Reversible Changes in the Composition of the Population of mtDNAs During Dedifferentiation and Regeneration in Tobacco

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ABSTRACT

Differences in the composition of the population of mtDNAs between green plants and calli of tobacco were detected by DNA filter hybridization analysis. The altered composition of the population of mtDNAs observed in calli returned to the composition typical of green plants during the process of regeneration. Quantitative assays revealed that the changes were associated with the differentiation and dedifferentiation of cells since the extent of the change in composition depended on the degree of differentiation of a population of cells. The sequence that accumulated in dedifferentiated cells was shown to be a product of recombination mediated by a 9-nucleotide repeated element, one of which is located at the 5' region of *atp6*. Although the recombinant sequence was not detected by a hybridization procedure in green plants, its presence was identified by a more sensitive polymerase chain reaction method. The recombination event was shown to result in a deletion that prevents reverse recombination. Therefore, the reversion from the altered composition to the normal state of the population of mtDNAs during regeneration is explained not by recombination but by the preferential amplification of subgenomic mtDNA molecules.

IN contrast to the mitochondrial genomes of animals, which are found as a single-size class, mitochondrial genomes of plants are found, in general, as a heterogeneous population of circular DNA molecules of various sizes (QUÉTIÉ and VEDEL 1977; reviewed by NEWTON 1988). It has been proposed that these multiple circular molecules, the major constituents of the genome, are interconvertible via reactions that involve repeated elements (PALMER and SHIELDS 1984; LONSDALE *et al.* 1984). Stoichiometric differences in mitochondrial DNA (mtDNA) molecules, related to the type of cytoplasm, have been detected in maize (SMALL *et al.* 1987). The evolution of plant mitochondrial genomes has been explained in terms of the recombination, segregation and fixation of subgenomic molecules (SMALL *et al.* 1989). The factors that trigger such rearrangements of the mitochondrial genome remain, however, to be characterized.

Mitochondrial genomes with altered structures have been detected in cultured cells or in regenerated plants of various species (reviewed by HANSON 1984). Cell culture is a powerful tool for the analysis of structural alterations of the mitochondrial genome. Cultured cells may tolerate a mixture of mutated and normal forms of mtDNA, while such a heteroplasmic state might cause a defective phenotype in normal tissues. The association of recombinational changes in the mitochondrial genome with a change in phenotype from sterile to fertile has actually been demonstrated in an analysis of regenerants from the callus of a cytoplasmic male-sterile, type-T line in maize (GENGENBACH *et al.* 1981; FAURON *et al.* 1992). Various types of cell-culture associated

alteration have been suggested, as follows: amplification of molecules present in substoichiometric amounts (VITART *et al.* 1992); complete loss of subgenomic molecules (RODE *et al.* 1987); and rearrangements that might have occurred *de novo* (SHIRZADEGAN *et al.* 1989). The involvement of nuclear genes in the control of such rearrangements has also been reported (HARTMANN *et al.* 1992). Recently, HARTMANN *et al.* (1994) have demonstrated, by utilizing polymerase chain reaction (PCR) amplification, that recombination products that were detected in a regenerated wheat plant by hybridization were already present at a low copy number in both parental plants and tissue cultures.

Among multiple patterns of tissue culture-induced mtDNA reorganizations, amplification and loss of mtDNA sequences during the passage from plant to callus and back again have been suggested based on hybridization experiments (MORÈRE-LE PAVEN *et al.*, 1992). However, the mechanism of this type of alteration has not been elucidated in detail. In the present report, we describe the molecular mechanism responsible for the reversible change in the composition of the population of mtDNAs that occur during the processes of dedifferentiation and regeneration, focusing on the restriction fragments on which *atp6* is located. The sequence that was specifically accumulated in dedifferentiated cells was shown to be the product of recombination that had occurred at the 5' region of *atp6*. This sequence was shown to be present in green plants at levels too low to allow detection by standard hybridization procedures. Data from quantitative assays suggested that the change in the population of mtDNAs was due to a shift in the equilibrium between the product of recombination and

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the progenitor form. Based on these results, a possible mechanism for the structural change and its reversion is proposed.

