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Research Article

A recombination point is conserved in the mitochondrial genome of higher plant species and located downstream from the *cox2* pseudogene in *Solanum tuberosum* L.

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Abstract

The potato (*Solanum tuberosum* L.) mitochondrial *cox3/sdh4/*pseudo-*cox2* gene cluster has previously been identified by heterologous hybridization using a *Marchantia polymorpha sdh4* probe. In our present study we used Southern blotting using *sdh4* and *cox2* probes to show that the *sdh4* and *cox2* genes are clustered in the mitochondria of potato, soybean and pea. Northern blotting revealed cotranscription of *sdh4* and *cox2* in potato but not in cauliflower, indicating that these genes are not clustered in cauliflower. A putative recombination point was detected downstream of the *cox2* pseudogene (pseudo-*cox2*) in potato mitochondrial DNA (mtDNA). This sequence corresponds to a 32 bp sequence which appears to be well-conserved and is adjacent to the terminals of some mitochondrial genes in *Citrullus lanatus*, *Beta vulgaris* and *Arabidopsis thaliana* and is probably involved in the genic rearrangements. It is possible the potato mtDNA pseudo-*cox2* gene was generated by recombination during evolution in the same way as that of several other mitochondrial genes and remains as an inactive partial copy of the functional *cox2* which was also detected in potato mtDNA.

Key words: mitochondrial DNA, rearrangement, plants, *Solanum tuberosum*, *cox2*.

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Introduction

Mitochondrial genomes vary greatly in size, being about 5.7 to 76.5 kb in protists (Gray *et al*., 1998; Gray *et al*., 1999), 16 to 19 kb in vertebrates, 17 to 176 kb in fungi and 16 to 2400 kb in land plants (including algae) (Ohta *et al*., 1998). Angiosperm mitochondrial genomes are the biggest and most complex relative to other eukaryotes (Newton, 1998), their large size being due not only to the fact that plant mitochondria encode two to three times more genes than animals and fungi but also to the frequent recombination events, sequence rearrangements producing pseudogenes (Burger *et al*., 2003) and the integration of DNA from the nucleus or chloroplast (Schuster and Brennicke, 1988). Mitochondrial insertions in the nuclear genome have also been reported (Stupar *et al*., 2001). There is great variation in genome size and gene organization given that mitochondrial organization is quite variable among species due to the large amount of mitochondrial recombination which produces subgenomic molecules or isomer forms of the master mitochondrial DNA (mtDNA) genome by intra and interspecific recombination mechanisms via repeated sequences in direct or inverted orientation (Lonsdale, 1984; Newton, 1988; Hanson and Folkerts, 1992; Suzuki *et al*., 1996). Repetitions of small size achieve intragenic recombination, which can result in the formation of new open reading frames (*ORFs*) or pseudogenes (Mackenzie and McIntosh, 1999).

The high level of recombination occurring in plant mtDNA results in great structural complexity of the mitochondrial genome; the advantage of this to the organelle perhaps being the maintenance of variation which results in different phenotypic subpopulations (Mackenzie and McIntosh, 1999). Recombination and amplification of sequences are responsible for the difference in size among mitochondrial genomes of the Bryophyte *Marchantia polymorpha*, algae and higher plants (Turmel *et al*., 2002; Städler and Delph, 2002) because in *Marchantia* duplications do not recombine whereas in higher plants some sequences are several kilobases in size and participate in homologous recombination which results in a complex arrangement of molecules (Wissinger *et al*., 1991). The fre-

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quency of homologous recombination is probably related to the abundance of repeated sequences able to recombine, large repetitions (0.7 to 120 kb) being considered responsible for the appearance of multipartite structures such as subgenomic circles while small repetitions recombine in response to stress and generate possible mutations, deletions and duplications as well as simple genic rearrangements (Nedelcu and Lee, 1998). Recombination may create chimeric genes and a duplicate repetitive region which contributes to genome expansion, as known to be the case in the mitochondrial genomes of cucumbers (Lilly and Havey, 2001).

