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The gene for the α-subunit of ATPase: a site of homologous recombination in plant mitochondrial DNA also functions in somatic hybrid cells

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Summary. Segments of mitochondrial DNA (mtDNA) carrying the gene for the α-subunit of F_1-ATPase (*atpA*) were detected by Southern hybridization with *atpA* from pea as probe. In the case of *Nicotiana langsdorffii,* we identified four fragments that are derived from combinations of two different 5′ and two different 3′ flanking regions of *atpA*. All four types share the coding region, suggesting that they result from homologous recombination in the coding region of *atpA*. By contrast, *N. glauca* generated only one analogous fragment, which indicated the existence of only a single type of *atpA* in *N. glauca.* In the case of somatic hybrids obtained by fusion between protoplasts from *N. langsdorffii* and *N. glauca,* analysis with *Eco*RI or *Hin*dIII detected three new fragments in addition to the parental fragments. These new fragments can be explained by homologous recombination within the coding region of *atpA*. Our results show that the coding region of *atpA* is involved not only in intragenomic homologous recombination but can also be involved in homologous recombination between two parental mitochondrial genomes of somatic hybrids.

Key words: Homologous recombination – ATPase α-subunit – Protoplast fusion – *Nicotiana*

Introduction

Mitochondrial genomes of higher plants are much larger and more variable in terms of size and constituent sequences than those of yeasts, fungi and animals, ranging from about 200 kb in *Brassica* to 2,400 kb in muskmelon (Ward et al. 1981). Their complexity can be explained in part by homologous recombination mediated by repeated sequences present in "master circles". Palmer and Shields (1984) suggested that in *Brassica campestris* recombination is responsible for the simultaneous presence of three structures, namely, a master circle of 218 kb and two smaller circles of 135 kb and 83 kb, which seem to be formed by recombination between a directly repeated sequence of 2 kb present in the master circle. It has also been shown that multiple circular molecules can be produded by several kinds of repeats in maize (Lonsdale et al. 1984) and in wheat (Quetier et al. 1985). By contrast, the mitochondrial genome of *Brassica hirta,* which lacks any such repeats, is found as only a single circle (Palmer and Herbon 1987).

Novel fragments different from either of the parental mitochondrial DNAs was revealed by restriction endonuclease analysis for the first time in *Nicotina* somatic hybrids obtained by protoplast fusion (Belliard et al. 1979). Such phenomena have since been observed in many parental combinations between species that include the Solanaceae, Umbelliferae, Cruciferae, Gramineae, Luguminosae and Rutaceae (reviewed by Ichikawa et al. 1989). Although such new patterns are postulated to be due to mitochondrial genome recombination, experimental proof of the existence of recombination in hybrid cells is very limited. So far, it has been shown that several different regions of mtDNA undergo intergenomic recombination in *Brassica napus* cybrids and that some of these regions are presumably also involved in intragenomic recombination (Vedel et al. 1986).

It has been reported that sequences that contain the gene for the α-subunit of F_1-ATPase are involved in the homologous recombination of mtDNA from maize (Issac et al. 1985), *Oenothera* (Schuster and Brennicke 1986) and pea (Morikami and Nakamura 1987). In pea, there are four types of this gene, with two different 5′ and 3′

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flanking sequences that share a common repeated sequence.

In this report we show that a homologous recombination site, within *atpA*, in the mtDNA of *N. langsdorffii* can function as the recombination site in somatic hybrids derived from cell fusions.

Materials and methods

Fusion of protoplasts and cell culture

Mesophyll protoplasts from *N. langsdorffii* and callus protoplasts from *N. glauca* were fused by the polyethylene glycol method and cultured as previously reported (Itoh and Futsuhara 1983). Two weeks after the fusion, the culture medium was replaced by hormone-free medium for selection of hybrids.

Isolation of total DNA and mtDNA

Three and one-half months after cell fusion, total DNA, which included organelle DNA, was isolated from 50–100 mg plant tissue as described previously (Honda and Hirai 1990). Leaves of the parental plants were also used for isolation of total DNA. MtDNA was isolated from leaves of *N. glauca* by the method of Kemble (1987).

Construction of a recombinant λ phage library of mtDNA from N. glauca

MtDNA from *N. glauca* was partially digested with *Sau*3AI and ligated with lamda *Fix*II vector as described in the instruction manual from the supplier (Stratagene cloning systems, Calif. USA).

