Male-sterility induction in transgenic tobacco plants with an unedited atp9 mitochondrial gene from wheat

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ABSTRACT Cytoplasmic male sterility in plants is associated with mitochondrial dysfunction. We have proposed that a nuclear-encoded chimeric peptide formed by mitochondrial sequences when imported into the mitochondria may impair organelle function and induce male sterility in plants. A model developed to test this hypothesis is reported here. Assuming that the editing process in higher plant mitochondria reflects a requirement for producing active proteins, we have used edited and unedited coding sequences of wheat ATP synthase subunit 9 (atp9) fused to the coding sequence of a yeast coxIV transit peptide. Transgenic plants containing unedited atp9 exhibited either fertile, semiferile, or male-sterile phenotypes; controls containing edited atp9 or only the selectable marker gave fertile plants. Pollen fertility ranged from 31% to 75% in fertile plants, 10% to 20% in semiferile plants, and <2% in male-sterile plants. Genetic and molecular data showed that the chimeric plasmid containing the transgene is inherited as a Mendelian trait. The transgenic protein is imported into the mitochondria. The production and frequency of semiferile or male-sterile transgenic plants conform to the proposed hypothesis.

Male sterility in plants leads to pollen abortion. This phenomenon is often observed in alloplasmic plants combining cytoplasmic and nuclear genetic material from two different species (1). Maternally inherited cytoplasmic male sterility (CMS) has been recently reviewed (2). Molecular studies on CMS have revealed the existence of modifications in mitochondrial DNA. In a number of CMS plants, the sterile phenotype is associated with the production of proteins arising from chimeric genes (3, 4); their incorporation into the mitochondrial membrane or into multiprotein enzyme complexes may lead to the impairment of mitochondrial function.

The hypothesis developed in this report is that the impairment of mitochondrial function may be obtained by introducing an altered subunit in the ATP synthase complex. The protein ATP9 represents a good candidate because it is involved in the proton channel of the ATP synthase. ATP9 is mitochondrial encoded in plants but nuclear-encoded in Neurospora (5) and mammals (6). A mitochondrially encoded protein can be introduced into nuclei and imported into mitochondria when fused to a targeting sequence (7, 8). An approach similar to the one described here has been used to introduce herbicide resistance into tobacco chloroplasts (9).

We have shown a discrepancy between the amino acid sequence of the ATP9 protein and the predicted sequence deduced from the gene. The ATP9 is translated from a posttranscriptionally modified mRNA by C → U transitions at some codons, by a process called RNA editing (10, 11).

Given that mRNA editing occurs in most plant genera (for a review, see ref. 12), one can suppose that proteins synthesized from unedited messengers may have a modified (inactive or less active) function. With this in mind, transgenic plants were constructed with genomic (unedited) and cDNA (edited) coding sequences of atp9 fused to a yeast mitochondrial signal sequence under the control of cauliflower mosaic virus (CaMV) 35S promoter. Protein targeting to plant mitochondria by a yeast presequence has been described (13). Transgenic plants were analyzed phenotypically with special attention to the male-fertility trait. Additionally, genetic and molecular analyses were performed to show the inheritance and the expression of alien genes. This use of a transgenic mitochondrial gene to induce the male-sterile phenotype has not been reported previously to our knowledge. This working model may be useful to understand the role of the mRNA editing process.

MATERIALS AND METHODS

Plant Material. Nicotiana tabacum cv. Petit Havana line SR1 (14) was maintained as an axenic shoot culture in hormone-free MSoo medium (15). Fully expanded leaves of 1-month-old plants were used for protoplast isolation.

Culture Medium. Protoplasts were cultivated on K3/H medium [1:1 (vol/vol) mixture of K3 (16) and H media (17) solidified with 0.8% SeaPlaque agarose]. Resistant calli were selected as described (18). Regenerated plants were rooted in T medium (19). All cultures were performed in the presence of hygromycin (20–40 mg/liter). For genetic analysis, seeds of transgenic plants were sown on MSoo medium containing hygromycin. Resistant plants were scored from 4-week-old seedlings.

Protoplast Isolation and Plant Regeneration. Leaf mesophyll protoplast transformation and selection of resistant colonies were performed as described (20). Regenerants were rooted and transplanted in soil until flowering.

Phenotype and Fertility Analyses. The plant phenotype was evaluated as the size of the plants, the number of nodes, and the color of leaves. The size and number of nodes were scored from soil surface to the emergence of the inflorescence. For generative organs, the shape and color of flowers including corolla, anthers, and pistil were analyzed.

The ability of pollen to germinate was determined from just-opened flowers. Pollen from two to four flowers was placed on 5% sucrose solution containing 3.75 ppm of H2BO3 (pH 5.8). The pollen grains were incubated at 28°C for 2–4 hr; >500 pollen grains were observed randomly for each line tested. The female fertility trait of male-sterile lines was determined by back-crossing with wild-type SR1 as the male parent. Fertility was determined according to the seed production per capsule; 5–20 capsules per plant were scored.