It is known that in the mitochondria of some plants integrated sequences originated in the nucleus or chloroplast because the insertion point of these sequences are randomly distributed in the mitochondrial genome (Stern and Palmer, 1984; Brennicke *et al*., 1993). Sequences encoding tRNAs or other genes may or may not conserve their expression ability (Breiman and Galun, 1990). During transfer between different cell compartments a gene sequence duplicates either by RNA synthesis and reverse transcription to produce cDNA which inserts itself in the new compartment or by transposition without intermediate RNA synthesis in which the original gene sequence is replicated and the DNA copy migrates to the new cell compartment where it installs itself and becomes a functional gene. However, these are hypothetical mechanisms of genic transference being that the real process remains unclear (Schuster and Brennicke, 1988; Ayliffe *et al*., 1998). Moreover, transfer of sequences from mitochondria to the nucleus have been well-studied and it is accepted that not only do the transferred genes produce pseudogenes in the donor genome but they suffer a high proportion of nucleotide substitution in comparison to the normally encoded genes, meaning that the neutral mutations occur in high frequency in these pseudogenes due to its gradual loss of functionality (Laroche *et al*., 1997).

The study reported in this paper concerned the cyclooxygenase (COX) pseudogene *cox2* occurring in the cyclooxygenase/succinate dehydrogenase (SDH) gene cluster *cox3*/*sdh4*/pseudo-*cox2* of potato mtDNA and other species. We investigated the relationship of the *cox3*/*sdh4*/pseudo-*cox2* gene cluster not only to its genomic localization and organization in some angiosperms species but also to its cotranscription profile and the relationship of the cluster to the 32 nt sequence (AAGCAATGCCCAAAGACT CCCATTTCTTTCTT) located downstream from it and which is conserved in the mitochondria of some species and appears to be involved in rearrangement and/or recombination events. This small sequence is presumed to be involved in the formation of pseudogene *cox2* and its consequent location downstream to *sdh4* in potato.

Material and Methods

Mitochondrial DNA (mtDNA) was isolated from etiolated seedlings of coix (*Coix lacryma-jobi*, cv Adlay), maize (*Zea mays*, cv AGF352), pea (*Pisum sativum*, cv Mikado), soybean (*Glycine max*, cv IAC-5), potato tubers (*Solanum tuberosum*, cv Binje) and cauliflower inflorescence (*Brassica oleracea*, from a local market). The mitochondria were lysed and the mtDNA separated by cesium chloride-ethidium bromide centrifugation, being after this digested using the *Bam*HI restriction enzyme as described by Siqueira *et al*. (2001). Standard procedures (Sambrook *et al*., 1989) were used for restriction enzyme digestions and agarose gel electrophoresis. Potato and cauliflower mitochondrial RNAs (mtRNAs) were prepared as described by Stern and Newton (1986), treated with DNAse I (Sigma, USA) to remove any residual DNA and then phenol-extracted and precipitated.

Restriction-digested mtDNAs were transferred to Hybond-N filters (Amersham, UK) by standard procedures (Sambrook *et al*., 1989). The DNA fragments used as probes were purified from gel-slices by electroelution and labeled by random hexamer priming. Homologous hybridizations were carried out using as probes the potato *sdh4* gene, exon 2 of the wheat *cox2* gene, and the potato *sdh4*/pseudo-*cox2* cluster against *Bam*HI-digested mtDNA from the angiosperms potato, cauliflower, maize, coix, soybean and pea and the posterior procedures being performed under the conditions described by Sambrook *et al*. (1989). The mtRNAs isolated from potato and cauliflower were treated with DNase I in order to eliminate DNA traces, fractionated by electrophoresis onto 1.2% (w/v) agarose gel (5 μg mtRNA per lane) and blotted onto Hybond-N filters. Probe labeling, hybridization, solutions to wash the membrane and the full procedures are described in Siqueira *et al*. (2001).

To detect similar sequences to the potato 32 nt sequence (*i.e.* downstream from pseudo-*cox2*) we used the BLAST program (www.ncbi.nlm.nih.gov/BLAST).

Results

Probes consisting of *cox2* gene (only exon 2) from wheat and *sdh4* and *sdh4*/pseudo-*cox2* genes from potato have been used in homologous hybridization in order to detect the conserved *sdh4*/pseudo-*cox2* genic cluster in the *Bam*HI-digested mtDNAs from potato, cauliflower, maize, coix, soybean and pea. Only one of the *cox2* exons from wheat was used device to the high conservation degree of this gene sequence in different plant species. Several bands showing the same sizes were obtained in the hybridizations performed with the three probes (*cox2*, *sdh4* and *sdh4/*pseudo-*cox2*, Figure 1). The *sdh4/*pseudo-*cox2* probe consisted of these two clustered genes so, the hybridization bands obtained with this probe corresponded to the sum of all bands obtained in hybridization approaches using the *cox2* or *sdh4* probes separately.