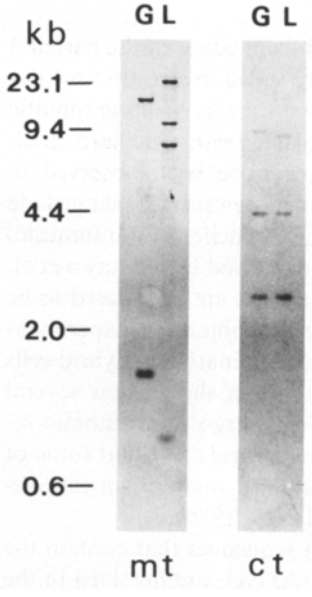

Fig. 1. Southern blot hybridization of total DNAs digested with *Bam*HI from *N. glauca* (*G*) or *N. langsdorffii* (*L*) and probed with the 1.5 kb *Hin*dIII-*Eco*RI fragment of mitochondrial (*mt*) *atpA* from pea or the 10-kb *Pst*I fragment that contains chloroplast (*ct*) *atpA* from rice

Southern hybridization

Hybridization was carried out as described elsewhere (Honda and Hirai 1990) and in accordance with the supplier's instructions (DIG-ELISA kit, Boehringer-Mannheim, Mannheim, FRG). In our experiments, we used the cloned gene for the α-subunit of the mitochondrial F_1-ATPase (*atpA*) from pea (kindly provided by Dr. A. Morikami of the School of Agricultural Sciences, Nagoya University) as probe. Most of the coding sequence of the gene, about 1.5 kb between the *Eco*RI site and *Hin*dIII site, was cut out of the plasmid vector for labeling with digoxigenin (Dig). In addition, a 1.5-kb fragment was divided into two parts by cleavage at the *Bam*HI site present at approximately the middle of the gene. The 5′-coding (*Eco*RI-*Bam*HI) and the 3′-coding (*Bam*HI-*Hin*dIII) regions were designated *atpA*-L and *atpA*-R respectively, and each was labeled separately as necessary. For a control experiment, the *Pst*I fragment (10 kb) that contained chloroplast (ct) *atpA* from rice was also used as a probe.

Extracted total DNA was digested with restriction enzymes in accordance with the manufacturer's recommendations (Takara Shuzo Co., Kyoto, Japan). The DNA fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to a nylon membrane (MAGNA nylon 66, MSI, Westboro, USA). After DNA had been bound to the membrane by formation of cross links with a UV transilluminator, hybridization and detection of fragments were performed as directed by the manufacturer of the kit mentioned above.

Results

Physical maps of mtDNA segments in the vicinity of atpA from two species of Nicotiana

It is known that the α-subunit of F_1-ATPase is encoded differently by mtDNA than by ctDNA. Homology between the genes is 59.4% at the level of the nucleotide sequences as indicated by data from maize (Issac et al. 1985; Rodermel and Bogorad 1987). First, we examined the possibility of detecting chloroplast *atpA* with mitochondrial *atpA* as probe because total DNA that contained mtDNA and ctDNA was used for hybridization in our experiments.

The fragment (1.5 kb) between the *Hin*dIII and *Eco*RI site of mitochondrial *atpA* from pea and the *Pst*I fragment (10 kb) of rice ctDNA that contained *atpA* were used separately to probe *Bam*HI digests of total DNAs from *N. glauca* and *N. langsdorffii* (Fig. 1). No identical fragments were detected with the two probes. There was no cross-reaction under the conditions used.

Total DNAs from the leaves of both parental plants and from a hybrid callus D-3 were digested with *Bam*HI, fractionated by electrophoresis and transferred to nylon membranes. Southern blot analysis with *atpA* as probe indicated that some species-specific fragments were present, i.e., two (15.0 kb and 1.5 kb) from *N. glauca* and four (approximately 23 kb, 10.5 kb, 8.2 kb and 0.9 kb) from *N. langsdorffii* (Fig. 2, panel T). We observed a new band of 4.7 kb in the hybrid callus in addition to parental fragments; in contrast, a 23-kb band specific to *N. langs-*

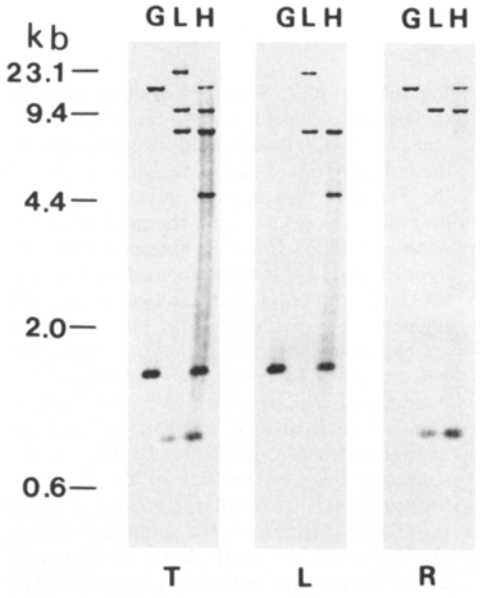

Fig. 2. Southern blot hybridization of total DNAs digested with *Bam*HI from *N. glauca* (*G*), the somatic hybrid (*H*) and *N. langsdorffii* (*L*) and probed with the 1.5-kb *Hin*dIII-*Eco*RI fragment of mitochondrial *atpA* from pea (*T*), the 5′ part (*L*) or the 3′ part (*R*) of the gene

