

Duplicated Sequence Elements and Their Function in Plant Mitochondria

Bernd Wissinger, Rudolf Hiesel, Werner Schobel, Michael Unseld, Axel Brennicke, and Wolfgang Schuster

Institut für Genbiologische Forschung, Ihnestraße 63, D-W-1000 Berlin 33, Bundesrepublik Deutschland

Z. Naturforsch. **46c**, 709–716 (1991); received July 22, 1991

A considerable portion of the plant mitochondrial DNA is derived from genome internal duplications. Many of these amplified sequences determine functions of transcription and processing. Among these are promoter regions, sequences defining the 3' ends of stable mRNAs, potential RNA processing sites and intron domains. Simultaneously, some of these repeated sequences can be active sites of recombination in plant mitochondria. Such duplicated control regions may simplify coordinate expression of different genes.

Introduction

The mitochondrial genomes of many plants contain various numbers of inverted and direct repeats recombining to different molecules [1, 2]. The recombinational activity of one direct repeat, as found for example in several *Brassica* species, results in three different molecules, two of which contain complementing parts of the master-circle DNA sequences. Less frequent recombination events result in the presence of molecules in substoichiometric copy numbers complicating estimates of genome complexity in plant mitochondria [3, 4]. The recombinationally active repeats occur independently of functional units, sometimes duplicating and/or disrupting coding sequences.

The numerous duplicated and repeated sequences that have been found in plant mitochondrial genomes are in only some instances connected with active recombination, while many other duplications are not involved in recombination processes. Many other functions, however, have been found connected with such sequence redundancies. To date duplicated sequence elements with signals for transcription initiation, transcript processing and 3' termini, translational start sites and intron domains have been identified. This review intends to discuss these functions located on repeated sequences and to show the potential of coordinating regulation in evolution by duplica-

tions and spreading of control elements through the mitochondrial genome of higher plants.

Gene copy number amplification

Some recombination events in plant mitochondrial genomes involve large repeated sequences of several kilobases in length, like the 12 kb repeat in maize [2]. Recombination of repeats containing complete coding sequences leads to different large-scale environments for the duplicated copies of a given gene. Divergent recombination and replication efficiencies lead to divergent copy numbers of individual genes in the mitochondrion [3, 4]. In such events entire cistrons can be duplicated and the potential controlling elements are still connected with their original genes and contexts [5]. Similar duplications of coding regions are found in chloroplast genomes which usually contain a large inverted repeat, active in recombination and encoding the ribosomal RNAs, some tRNAs and protein genes [6, 7].

The rRNA genes in mitochondria are amplified on recombinationally active repeats, for example in the wheat mitochondrial genome [5]. Here we will not further consider the effects of these differential gene amplifications but rather focus on the dissociations of genes and control elements with promoter function, translational initiation sites and processing signals.

Promoter sequences

The putative plant mitochondrial promoter sequence itself is defined as in prokaryotes and all other genetic systems as a conserved sequence ele-

Reprint requests to A. Brennicke.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0900–0709 \$ 01.30/0



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ment. Sequence similarity of short elements, however, is distinct from the duplications considered here, which are identical or nearly identical in their different copies of larger sequence blocks.

The plant mitochondrial promoter sequence seems to be conserved at least in some species, notably in wheat and most dicots [4, 8]. Analysis and definition of a promoter site is most advanced in wheat where both *in vitro* capping of *in vivo* primary transcripts and *in vitro* transcription identified the same sequence element for a specific gene [9]. This sequence element of 6–9 nucleotides is also found at the 5' termini of many dicot transcripts, suggesting these to be derived from transcription initiation [10]. An exception appears to be the maize mitochondrion, where no obvious conserved sequence elements have been identified near the *in vitro* capped termini [11].

A large sequence block containing a putative promoter region in *Oenothera* mitochondria is duplicated and is found upstream of the gene encoding subunit I of the cytochrome oxidase complex (coxI) and the orfB/coxIII cistron (Fig. 1) [10].