MATERIALS AND METHODS

Callus culture and regeneration of plants: Calli of *Nicotiana tabacum* L. var. Burley 21 were induced from stem tissue of the plant. Segments of 2-week-old stem tissue were cultured on LS medium (LINSMAIER and SKOOG 1965) that contained (per liter) 3 mg indoleacetic acid (IAA), 3 mg 1-naphthaleneacetic acid (NAA), 0.1 mg kinetin and 3% (w/v) sucrose. The calli were maintained in the dark at 25° and were subcultured at 3-week intervals. To induce regeneration of shoots, pieces of 1-year-old calli were cultured on LS medium that contained 0.1 mg/liter IAA, 1.0 mg/liter kinetin and 3% (w/v) sucrose in the light. After formation of shoots, regenerants were transferred to hormone-free LS medium for formation of roots. All media were solidified with 0.2% (w/v) GELRITE (Scott Laboratories Inc.).

Isolation of nucleic acids: mtDNA was isolated from young leaves and calli essentially as described by UMBECK and GENBACH (1983) with the exception that tissues were homogenized in a Waring blender in an extraction buffer that contained 0.4 M sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% bovine serum albumin and 0.2% 2-mercaptoethanol.

Total DNA was isolated from whole green plants, calli, green calli and regenerated plants by the method of KANAZAWA and TSUTSUMI (1992).

Total RNA was isolated from plants and calli as described by KAWAKAMI and WATANABE (1988).

Construction and screening of an mtDNA library: mtDNAs from green plants and calli of *N. tabacum* were partially digested by *Sau3AI* and completely digested by *EcoRI*, respectively, and ligated into λ Fix II (Stratagene) and pBluescript II phagemid (Stratagene), respectively. The resulting clone banks were screened by either plaque or colony hybridization. The 3.2- and 4.8-kb *EcoRI* fragments that contained *atp6* were cloned using rice *atp6* as a probe. A 4.2-kb *EcoRI* fragment was cloned using the *EcoRI-PstI* fragment, located in the 5' region of *atp6* on the cloned 4.8-kb fragment, as a probe.

Gel blot analysis and quantitative assay: Total DNA digested by restriction endonucleases or total RNA was fractionated by electrophoresis on a 1% agarose gel. Nucleic acids were transferred to nylon membranes and allowed to hybridize with labeled probes. The probes were labeled either with [α -³²P]dCTP using a Multiprime labeling kit (Amersham Corp.) or with digoxigenin using a DIG DNA labeling and detection kit (Boehringer Mannheim). Washing of membranes was performed at a final stringency of $0.1 \times \text{SSC}$, 0.1% sodium dodecyl sulfate at 65° for 30 min. For quantitative assays, membranes were exposed overnight to imaging plates (Fuji Film, Japan) and radioactivity was quantitated with a Bio-imaging analyzer (Fujix BAS 2000; Fuji Film).

Amplification by PCR: PCR was performed using a DNA-amplification system (Perkin-Elmer). Each cycle of PCR consisted of denaturation for 1 min at 94°, annealing for 2 min at 50°, and extension for 2 min at 72°. This cycle was repeated 30 times. The primers for PCR were chosen to amplify the recombination junction: 5'-GGCCAGCAAGAAGGAATG-3' and 5'-GCCTCAAGCCGATAAGTC-3' for the sequence on the 4.8-kb *EcoRI* fragment; and 5'-CTCTGTTGATGCTTGGCC-3' and 5'-AGAATAGCGTGATGGCCG-3' for the putative sequence on the 2.6-kb *EcoRI* fragment. Each primer was designed to be of the same length (18-mer) and the same GC content (55.6%).

FIGURE 1.—Differences in mtDNA extracted from green plants, calli, green calli and regenerated plants. (A) Southern blot analysis. Total DNA from each type of material was digested with *EcoRI*, and after electrophoresis the gel blot was probed with ³²P-labeled cloned *atp6*. (B) Relative amounts of the 3.2- and 4.8-kb *EcoRI* fragments on which *atp6* was located, as measured by the radioactivity of bands on the hybridized blot. The data for calli and green calli are mean values obtained from five and three subcultured lines from a single explant, respectively, and those for regenerated plants were obtained from six regenerated plants.

