whether splicing precedes editing, or vice versa, or whether there is no strict order of processing at such sites. Editing is usually an early step in RNA maturation, as evidenced by editing of unspliced (or partially spliced) precursors (cf. Sutton et al., 1991; Yang and Mulligan, 1991), however some exon sites located either immediately upstream or downstream of an intron remain unedited in precursors, unlike more distal sites (cf. Li-Pook-Than et al., 2007; see Fig. 3 underlined). This suggests that an editing recognition *cis*-element is created by exon–exon ligation or alternatively, perhaps steric hindrance limits access of editing machinery to sites near intron junctions (cf. EBS–IBS and γ – γ' interactions in group II introns, Fig. 1). The importance of the EBS–IBS base-pairing interaction in wheat *cox2* splicing is sup-

ported by site-directed mutational analysis using an *in organello* expression system (Farré and Araya, 2002). Interestingly, growth at low temperature has been seen to suppress editing of the IBS1 site of *cox2* exon 1 (concomitant with elevated levels of unspliced precursors) in wheat (Kurihara-Yonemoto and Handa, 2001; Fig. 1 underlined). In genes with multiple *cis*-splicing introns, it also appears that splicing efficiency may vary among introns (cf. wheat *nad7* sluggish intron 2; Li-Pook-Than et al., 2004).

Ribozymic group II introns are excised as lariats (with a tail of 6–7 nt), and while some plant mitochondrial introns follow this two-step transesterification splicing pathway, others do not (Li-Pook-Than and Bonen, 2006). For example, the wheat *nad1* intron 2 (with a short tight dVI helix lacking a bulged adenosine) was found to have several discrete physical forms, including full-length circularized molecules as well as ones with an extra non-encoded 7 nt block, but no conventional lariats. Surprisingly, the wheat *cox2* excised intron exhibited yet other structures, with linear polyadenylated molecules as well as heterogeneous 3' truncated circular forms being observed. In contrast, among 17 barley chloroplast group II introns, all were seen to be excised as classical lariats except the *trnV* intron (which lacks a bulged adenosine in dVI) and it showed a linear form consistent with a hydrolytic pathway (Vogel and Börner, 2002). As yet there are just a few other reports of non-lariat-type group II splicing *in vivo*, for example in bacteria (cf. Granlund et al., 2001; Molina-Sanchez et al., 2006) or in mutant yeast strains (Murray et al., 2001). It is notable that the abundance of excised intron RNAs shifts during embryo-to-seedling development (cf. Carrillo and Bonen,

1997) and this may reflect different splicing pathways, or perhaps differences in the availability of intron turnover machinery (cf. analogous to lariat debranching enzyme in the nucleus).

Thus it appears that mitochondrial introns in flowering plants have become degenerate in structure and that they exploit novel (and diverse) splicing mechanisms with an apparent shift away from group II-type ribozymic splicing biochemistry to hydrolytic (and other) pathways. It will be interesting to learn if RNA binding proteins (and/or small RNAs) compensate for weakened RNA helices. In this regard, it would parallel a model for the origin of nuclear pre-mRNA introns from group II introns, whereby RNA structural information was dispersed into small RNAs, which evolved into the present-day spliceosomal snRNAs (cf. Sharp, 1991).

5. Splicing machinery for group II intron excision in plant mitochondria

At the present time, relatively little is known about the splicing machinery in plant mitochondria, although it is expected to be complex and involve numerous nuclear-encoded factors (some intron-specific and some generalized) based on data for fungal mitochondrial and plant/algal chloroplast group II splicing (reviewed in Lehmann and Schmidt, 2003; Lambowitz and Zimmerly, 2004). Although bacterial ribozymic group II introns typically each encode their own specific RNA maturase for splicing *in vivo*, the mitochondrial genomes of flowering plants encode just one intronic *mat-r* ORF and it lacks an identifiable initiation codon. However, comparative sequence analysis (as well as editing data) strongly supports the view that it is under functional constraint (cf. Thomson et al., 1994). Moreover promoters have been identified both upstream of this intronic ORF as well as within it in wheat (Farré and Araya, 1999). Interestingly, four homologues of *mat-r* have also been identified in the nucleus in *Arabidopsis* and rice (Mohr and Lambowitz, 2003), and recently one of these (namely, nMat1a) has been found to be important for *nad4* splicing from *Arabidopsis* mutational analysis (Nakagawa and Sakurai, 2006). Notably, this correlates with a tobacco mutant that was found to be defective in the splicing of *nad4* intron 1 but not other introns (Brangeon et al., 2000). Since *nad4* intron 1 is quite similar in core sequence to *nad1* intron 4 (cf. Haouazine et al., 1993) and the former lacks an intronic ORF, it is perhaps not surprising that a nuclear-encoded nMat-r be required for its splicing. Recent GFP localization studies and mutational analysis support the view that other nMat-r homologues also play a role in mitochondrial splicing (O. Ostersetzer, personal communication).