Potato mtDNA hybridized with the *cox2* probe resulted in three bands of which the largest produced a weak

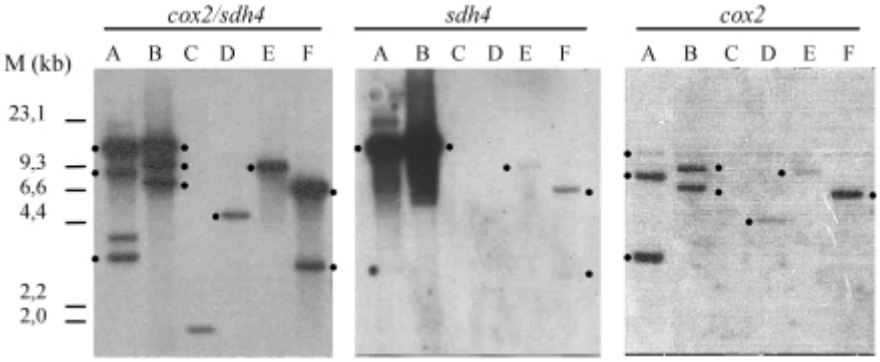

Figure 1 - Hybridizations between mtDNA from some plant species and *cox2/sdh4*, *sdh4* and *cox2* probes (indicated at the top of the autoradiographs). Legends: A = potato, B = cauliflower, C = maize, D = coix, E = soybean, F = pea, M = molecular size markers (λ*Hind*III/φx*Hae*III). The black points indicate bands of the same size.

autoradiographic signal representing a fragment of about 10 kb. This is the same fragment that appeared in hybridizations using the *sdh4* probe and corresponds to the *cox2*-containing fragment located downstream from the potato *sdh4* gene. It thus appears that both the *cox2* and *sdh4* genes are located in the same *Bam*HI fragment detected by *cox2* and *sdh4* probes in the performed hybridizations. This same 10 kb fragment has appeared in previous hybridization studies with the *cox3* probe, indicating that the *cox3* gene is located on the same *Bam*HI fragment that contains the *cox2* and *sdh4* genes, in a clustered arrangement of genes (Siqueira *et al*., 2001). The other two bands showing a strong autoradiographic signal may each correspond to two copies of the *cox2* gene in the potato mtDNA. There is also the possibility that the *Bam*HI enzyme cut the *cox2* gene in two fragments if there is a restriction site for this enzyme inside this gene. In this case, each part of the digested *cox2* gene strongly hybridized with the *cox2* probe.

Hybridization performed with cauliflower mtDNA resulted in two bands with a strong intensity in the autoradiography, but neither of which corresponded to the ~12 kb fragment appearing in hybridizations with the *sdh4* probe. This demonstrates that in cauliflower mtDNA the *cox2* gene is located far from the *sdh4* gene and does not form a genic cluster in this plant. These two bands may point to the existence of two copies of the *cox2* gene in the mitochondrial genome or they may reflect again the existence of a *Bam*HI restriction site inside the *cox2* gene. This is supported by the same fact that may occur in potato: each *Bam*HI fragment containing a piece of *cox2* gene strongly hybridized with the used probe.

Soybean mtDNA resulted in only single band of ~8 kb size in the hybridizations with the *sdh4* and *cox2* probes. Probably, this fragment is corresponding to an intact *cox2* gene, indicating that in soybean the *cox2* gene is located close to the *sdh4* gene.

Pea mtDNA also produced one intense band (~6.6 kb) when hybridizing with the *cox2* probe and this band is corresponding to the same band that appeared in hybridization with the *sdh4* probe, indicating that these genes are located next between them.

Maize and coix mtDNAs produced bands in the hybridization with the *cox2* probe that were not appeared in the hybridization with the *sdh4* probe, indicating that in these plants the *cox2* and *sdh4* genes are located in distant positions along the mitochondrial genome. Moreover, in these species, this result indicates that there is only one *cox2* sequence without any copies.