DNA sequence analysis: DNA fragments were subcloned into pUC19. DNA sequencing was performed with a DNA sequencer (Applied Biosystems). DNA sequencing data were analyzed using the GENETYX program (Software Development Corporation, Japan).

RESULTS

Reversible changes in mtDNA population: We screened for changes in the mtDNA population by comparing the Southern hybridization profiles of total DNA from green plants and calli that had been probed with several fragments of mtDNA (data not shown). In this study we focused on one clear-cut alteration, a polymorphism that was detected when *atp6* was used as the probe (Figure 1A). The alteration was detected as follows: the *atp6* probe hybridized only with the 3.2-kb *EcoRI*-generated fragment from the DNA from green plants, whereas, in the DNA from calli, a prominent 4.8-kb hybridizing signal was newly recognized while the 3.2-kb fragment hybridized with the probe at a reduced level. We monitored the heteroplasmic state observed in dedifferentiated cells during the process of plant regeneration. All of six analyzed regenerated plants gave profiles similar to that of green plants although some of them retained the 4.8-kb hybridizing fragment at a low level. Green calli, being intermediate between calli and regenerated plants, gave an intermediate pattern of hybridization. Since green calli and regenerated plants can

FIGURE 2.—Comparison of the 3.2- and 4.8-kb *EcoRI* fragments. (A) Restriction maps of the 3.2- and 4.8-kb *EcoRI* fragments. The abbreviations are: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I. The distance from the *Bam*HI site is indicated in base pairs. (B) Comparison of the nucleotide sequences of the 4.8-kb (top) and 3.2-kb (bottom) fragments in the region of the rearrangement. Identical bases are indicated by asterisks. The sequences are numbered from the *Bam*HI site on each fragment.

be considered to be partially and almost completely differentiated, respectively, these results suggested the dependence of the alteration in mtDNA population on the extent of dedifferentiation. We analyzed the fluctuations in levels of signals from the 3.2- and 4.8-kb fragments quantitatively by Southern hybridization (Figure 1B) with the 1.8-kb *Bam*HI-*Eco*RI fragment that contained *atp6*, a region common to both fragments, as probe (Figure 2A, see below). This assay clearly showed the association of the alteration with the differentiation and dedifferentiation of cells. A few, more weakly hybridizing signals were detected in the low molecular weight region of the blots after lengthy exposure. However, these signals did not differ among the experimental materials (not shown).

Molecular properties of the alteration in the mtDNA population: To characterize the reversible change in the composition of the population of mtDNAs, we cloned the 3.2- and 4.8-kb *Eco*RI fragments of mtDNA and analyzed them. The restriction maps of these fragments showed that they were identical between the *Eco*RI and *Bam*HI sites located in the 3' and 5' region of *atp6*, respectively (Figure 2A). The map of this region was also identical to that of tobacco *atp6* that had previously been analyzed by BLAND *et al.* (1987). The difference at an *Eco*RI site was detected in the upstream

FIGURE 3.—Origin of the novel 4.8-kb *Eco*RI fragment that accumulated in dedifferentiated cells. Regions derived from the 3.2- and 4.2-kb fragments are indicated by open and stippled boxes, respectively. Repeated units are indicated by black boxes.

region of *atp6*. The nucleotide sequences of the region 5' of the *Bam*HI site of both fragments were analyzed and these fragments were found to share the same sequence for 795 bp from the *Bam*HI site (Figure 2B). Thus, the 4.8-kb fragment appeared to be the product of a recombination event that involved at least the 5' region of *atp6* in the 3.2-kb fragment. To identify the sequence that underwent recombination with the sequence on the 3.2-kb fragment, a mtDNA library was screened with the sequence that was located on the 4.8-kb fragment but not on the 3.2-kb fragment as the probe, namely, the *Eco*RI-*Pst*I fragment located in the 5' region of *atp6* on the cloned 4.8-kb fragment. As a result, a 4.2-kb *Eco*RI fragment was cloned (Figure 3). Southern analysis showed that this fragment was the single candidate for the counterpart in the recombination and, moreover, that the level of the 4.2-kb fragment was lower in calli than in green plants (see Figure 5A). This analysis also revealed that the predicted 2.6-kb *Eco*RI fragment was missing from both green plants and calli, suggesting that the recombination event was accompanied by a deletion.