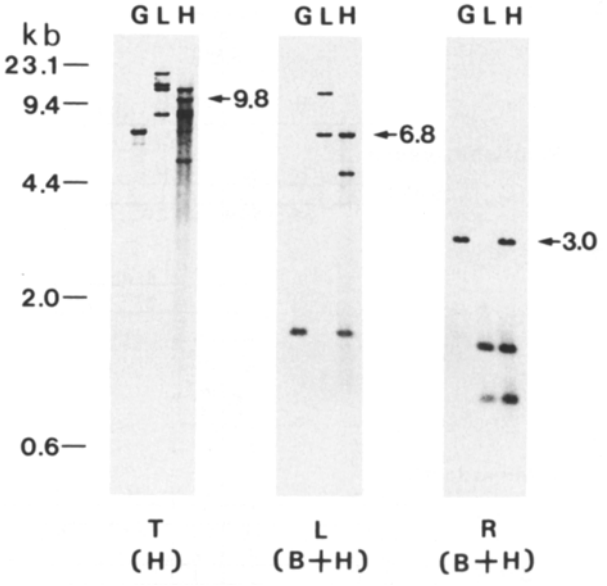

Fig. 3. Southern blot hybridization of total DNAs digested with *Hin*dIII (indicated by *H* below the panel) or *Bam*HI-*Hin*dIII (*B* + *H* below the panel) from *N. glauca* (*G*), the somatic hybrid (*H*) and *N. langsdorffii* (*L*) and probed with the 1.5 kb *Hin*dIII-*Eco*RI fragment of mitochondrial *atpA* from pea (*T*), the 5′ part (*L*) or the 3′ part (*R*) of the gene

dorffii had disappeared in the hybrid. Subsequently, *atpA*-L and *atpA*-R were used as hybridization probes to the same membrane as used before (Fig. 2, panels L and R). Results showed that all fragments detected by *atpA* were evenly distributed between those detected by these two probes. For example, in *N. langsdorffii* bands of 23 kb and 8.2 kb were found with *atpA*-L, while bands of 10.5 kb and 0.9 kb were found with *atpA*-R. There was no overlap of fragments between those detected by *atpA*-L and *atpA*-R, respectively. These results demonstrated that a *Bam*HI site located approximately in the middle of the gene is conserved between two species of *Nicotiana* as well as in pea and that the number of copies of *atpA* is four in *N. langsdorffii* and one in *N. glauca*.

Recombination of the mitochondrial genome near atpA in N. langsdorffii

It was assumed that there were four versions of the segments that carried *atpA* present in the mitochondrial genome of *N. langsdorffii*, because two different 5′-half and two different 3′-half segments should give rise to four versions by homologous recombination around *atpA*, as reported in *Oenothera* (Schuster and Brennicke 1986) and pea (Morikami and Nakamura 1987). To confirm this assumption, *Hin*dIII was used instead of *Bam*HI. Hybridization of DIG-labeled *atpA* to *Hin*dIII digests of total DNAs detected four fragments from *N. langsdorffii*, supporting the hypothesis that recombination actually occurs near the gene [Fig. 3, panel T (H)].

From results described above and Southern blots of *atpA*-L and *atpA*-R to total DNAs digested with *Bam*HI-*Hin*dIII [Fig. 3, panels L (B + H) and R (B + H)] or *Eco*RI and *Bam*HI-*Eco*RI (data not shown), we constructed a simple physical map of the *atpA* region in mtDNA from *N. glauca* and *N. langsdorffii* (Fig. 4 A and C, respectively).

We also constructed a phage library of mtDNA from *N. glauca* and screened recombinant phages with mitochondrial *atpA*. The physical map of one of the positive λ clones (designated as λ 829) agreed with that obtained from the hybridization of digested total DNA to *atpA* (Fig. 4 A). Therefore, the physical maps of mtDNA around *atpA* from *N. glauca*, shown in Fig. 4, were confirmed. And those of mtDNAs from *N. langsdorffii* and the hybrid, which were constructed by the same procedure, were proved to be reliable.