The promoter element is located almost in the centre of this repeated sequence of 657 bp, with more than 200 nucleotides upstream being identical between the two genes. Potential transcription signals located in these 200 nucleotides preceding the promoter are thus identical for the two subunits of this complex. Four copies of the repeated element are found in the mitochondrial genome with two different types of flanking sequences, showing this region to be active in homologous recombination.

The 3' portion of the duplicated sequence element downstream of the putative promoter region is once more repeated upstream of the open reading frame encoding subunit 6 of the ATPase (atp6) in *Oenothera* mitochondria [12]. Transcripts of the atp6 thus contain the same non-coding 5' sequences as the mRNAs coding for coxI and orfB/coxIII.

Partial copies of this sequence element have also been found in other plant species in corresponding locations upstream of different genes. Almost one hundred nucleotides of the sequence block are

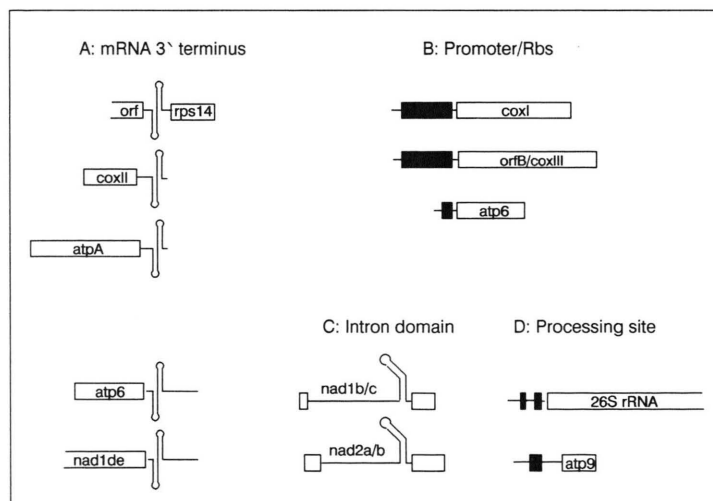


Fig. 1. Several duplicated sequences with specific functions have been found in the mitochondrial genome of *Oenothera*. The signals contained in these motifs are shared and used by several different genes. The amplified regions are indicated schematically as potential secondary structures at mRNA 3' termini (A) and as intron domains (C), or by black bars for duplicated putative promoter and ribosome binding sites (rbs, B) as well as potential processing signals (D). Abbreviations of plant mitochondrial genes: orf = open reading frame of unidentified function, rps 14 = small ribosomal protein 14, coxII = cytochrome oxidase subunit II, atpA = alpha subunit of the ATPase, atp6 = subunit 6 of the ATPase, nad1de = exons d and e of the NADH dehydrogenase subunit 1, coxI = cytochrome oxidase subunit I, orfB = open reading frame B encoded upstream of coxIII, coxIII = cytochrome oxidase subunit III, nad1b/c = intron between exons b and c of NADH dehydrogenase subunit 1, nad2a/b = intron between exons a and b of NADH dehydrogenase subunit 2, 26S rRNA = large ribosomal RNA, atp9 = ATPase subunit 9.

more than 90% identical upstream of the tobacco *rps13* [13] and of the *Petunia* *coxII* genes [14]. These duplications, however, do not include the putative promoter region with the mapped transcript termini, but are well within the transcribed leader sequence that may contain translation signals (see also below).

A different type of putative promoter duplication is found in cytoplasmic male sterile (cms) lines of maize, radish, *Petunia* and other species [15]. Several mosaic genes in different species make use of upstream promoter and translation initiation sequences from different genes. The T-urf13 gene of the maize T-cytoplasm, for example, contains the potential promoter region of subunit 6 of the ATPase [16]. A mosaic gene in a male sterile *Petunia* line uses the potential promoter sequence of subunit 9 of the ATPase [17].