Northern blots containing total mtRNA from potato and cauliflower were performed in order to detect *sdh4*/pseudo-*cox2* cotranscription. MtDNAs from these plants were hybridized against the same probes used in the previous hybridizations (Figure 2). The results show that in potato, there is a 4.4 kb transcript common to the *sdh4* and *cox2* probes; a fact that indicates cotranscription of these genes. It is noteworthy again that the transcripts observed in

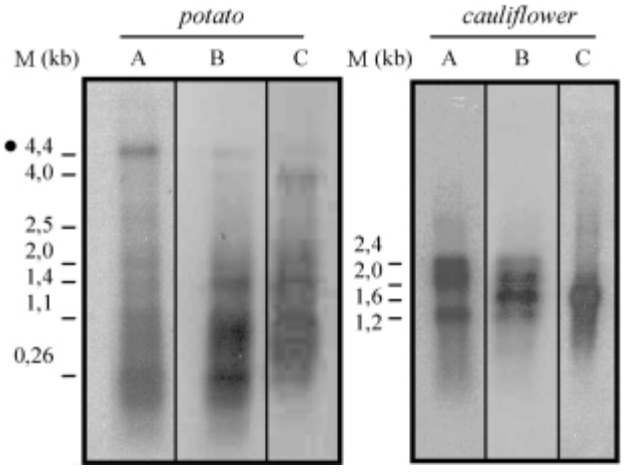

Figure 2 - Hybridization between mtRNA from potato and cauliflower with the following probes (indicated at the top of the pictures): A = *sdh4*, B = *sdh4/cox2* and C = *cox2*. The numbers on the left of the pictures indicate the transcripts size in kb based on an Invitrogen 0.24 to 9.5 kb RNA Ladder molecular size marker (M). (•) indicates the common transcripts for *cox2* and *sdh4* probes. The observed transcripts in the hybridization with the *cox2/sdh4* probe correspond to the same transcripts observed in the separate hybridizations with *sdh4* and *cox2*.

the hybridizations with the *sdh4*/pseudo-*cox2* probe correspond to the same obtained in each separate hybridization with the *sdh4* or *cox2* probes. In the hybridization with cauliflower mtRNA a single 1.6 kb band was obtained with the *cox2* gene which did not appear in the hybridizations with the *sdh4* probe, indicating that in these plants, cotranscription does not occur between *sdh4* and *cox2* and that *sdh4* and *cox2* genes are not clustered. This observation is in agreement with the conclusions obtained from the Figure 1.

Downstream from the partial *cox2* sequence, itself placed downstream from the *sdh4* gene in the potato, there is a small sequence (Figure 3.A) corresponding to 32 nucleotides (AAGCAATGCCCAAAGACTCCCATTTCTT TCTT GenBank: AF280607) which are highly (93-100% similarity) conserved in other mitochondrial genomes. Employing the BLAST program for sequence identity search, it was revealed the presence of this small sequence in other mtDNAs besides potato (Figure 3.B). This sequence was found in watermelon (*Citrullus lanatus*) (AF288042), sugar beet (*Beta vulgaris*) (Kubo *et al*., 2000), *Arabidopsis thaliana* (Unseld *et al*., 1997) and *Brassica napus* (GenBank: AP006444) and seems to be involved in mitochondrial genome rearrangement. In *Citrullus,* this sequence locates upstream to *nad9* gene. In sugar beet the same sequence is located in different regions upstream to the *atp6* and *atpA* genes, to a direct repeat of the mitochondrial genome and downstream to the *rps7* gene and *cox2* pseudogene. In *A. thaliana,* this sequence is located upstream to *nad9* and downstream to the *rpl16 and atp9* genes, while in *B. napus* the sequence appears upstream to c-type cytochrome synthesis gene (*ccmFN2* or *cycK*) and downstream to the *nad9* gene. These results indicate conservation of this sequence between some plant species. In some cases, the 32 nt potato sequence is not totally conserved but appears as a partial sequence in various plants. In sugar beet and some other plants there are several small fragments that share partial homology with the potato fragment (data not shown) which may be involved in recombination events in the mitochondrial genome of these species throughout evolution.

Discussion

Our hybridization results (Figure 1) show the presence of the *cox3*/*sdh4*/pseudo-*cox2* cluster (Siqueira *et al*., 2001) in potato mtDNA but this sequence was not identified in the other plants analyzed in this study (cauliflower, coix, maize, pea and soybean), which is another example of the complexity and diversity of plant mitochondrial genomes due to their inherent dynamism and high level of recombination. The *sdh4*/*cox2* cluster (the pseudo-*cox2* cluster) was encountered in potato, pea and soybean but not in cauliflower, coix and maize, where these genes were encountered not as a cluster but separately. Moreover, if in potato the putative single copy of *cox2* was not cut by *Bam*HI there is a possibility that the potato genome contains a second copy of this gene.