Recombination of two mitochondrial genomes occurred near atpA in somatic hybrids

Analysis with *Hin*dIII revealed that the hybrid had three novel fragments in addition to three parental fragments [Fig. 3, panel T (H)]. The length of the new *Hin*dIII fragment (9.8 kb) was the sum of the lengths of the *N. langsdorffii*-specific 5′ *Bam*HI-*Hin*dIII fragment [6.8 kb, in

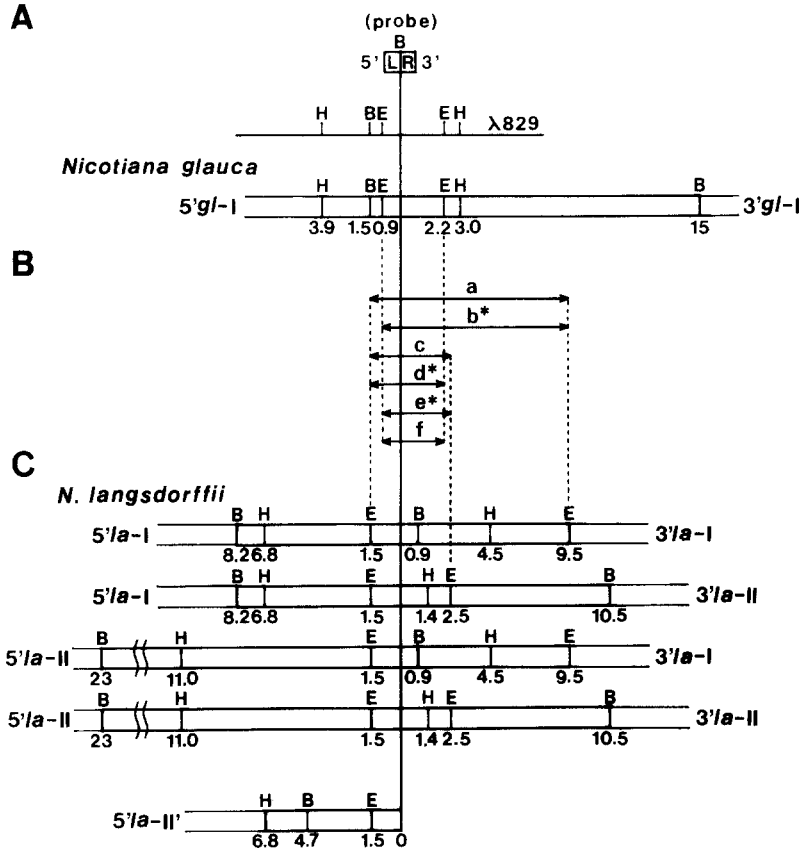

Fig. 4A–C. A, C Simple restriction maps in the vicinity of *atpA* from *N. glauca* (**A**) and *N. langsdorffii* (**C**). Probes used for hybridization are indicated *above* the maps. *L* and *R* refer to the 5′ and 3′ part of *atpA*, respectively. The map of λ 829 cloned from the mtDNA of *N. glauca* is added to those obtained from the hybridization analyses performed with the total DNAs obtained from the two species. All segments are divided by the *Bam*HI site in approximately the middle of the *atpA* gene and are indicated, respectively, by *three letters* beside each segment, such as 5′ *1a*-I. *Vertical* lines on each divided segment indicate the nearest restriction site to the *Bam*HI site described above and the size of the fragment (kb): *B Bam*HI, *H Hin*dIII, *E Eco*RI. A newly postulated fragment in the somatic hybrid is also indicated as 5′ *1a*-II′. **B** *Eco*RI fragments detected with *atpA* in the somatic hybrid. The borders of each fragment correspond to *vertical lines* for *Eco*RI in **A** and **C**. Designations of each fragment (*a–f*) refer to those in Fig. 5. New fragments appearing in the case of the somatic hybrid are marked by asterisks (*)

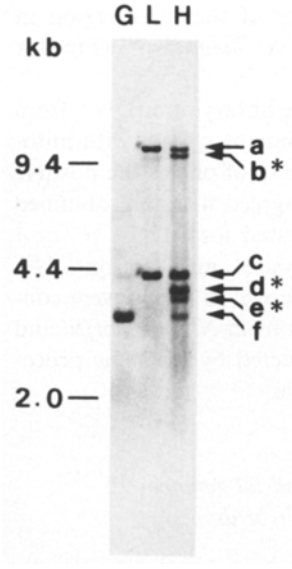

Fig. 5. Southern blot hybridization of total DNAs digested with *Eco*RI from *N. glauca* (*G*), the somatic hybrid (*H*) and *N. langsdorffii* (*L*) and probed with the 1.5-kb *Hin*dIII-*Eco*RI fragment of mitochondrial *atpA* from pea. Designations (*a–f*) are equivalent to those in Fig. 4B. New fragments from the somatic hybrid are marked by asterisks (*)