The promoter sequences are in both cases duplicated. They are found once in their original location and as a second copy upstream of the mosaic gene. Other cms associated recombinations do not possess amplified putative promoter regions but make use of the original copy and have altered the protein coding region by recombination [18]. No effect, at the level of transcription by amplification of the promoter sequences, is detected in blot analyses of mRNAs, suggesting that potential specific transcription factors of these genes are not out-competed by the duplications.

Translation initiation elements

No homologous sequence elements have been observed in the untranslated leader regions of different genes besides large duplications, suggesting either gene-specific translational control as in yeast mitochondria [19], or a general lack of regulation at this level in plant mitochondria, with as yet unidentified mechanisms initiating translation.

The cms-associated amplifications and rearrangements of the two examples given above, T-urf13 and the *Petunia* rearrangement, cover several hundred nucleotides upstream of the AUG initiation codon and thus include not only transcription initiation sites, but also the translation initiation regions, potentially necessary translational control signals, and the ribosome binding sites.

A sequence of 122 nucleotides from the coding region of ATP6 is duplicated in maize mitochondria

and is found as a second copy just preceding the AUG of the intact *coxII* gene [20]. The ribosome binding site and specific signals for translation of *coxII* must, rather fortuitously, be contained in this original reading frame sequence.

The sequence element amplified upstream of *coxI* and *orfB/coxIII* in *Oenothera* and partially also upstream of the *atp6* gene terminates just three nucleotides upstream of the *coxI* initiation codon, five nucleotides before the *orfB* AUG codon and two nucleotides upstream of the *atp6* reading frame (Fig. 1) [12]. The ribosome binding sites and other potential translation start signals for these open reading frames must thus be contained in the element and will be identical for the three genes.

Processing signals

Two conserved sequence elements have been observed at putative processing sites [21]. The strongest case of processing at these locations can be made for the 5' termini of the mature ribosomal RNAs, which have to be efficiently generated by either processing or *de novo* initiation. The 5S rRNA 5'-terminal region shows similarity with the putative promoter sequence discussed above, while the mature sequences of both 18S and 26S rRNAs are more likely to be generated by processing of precursors. Larger, potential precursor molecules have been identified and mapped for both large rRNAs, in a monocot and a dicot species [21, 22].

The same sequence elements conserved at the termini of the rRNAs have also been found at some mRNA termini. Here they are likewise found only where larger, potential precursor mRNA molecules for the same region are present [21]. Most striking is the example of sequence similarity at the 26S rRNA and a short *atp9* transcript in *Oenothera* and *Petunia* mitochondria [21]. The divergence between the two locations is mainly a deletion/insertion of 61 nucleotides in the sequence element of this putative processing signal. These two elements have consequently been suggested to be derived from a duplication inactive in recombination and subsequent sequence divergence. The two copies of the *atp9* gene in *Petunia* mitochondria diverge just upstream of this element, which is therefore located just at the border of the duplicat-

ed gene. Other members of this sequence element family, such as the more than 20 nucleotides identical upstream of the tobacco *atp9* and *atp6* open reading frames, might likewise derive from duplications.

tRNA Fragments as processing signals?

Other processing sites in large transcripts of plant mitochondria may make use of the processing activities recognizing tRNA structures [23]. Parts of several tRNAs and sequence elements that can be folded into tRNA-like secondary structures (t-elements) have been found in several species. The t-elements constitute, at least in wheat mitochondria, a class of short repeats observed in several different locations of the genome [23]. Interestingly these tRNA elements can be recognized and correctly processed by the mitochondrial RNAase P and the 3'-processing activity [23].

A possible role of these sequences in RNA maturation as processing signals has consequently been discussed [23]. Their location close to, and in the same orientation as, actively transcribed genes is a further argument for their functional involvement as processing sites. Mitochondria in mammalia, lower eukaryotes and fungi are known to employ tRNAs as processing signals [24, 25]. Mammalia make extensive use of the tRNAs interspersed between the protein coding regions around the mitochondrial genome, and a tRNA punctuation model has been developed for this mode of transcript maturation [25].