Based on recent reports of the involvement of PPR proteins (pentatricopeptide repeat-type RNA binding proteins) in a variety of RNA processing events in plant organelles (reviewed in Andrés et al., 2007; Shikanai, 2006) and the huge size of this nuclear multi-gene family

with over 400 members in each of *Arabidopsis* and rice, it is anticipated that some will be involved in splicing. Indeed a PPR protein has been shown to be essential for maize chloroplast *rps12* trans-splicing (Schmitz-Linneweber et al., 2006) and an *Arabidopsis* PPR mutant defective in mitochondrial splicing has also recently been identified (I.D. Small, personal communication). In addition, RNA binding proteins which perform other roles in organellar RNA metabolism (such as RNA helicases and tRNA maturation enzymes, reviewed in Lehmann and Schmidt, 2003) might be recruited to serve as (multifunctional) splicing factors, based on observations for yeast mitochondrial and plant chloroplast group II splicing. In this regard, an *Arabidopsis* Mg²⁺ ion transporter was found to complement a yeast mitochondrial MRS2 splicing mutant (Schock et al., 2000). Plant mitochondrial splicing factors are predicted in at least some cases to be plant-specific, based on heterologous potato–wheat *in organello* expression experiments (Choury et al., 2005). Studies in maize chloroplast have shown that some machinery is restricted to the splicing of a particular group II introns (cf. Ostersetzer et al., 2005) whereas other factors assist in the excision of multiple ones (cf. Ostheimer et al., 2005). If maturation of the chloroplast *psaA* mRNA in *Chlamydomonas*, which involves two trans-splicing events requiring 14 different nuclear-encoded factors (cf. Perron et al., 2004; Merendino et al., 2006) is any indication, then the splicing machinery in plant mitochondria will be very complex. It may well also have a dynamic nature, with various nuclear-encoded RNA binding proteins being recruited, then later discarded and replaced by other splicing factors over evolutionary time.

6. Future perspectives

Since the time that the first plant mitochondrial intron was identified more than 25 years ago (cf. maize *cox2*; Fox and Leaver, 1981), there has been an impressive accumulation of intron sequence data from a wide variety of land plants. However, it has been challenging to study splicing in plant mitochondria in the absence of *in vitro* self-splicing or plant mitochondrial transformation systems. The development of an *in organello* electroporation system to introduce constructs into isolated mitochondria in wheat (Farré and Araya, 2002), maize (Staudinger and Kempken, 2003) and potato (Choury et al., 2005) provides a powerful tool which is likely to provide further new insights into splicing (and editing) mechanisms. In addition, the characterization of mutants (particularly in *Arabidopsis* and more particularly for PPR gene candidates) offers exciting prospects for elucidating the nature of splicing (and editing) machinery.

The complex picture that is emerging for plant mitochondrial introns and their idiosyncrasies prompts a number of questions. Why do they deviate from the conventional group II structure and how does this impact on splicing? How diverse are the splicing mechanisms and

how complex is the splicing machinery? Do *trans*-splicing introns require extra machinery for correct partner recognition? How are splicing and editing coordinated? Might mitochondrial splicing/editing play a regulatory role during plant development or environmental stress? This notion is prompted by the intriguingly high bias of introns in *nad* genes, along with the presence of alternative respiratory pathways in plants that allow complex I (NADH dehydrogenase) to be by-passed. It is clear that there is much still to be learned about splicing and the behavior of introns in plant mitochondria.

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