Fig. 3, panel L (B + H) and 5′ *1a*-I in Fig. 4C] and the *N. glauca*-specific 3′ *Bam*HI-*Hin*dIII fragment [3.0 kb, in Fig. 3, panel R (B + H) and 3′ *g1*-I in Fig. 4A]. An analysis with *Eco*RI also supported the hypothesis that recombination between two species had occurred at *atpA* (Figs. 4B, 5). In addition to parental fragments (designated a, c and f), three novel fragments (designated b, d and e) were found in the hybrid. All of the new *Eco*RI fragments can be explained by recombination at *atpA*, as shown in Fig. 4B. We analyzed 12 calli: 8 of them showed the same pattern as that of D-3. Therefore, we concluded that these fragments were generated from recombination between two mitochondrial genomes.

Fusion also caused the loss of *N. langsdorffii*-specific fragments in hybrids. The largest *Bam*HI fragment (23 kb, Fig. 2) and two large *Hin*dIII fragments (15.5 kb and 12.4 kb, Fig. 3), which were all found in *N. langsdorffii*, had disappeared in the hybrid. These fragments were derived from one of the 5′-half segments of *N. langsdorffii* (5′ *1a*-II in Fig. 4C). Although little information about this phenomenon could be obtained, we assume that it is the result of a recombination event different from that observed near *atpA*. However, one should ex-

pect the presence of another 5′-half segment in place of 5′ *1a*-II in the hybrid because of the appearance of a 4.7-kb *Bam*HI fragment (Fig. 2). Therefore, the hypothetical 5′ *1a*-II′ is also shown in Fig. 4C.

Discussion

Rearrangements of mtDNA found in somatic hybrids or cybrids have been extensively reported by many groups (see review by Ichikawa et al. 1989). These changes have usually been explained by recombination. However, it is possible that novel fragments could be generated from variations in either of the parental mtDNAs, although where or how these new fragments are produced has not been demonstrated in any detail. In this report, we have shown that the coding region of mitochondrial *atpA* is a suitable site for homologous recombination between two molecules of mtDNA. It is intriguing that this region is also involved in intragenomic homologous recombination in *N. langsdorffii,* as has been previously reported in other plants, namely, maize, *Oenothera* and pea. Therefore, this region should also be an active site of inter- and intragenomic homologous recombination in somatic hybrids.

This result stands in contrast to the *atp9*-mediated recombination found in a *Petunia* hybrid (Rothenberg and Hanson 1987). Unlike the *atpA* reported here, *atp9* does not participate in intragenomic recombination within the parental mitochondrial genome. The frequency and/or mechanism of recombination might be different for *atpA*-mediated and *atp9*-mediated recombination.

These two kinds of recombination did, however, give similar results with respect to newly generated DNA sequences. Not all possible combinations between 5′-half and 3′-half segments were detected in the hybrid. According to our data, analysis with *Hin*dIII revealed not nine ($5' : 3 \times 3' : 3$) but six ($5' : 2 \times 3' : 3$) fragments in the hybrid [Fig. 3, panel T (H)]. The *Hin*dIII site (11.0 kb) on a segment of *N. langsdorffii* (5′ *1a*-II) disappeared, as did a *Bam*HI site (23 kb) on it (Fig. 2, panel T); but a novel *Bam*HI site (4.7 kb) was detected instead of the lost *Bam*HI site (23 kb). On the basis of these results, we had to postulate the presence of a new segment, 5′ *1a*-II, in the hybrid (Fig. 4C). These phenomena can probably be explained by other recombination events or by the sorting out of particular molecules, which seems to be caused by the stress of tissue culture or by the absence of origins of replication on the molecules.

Previous data from maize, *Oenothera* and pea showed that sequences outside of the coding sequence of *atpA* are quite different among several types of *atpA,* although all of them share some part of the common coding sequence of *atpA*. The same situation was found in the two species of *Nicotiana* employed in our experiments. Three novel fragments containing the coding sequence of *atpA* were detected by analysis with *Hin*dIII or *Eco*RI (Figs. 3 and 5). Their appearance cannot be explained unless intergenomic recombination occurs at the homologous region. Therefore, we can conclude that the coding region of *atpA* actually mediates intergenomic "homologous" recombination. Since this site is also shown to be involved in intragenomic recombination in *N. langsdorffii,* we can expect that *atpA* is involved in two types of homologous recombination events.

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