RNA precursor cleavage at tRNA borders may thus be a feature commonly used in mitochondria of all species. In plant mitochondria tRNAs may have been duplicated and may then have diverged in their primary sequence. The minimal structures required to maintain the processing recognition function are now found at the processing sites [23].

mRNA 3' Termini

Complex secondary structures can be folded from the sequences immediately preceding most of the mapped mRNA 3' termini in plant mitochondria [26]. These structures consist of single or double hairpins analogous to sequence elements found preceding chloroplast mRNA 3' termini [27]. Despite the similar secondary structure arrangements little primary sequence conservation is usually

observed among the different plant mitochondrial 3'-terminal sequence regions.

Some of the sequences preceding mRNA 3' termini, however, have been amplified and are now duplicated at corresponding locations of different genes in several species. In maize about 35 nucleotides are repeated at the transcript termini of the *coxI*, the *T-urf13* and one of the *S-2* plasmid encoded transcripts. A related sequence in *Oenothera* is located just preceding mapped 3' termini of the *atpA*, *coxII* and an open reading frame upstream of the gene coding for the small ribosomal protein 14 (rps 14) [26, 28]. Two elements of 50 nucleotides each are duplicated downstream of the first two genes, *atpA* and *coxII*, preceding the 3' termini. The distances between the termination codons of the open reading frames and the repeated elements vary by less than 100 nucleotides between the different genes.

Another sequence with the potential to fold into hairpin structures preceding mRNA 3' termini is identical downstream of the genes encoding *atp6* and subunit 1 of the NADH dehydrogenase (*nad1*) in *Oenothera* [12, 29]. This sequence identity is due to a recombination event downstream of the two open reading frames within the sequence covered by the mRNAs, resulting in identical sequences for several kilobases downstream of the two genes, including the mRNA 3' termini. The *atp6* gene in *Oenothera* mitochondria is thus flanked on both sides by sequences repeated and used elsewhere in the genome.

These observations of redundant sequences preceding mature mRNA 3' termini suggest that amplified sequence elements have the same function at different locations in plant mitochondrial gene expression.

Intron domains

All intron sequences identified to date in plant mitochondria belong to the organellar group II intron family [30]. Besides the normal *cis*-splicing intervening sequences, five disrupted intron sequences have been found that link separate mRNA molecules in *trans via* intermolecular interactions between the intron sequences [29, 31].

The terminal domains V and VI of these introns encode major catalytic functions of the intron sequences, as deduced from *in vivo* and *in vitro* ex-

periments with fungal mitochondrial introns [32]. Individual nucleotide positions in these sequence regions are therefore well conserved among otherwise very different introns and species, generally allowing recognition of their relationship and classification into distinct groups. The similarity of these sequences, however, is easily distinguishable from the sequence amplifications considered here, which involve larger, identical sequence stretches at different locations.

An example of a duplicated sequence constituting part of an intron structure has been found in *Oenothera* mitochondria. 48 nucleotides are identical in two otherwise unrelated introns of two genes encoding subunits of the NADH dehydrogenase complex (Stefan Binder, Anita Marchfelder, Axel Brennicke, and Bernd Wissinger, unpublished results). This repeated sequence contains domain V and adjacent nucleotides, allowing the two introns (intron b/c of nad 1 and intron c/d of nad 2) to use the identical domain V activity for splicing of this intron. Intron domain V is essential for intron excision and may play a crucial role in the actual splicing and exon ligation process. Intriguingly, domain V has been shown by *in vitro* experiments with a yeast group II intron to make its essential contribution for splicing also in *trans* [33]. These observations open the door for speculations as to the duplication and integration process of this intron sequence and to its mobility at the RNA level. Further data on other intron sequences will be required to identify their interrelationship and phylogeny, which appear to be distinct, however, from this identical duplication of 48 nucleotides, as judged from the 13 intron sequences identified so far in plant mitochondria.