As mentioned above, the *sdh4*/*cox2* cluster is absent from the mitochondrial genome of cauliflower, coix and maize, where there were single bands for each gene indicating that they were unclustered, but, however, another approach is necessary in order to detect the presence or absence of *cox2* in the nucleus. If this gene is encountered in this compartment, this could indicate that genic transfer occurred during evolution and perhaps the mitochondrial copy could have been inactivated and replaced by a pseudogene. Multiple transference processes have been described for structural and respiratory chain genes in plant mitochondria, especially for the respiratory chain genes *sdh4* (Adams *et al*., 2001) and *cox2* (Kanazawa *et al*., 1998; Adams *et al*., 1999; Subramanian *et al*., 2001). However, as was observed by us in pea and soybean, the cited studies gave no evidence for the evolution of a recombined sequence or site that could have allowed the separation of *cox2* and *sdh4* or of fragments of these genes (pseudogenes) dispersed in the rest of the genome.

We found that in maize and coix the *cox2* and *sdh4* genes are dispersed in the mitochondrial genome and appear as a single copy, indicating the absence of a recombination event that placed these sequences in close together or created pseudogenes. In cauliflower mtDNA, however, the *cox2* probe revealed two bands which may corresponding either to *cox2* cut by *Bam*HI or two copies of the *cox2* gene, although the *sdh4*/*cox2* cluster was absent. Adams *et al*., (1999) has shown that in some plant species the *cox2* gene has been transferred from the mitochondria to the nucleus while in other species it remains in the mitochondria or still it can exists like a functional nuclear copy in addition to a pseudogene that remains located in the mitochondria. Moreover, Lupold *et al*. (1999) reported that rearrangement of sequences influences the activity of *cox2* promoters in maize mitochondria.

Northern Blotting performed with total mtRNA from the potato and cauliflower against *sdh4* and *cox2* probes indicated that cotranscription of the *sdh4*/pseudo-*cox2* cluster occurred only in potato and not cauliflower (Figure 2). This observation was to be expected because *sdh4* and *cox2* are separated in cauliflower (Figure 1) which means that cotranscription between these genes is not to be expected. In cauliflower mtDNA there were no transcript bands common to both the probes, while in potato there was a common 4.4 kb transcript that was obtained with both probes. Unseld *et al*. (1997) stated that cotranscription of mitochondrial genes in plants is a frequent event because many mitochondrial genes are clustered.

The 32 nt sequence we detected alongside pseudo-*cox2* sequence is a further example of the small sequences commonly involved in the frequent rearrangements which occur in the mitochondrial genome of plants. The conservation of the this sequence in some plant species may point to