The recombination junction was identified by sequence analysis to be a 9-nucleotide repeated sequence (Figure 4A). The recombination event may have occurred within only a 4-bp sequence since one mismatched pair was observed at the center of the 9-nucleotide repeat. A CCCCTCCCC motif, identical to that found at recombinational hot spots in mtDNA of *Neurospora* (ALMASAN and MISHRA 1991), was located in this region. In addition to the similarity in repeated elements, the sequence around the recombination junction on the 3.2-kb fragment could also form a stem-loop secondary structure (Figure 4B), as is also the case in

FIGURE 4.—Nucleotide sequences and secondary structure of recombination junction. (A) Nucleotide sequences of the 3.2-, 4.2- and 4.8-kb *EcoRI* fragments that surround the recombination junction. Vertical lines are drawn between homologous bases of the 3.2- and 4.2-kb fragments within the junction sequence. (B) Potential secondary structure at the recombination junction of the 3.2-kb *EcoRI* fragment. A wavy line highlights the 9-nucleotide repeat element.

Neurospora. The recombinant 4.8-kb fragment was not identified in DNA from green plant cells by Southern analysis, as shown in Figure 1. However, we postulated that it might be present at a very low level, below the limits of detection of the hybridization procedure. To check this possibility, an experiment involving PCR was performed, using primers designed to amplify the recombinant sequence. A fragment of the expected size was amplified from green plants, as well as from calli, indicating that the recombinant sequence did exist in green plant cells (Figure 5B). We examined the result of PCR for fear that the recombinant sequence could be an artifact that was generated in the test tube during amplification. We ran a control reaction using the cloned 3.2- and 4.2-kb fragments and found that the recombination did not occur under our PCR conditions. Furthermore, *EcoRI*-digested total DNA of green plants was separated by gel electrophoresis and only 4.8-kb fraction was used as a template for PCR. In consequence, a fragment of the expected size was amplified using this template, confirming the presence of recombinant sequence in the mtDNA of green plants (not shown). The possibility of the presence at a very low amount was also examined for the putative recombination-related sequence on the 2.6-kb fragment. In this case, however, no amplification product of predicted size (298 bp) was obtained (not shown), suggesting that the recombination event was accompanied by a complete deletion.

Transcription of *atp6* was examined by Northern analysis. Transcripts of identical size were detected in total RNA from green plants and calli (Figure 5C), indicating that the alteration had no effect on the pattern of transcription of this gene.

DISCUSSION

From our analysis of mtDNA in green plants and calli, we postulated that the 3.2- and 4.8-kb fragments were located on different molecules for the following two reasons: first, the sequence of the 4.8-kb fragment was the product of recombination between the 3.2- and 4.2-kb fragments; and second, the relative levels of the 3.2- and 4.8-kb fragments varied with respect to one another, as shown in Figure 1. The 3.2- and 4.2-kb fragments are probably located on the same circular molecule, a

FIGURE 5.—Characterization of recombination. (A) Southern blot analysis showing the deletion that occurs as a result of the recombination between the 3.2- and 4.2-kb fragments. Total DNA from green plants (lane 1) and calli (lane 2) was probed with a digoxigenin-labeled 1.0-kb *BglII* fragment located on the 4.2-kb *EcoRI* fragment. (This 1.0-kb fragment is the rightmost *BglII* fragment shown on the 4.2-kb *EcoRI* fragment in Figure 3.) (B) Amplification by PCR of the product of the recombination between the 3.2- and 4.2-kb fragments from green plants (lane 1) and calli (lane 2). PCR was performed using primers that had been designed to amplify the recombinant sequence that was based on the sequence of the 4.8-kb fragment. ϕ X: ϕ X174 DNA was digested with *HaeIII* to generate size markers. (C) Northern blot analysis of transcripts of *atp6*. A gel blot of total RNA (10 μ g) from green plants (lane 1) and calli (lane 2) was probed with the digoxigenin-labeled 1.8-kb *BamHI-EcoRI* fragment that contained *atp6*.