Gene fragments

Numerous fragments of protein-encoding genes, but also of structural RNAs, have been found in the mitochondrial genomes of all higher plant species investigated in this respect. All possible transitions and combinations have already been observed, from the duplication of entire cistrons, of entire open reading frames without the complete flanking sequences, or of an open reading frame where only one nucleotide is missing down to small duplications of only 20 nucleotides barely detectable by their similarity.

Some of these duplications are perfectly conserved, others are no longer active in recombination and copy correction and have drifted away from the original sequence. Many of these sequences most likely originate from recombination and duplication in the genomic DNA molecules, but some sequence arrangements are suggestive of having arisen from reverse transcription of RNA molecules.

A striking example of such an arrangement is found in the above mentioned mosaic open reading frame of *Petunia*, where several sequences of the coxII open reading frame are joined to other sequences [17]. The coxII fragments are sequences derived from the first and second exons that are connected without the intervening sequence. The upstream exon sequence terminates at a recently recognized cryptic splice site joined exactly to the 5' nucleotide of the downstream exon [34]. This connection could be explained by being derived from a transcript spliced at the cryptic site and integrated into the DNA through a reverse transcription step. Reverse transcription of a spliced RNA should incorporate processed RNA editing sites from this region into the DNA product to distinguish this mode of amplification from a DNA recombination mechanism *via* small sequence similarities.

A sequence duplication of a coxII gene fragment in *Oenothera* mitochondria, however, does show a nucleotide alteration that has been shown to be an editing site in the intact coxII sequence in this species (Wolfgang Schuster, unpublished results) and is thus another candidate for being derived *via* a reverse transcription step.

This coxII fragment is located in a highly rearranged region of the *Oenothera* mitochondrial genome, directly adjacent to a pseudogene copy of the atpA gene and downstream of fragments from ribosomal protein genes that are encoded in other parts of the mitochondrial genome (Wolfgang Schuster, unpublished results).

Gene fragments and RNA editing

RNA editing in plant mitochondria alters the primary mRNA sequence transcribed from the DNA by numerous C to U and more rarely U to C transitions [35–37]. Both, biochemical mechanism and specificity determinants are as yet unclear. Comparison of the sequences surrounding editing

sites in different plant species has so far revealed no clear features in primary or secondary structures that could play a role in marking a specific site for editing. Direct comparison of editing patterns between two plant species shows several sites to be edited in one but not the other species in spite of identical sequences surrounding such a site for sometimes more than 20 nucleotides on either side [38, 39].

These observations have stimulated discussion of other nucleic acid determinants conferred by additional, separate RNA molecules like in trypanosome mitochondria, where specificity of the RNA editing events is carried by small RNA molecules of antisense orientation, the so-called "guide RNAs" [40]. If such guide RNAs are also involved in RNA editing in plant mitochondria, they will most likely be encoded in the mitochondrial genome.

The published regions of sequence homology with coding regions, however, are either completely identical with the genomic sequences or have diverged in nucleotides other than the editing sites. The only gene fragment identified so far with a nucleotide alteration at an editing site is the fragment of the *coxII* open reading frame in *Oenothera* mitochondria. This sequence contains one nucleotide alteration that would specify an A residue in the antisense orientation where the primary transcript of the gene contains a C residue that has been shown to be edited. An additional mismatch between the antisense RNA sequence and the mRNA is found adjacent to this nucleotide. Other sequence divergencies are also found in the vicinity, rather suggesting this sequence to be derived through a reverse transcription step from the mRNA and subsequent divergence of the primary sequence. The search for duplicated gene fragments as potential candidates for the RNA editing specificity has so far been unsuccessful and will have to be pursued with different approaches.