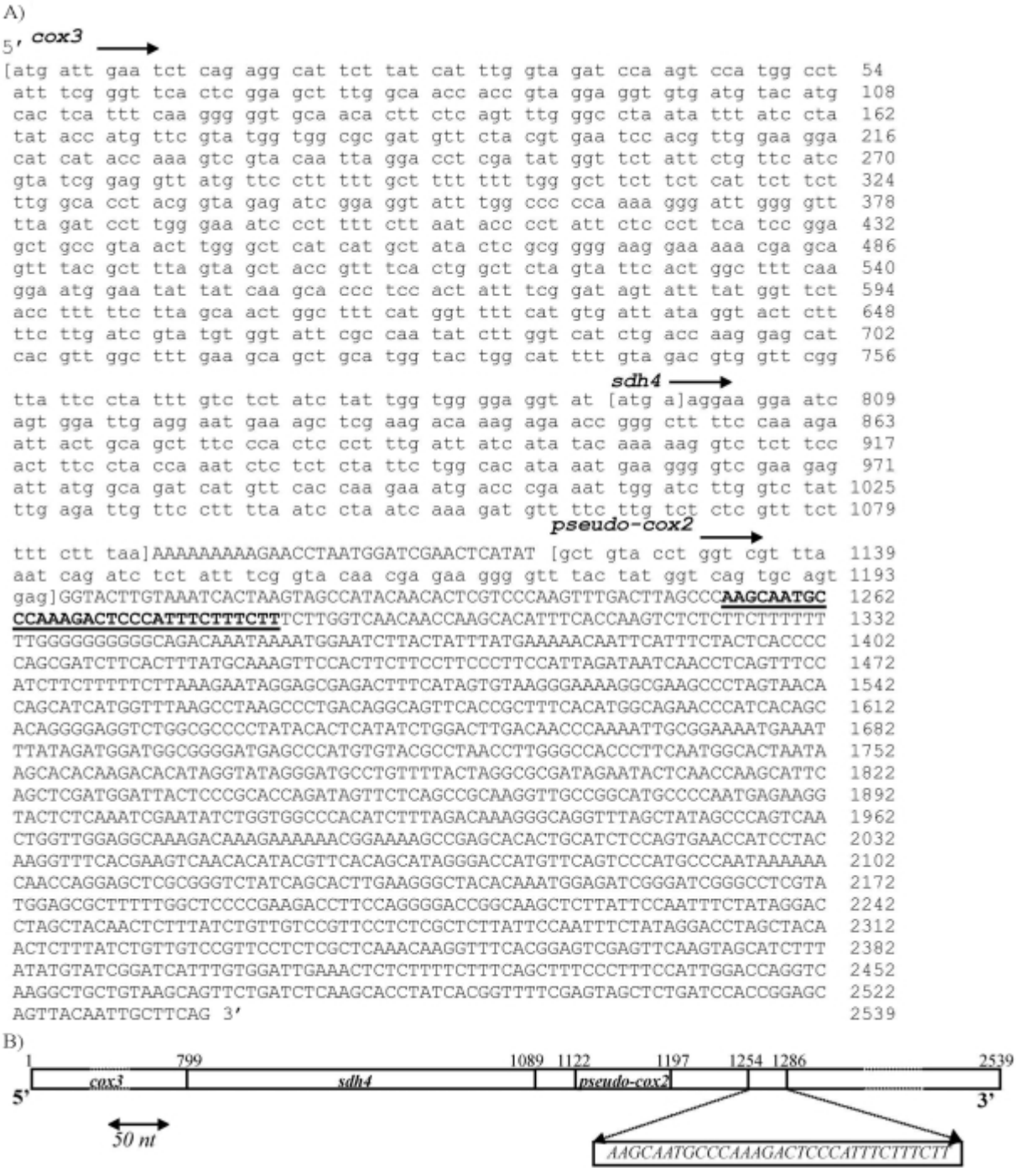

Figure 3 - Diagrams (not to scale) showing the position of the conserved potato mitochondrial sequence. (A) Linear nucleotide potato sequence. The *atga* sequence at site 795 indicates the *atg* start codon for *sdh4* and the *tga* stop codon for *cox3*. Nucleotide numbers are shown on the right and square brackets indicate the start/stop for each gene. The 32 nt recombinatory sequence conserved in some genomes is underlined and in bold. (B) Representative diagram showing the total sequence analyzed in this research. Numbers above the bar indicate the start and stop positions for the *cox3* and *sdh4* genes, pseudo-*cox2* and the 32 nt conserved sequence (in evidence below the diagram). Each cm in the bar corresponds to 50 nt.

it having a useful function, its association with various genes in diverse plant species reflects its ability to recombine during evolution. In *Beta vulgaris*, p. ex. these conserved sequence appears to be dispersed along the genome in association with different genes (Kubo *et al.*, 2000) and may be involved in the rearrangement process. In potato this sequence may be responsible for the interruption of the *cox2* sequence and could be associated with the relatively high recombination rate of the *cox2* genic region, which may result in a partial *cox2* sequence located downstream from the *sdh4* gene. Ceci *et al*. (1993) have reported that in sunflower a recombination event occurred downstream

from the *sdh4* gene and that this event inserted a 417 bp chloroplast DNA fragment which included a ct-tRNA^{Val} sequence. This recombination event took place four nucleotides upstream from the insertion point of the *cox2* partial sequence in potato mtDNA, illustrating that this region seems to be a `hot spot' for recombination events in various species. An interesting fact is that BLAST analysis (data not shown) showed that in *Arabidopsis* and sugar beet mtDNA the *cox2* pseudogene is conserved adjacent to the 32 nt sequence just as in potato mtDNA.

Our analysis of the *cox2* gene and the associated recombinatory sequence is another example of the very complex and dynamic structure of plant mitochondrial genomes. Further analysis should be carried out with the plant species investigated by us to establish whether or not transcription initiation and processing sites exist and to determine the transcript promoter region and also to verify the presence or absence of the *cox2* sequence in the nucleus.

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