configuration that allows a loop-out type of rearrangement, since deletion occurred upon recombination between the sequences on these fragments. Southern analysis with *atp6* as probe suggested the existence of an equilibrium between the molecules on which the 3.2- and 4.8-kb fragments are located, respectively (Figure 1). This possibility is supported by the results of rehybridization of the same filter with other probes for mitochondrial genes. In these cases, the ratio of the sum of the amount of the 3.2- and 4.8-kb fragments to the amount of other fragments was almost constant (not shown). Such an observation is very similar to the case of *atpA* in maize, which has been shown to be located in at least four genomic environments and to have varied quantitatively during evolution (SMALL *et al.* 1987).

From the above findings, we propose two models that most simply explain the mechanism responsible for the changes in the mitochondrial genome during dedifferentiation and regeneration. In one model, the recombination between the 3.2- and 4.2-kb fragments is en-

FIGURE 6.—Possible mechanisms for the generation of a change in the composition of the population of mtDNAs and the reversal of such a change. (A) Promotion of recombination within a molecule of mtDNA. This model cannot explain the reversal of the change since one of the products of recombination is missing. (B) Selective amplification of a recombinant or of its progenitor forms of mtDNA molecules.

hanced in dedifferentiated cells, as shown in Figure 6A. As a consequence, the amount of molecule A is decreased while molecule B forms a majority in the population of mtDNAs in calli. The sequence located on the hypothetical molecule C is lost as a result of the recombination since both Southern analysis and amplification by PCR revealed the absence of the 2.6-kb fragment that should be located on molecule C. This sequence probably has a disadvantage in replication, for example, lack of an origin of replication. The observed recombination event, via a short repeat, that was accompanied by a deletion is quite similar to those reported to occur in the mitochondria of other organisms, such as yeast (DE ZAMAROCZY *et al.* 1983), *Neurospora* (ALMASAN and MISHRA 1991), and human (SHOFFNER *et al.* 1989), as well as in nonchromosomal stripe (NCS) mutants in maize (NEWTON *et al.* 1990) and chloroplast mutator (*CHM*) of *Arabidopsis* (MARTÍNEZ-ZAPATER *et al.* 1992). Illegitimate recombination, which is considered to be mediated either by slipped mispairing of short direct repeats during replication (ALBERTINI *et al.* 1982) or by the topoisomerase-catalyzed breakage and rejoining of DNA (SHUMAN 1989), may be responsible. The second model that provides an explanation for our results involves the preferential amplification of molecule A or molecule B due to selective replication and/or transmission during mitochondrial division. Amplification by PCR revealed that the recombinant 4.8-kb fragment was already present at very low levels in green plant cells (Figure 5B). The recombination event may have occurred during

evolution of this plant species, or it might occur infrequently in green plant cells. Thus, the recombination event described above is not necessarily required *de novo* for the accumulation of molecule B in dedifferentiated cells.

The proposed shift in equilibrium can be explained by either of the two models. The events that occurred during the process of regeneration cannot, however, be explained by the *de novo* recombination model since no significant amount of molecule C was found that could drive recombination in the reverse direction. Accordingly, the mechanism responsible for the alteration in the reverse direction is probably explained by the second model.

In animals, it has been shown that accumulation of mutated mtDNA molecules above a threshold level causes mitochondrial dysfunction if the mtDNA molecules are defective (reviewed by WALLACE 1992). The sorting out of recombinant molecules during the process of regeneration suggests that the state represented in the high level of the 4.8-kb fragment may not be suitable for plant growth, and possibly not for optimal mitochondrial function. The composition of the population of mtDNAs may be less strictly regulated in the dedifferentiated cells. In this regard, we recall that certain human cells can survive even without mtDNA when cultured in a medium rich in glucose, supplemented with pyruvate and uridine, with the synthesis of ATP depending exclusively on glycolysis (KING and ATTARDI 1989). Likewise, perhaps plant tissue culture medium permits the growth of cells which lack normal mitochondrial function.

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