Conclusions

These examples of genomic duplications are given to accentuate one of the potential biological advantages of the high recombination rate in plant mitochondria, the multiple use of the same signal sequence elements in different functional genomic contexts. Fig. 1 summarizes some of the examples of duplicated sequence elements described above in detail.

The size of plant mitochondrial genomes favors such random duplications and recombinations to result in functional arrangements of controlling and coding sequences. The genome is large enough to tolerate 'wrong' or disadvantageous locations of such sequences and small enough to raise the chance of a useful context to significant levels. The multiple copies of the genome will presumably eliminate, without deleterious consequences, non-sense recombinations, such as signals for transcript-termini between promoters and downstream encoded essential open reading frames.

Important in these considerations especially of transcriptional signal duplications are observations of the transcriptional activities of the plant mitochondrial genome. These investigations suggest that large parts of the genome are transcribed; estimates range from 70 to 90% of the entire genome complexity in different species [41]. This high rate of transcription may be due to such amplifications of putative promoter sequences, as described above, or may suggest rather indiscriminative initiation of transcription by the mitochondrial RNA polymerase and associated factors. *In vitro* experiments, however, identified clear initiation sites [9] and thus support the hypothesis of multiple, distinct promoters, some of which were created by recombinational amplification.

The advantages of such random amplification of promoters (and other signals) are obvious; identical signals can use identical recognition systems and therefore simplify the machinery required for control. Especially the control of expression of the genes encoding different subunits of the same or connected complexes will be simplified by common expression signals, such as the duplicated promoters for subunits I and III of the cytochrome oxidase in *Oenothera* mitochondria. Processing signals using the very active enzymes of tRNA maturation may ensure efficient processing at these sites without requiring additional specificities.

The accidental amplification of, for example, promoters may lead to the transcription of regions not encoding any required function, such as most of the chloroplast and nuclear sequences found to be integrated in plant mitochondrial genomes. The potential for transcribing new sequences, such as tRNA genes imported from the chloroplast, and to use the encoded function in the mitochondrion

must be worth the price of transcribing unnecessary sequences. The expense involved in large-scale transcription of the genome appears to be paid for by the possibility of fine-tuning posttranscriptional control. Rapid turnover of some transcripts in plant mitochondria suggests an extensive control function of posttranscriptional regulation similar to chloroplasts, where transcript requirements are accounted for by differential regulation of mRNA stability with numerous specific and unspecific control mechanisms [27]. Similar regulated degradation could be involved in the generation of different tissue and development specific abundancies of plant mitochondrial transcripts. Additional control may be exerted at the level of translation where only a fraction of the available mRNAs might be selected for translation. Extensive regulation at this level has been found in yeast mitochondria with numerous nuclear encoded factors involved in translational activation [19]. Investigation of the translation process in plant mitochondria is just beginning; even the significance of potential ribosome binding sites still needs to be determined and it may well be that like in yeast a plethora of factors will turn out to be involved in a number of regulatory steps.

Economy in biological systems is often abandoned in exchange for an advantage in flexibility

by regulatory possibilities. RNA editing may turn out to be an extreme case against economical reason. By simple logic this process is not really required for function if the mature mRNA sequence is encoded in the genome [42]. RNA editing consumes additional energy, requires time beyond the transcription process, and may occupy additional coding space.

The possibility to coordinate regulatory functions may be the *raison-d'être* for duplicated signal sequences. Identical control sequences offer the easiest and most straightforward way of ensuring tight coordinated control over the nearby structural information and may for this reason have come into such extensive use in plant mitochondrial genomes. Similar recombinational amplifications may also play a role in larger genomes, but would there meet the difficulty of appropriate location. Further investigations will be required to locate similar duplications in genomes with complexities as high as chromosomal DNAs.

Acknowledgements

Work in the authors' laboratory is supported by grants from the Deutsche Forschungsgemeinschaft and the Bundesministerium für Forschung und Technologie.

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