Genetic Analysis. Genetic segregation of the hygromycin phosphotransferase (aph) gene was determined in offspring (200–500 seedlings). Back-crossing was performed by emas-

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calculation just before flower opening when anthers were still closed. Capsules or seeds were treated as described (21). Seeds were sown on MSoo supplemented with 40 mg of hygromycin per liter. Seedlings were scored for hygromycin resistance 1 month after sowing.

Plasmid DNA. The recombinant plasmids used in these experiments are summarized in Fig. 1A. The atp9 coding sequences were obtained from the cDNA corresponding to the edited and unedited forms of wheat mitochondrial mRNA (11). The atp9 was fused to a 303-base-pair (bp) EcoRI/Kpn I from plasmid 19.4 (22) including codons 1–62 from yeast coxIV. The resulting fragment, recovered by HindIII digestion, was ligated into the Sma I restriction site of PDH51 plasmid (23). The hygromycin gene was inserted into the HindIII site of PDH51, pEA903 (edited form of atp9), and pEA904 (unedited form), giving rise to pH1, pH2, and pH5, respectively. All of the genes were under the control of the CaMV 35S promoter and the terminator of gene VI from CaMV.

**PCR Analysis.** The oligonucleotide primers used for amplification were (i) 5'-CCTAGTCATCTTATAAG-3', spanning codon 3–9 of the coxIV presequence; and (ii) 5'-TATGGCTAAGACATGAGCG-3' located at the CaMV terminator gene VI (45 bp upstream of the polyadenylation signal).

Total DNA was isolated from 10 g of leaf tissue essentially as described (24). One microgram of DNA was amplified in a final volume of 100 µl by using 0.5 units of Taq I polymerase (Stratagene), 0.18 mM each dNTP, and 100 pmol of each amplification primer. The denaturation step was at 95°C for 1 min, the annealing step was at 52°C for 2 min, and the polymerization step was at 72°C for 1 min. Twenty-five cycles were performed. Samples were electrophoresed through 1.5% agarose gel and transferred to Hybond-N+ (Amersham) as described (24). Filters were prehybridized at 42°C in 50% deionized formamide containing 5× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 8× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.02% Ficoll), and 0.5% SDS. The blots were hybridized with a 32P-labeled EcoRI/HindIII atp9 300-bp coding sequence.

**RNA Extraction and Gel Blot Analysis.** RNA was extracted from leaves. Five grams of tissue was ground with a pestle and mortar in liquid nitrogen. Frozen powder was extracted with 5 ml of phenol/chloroform/isooalcohol, 25:24:1 (vol/vol), and 5 ml of TNES + DTT (0.1 M NaCl/10 mM Tris-HCl, pH 7.5/1 mM EDTA/0.1% SDS containing 2 mM dithiothreitol). The aqueous phase was extracted twice with an equal volume of chloroform/isooalcohol, 24:1 (vol/vol), and RNA was precipitated with an equal volume of 4 M lithium chloride at 0°C overnight. The RNAs were dissolved in diethyl pyrocarbonate-treated water. RNA concentration was measured as A260. The poly(A)+ RNAs were purified by oligo(dT)-cellulose affinity chromatography (25). Twenty micrograms of total RNA and 1 µg of poly(A)+ RNA were electrophoresed in formaldehyde/formamide 1.5% agarose gels as described (25) and transferred to nylon membranes Hybond-N+ (Amersham). Hybridizations with the atp9 probe was performed as described above.

**Isolation of Proteins and Protein Gel Blot Analysis.** A Xba I/Kpn I fragment containing the yeast coxIV codons 21–54 was isolated from plasmid 19.4 (22). This fragment was ligated to the plasmid pGEX-A (26) in frame with the glutathione S-transferase open reading frame. The fusion protein was induced after transformation of Escherichia coli DH5α cells and purified as described (26). The fusion protein was used as antigen to produce anti-COXIV antibodies in rabbits. Leaves of greenhouse-grown plants were used for cell fractionation as described (27). One hundred micrograms of cytosolic and mitochondrial proteins were fractionated by urea/SDS/PAGE (28). Proteins were electroblotted onto Immobilon-P (Millipore) membrane (29). Immunoreaction was performed by using a 1:500 dilution of anti-COXIV antisera according to Darley-Umar et al. (28). Peroxidase anti-rabbit IgG-conjugated antibody was used to visualize cross-reactivity.

**RESULTS**

**Transformation and Culture.** Plasmid constructs used for protoplast transformation are shown in Fig. 1A. The wheat atp9 coding sequence is very similar to tobacco atp9 (30), with three conservative changes at positions 8, 20, and 22. Five major changes between pH 2 and pH 5 constructs arose from editing events at positions 28, 45, 64, and 71 and the creation of a stop codon. The "unedited" protein is more...
hydrophilic with a six-residue extension at the carboxyl terminus (Fig. 1B).

Tobacco protoplasts from leaves of the SR1 line were transformed with plasmids pH1, pH2, and pH5 (Fig. 1A) by direct gene transfer (21). The protoplast-derived cells were placed under selective conditions. The frequency of regeneration of pH1, pH2, and pH5 transformant lines was generally >50%. Best growing green plants were transferred to soil.

**Phenotype Analysis of Transgenic Plants.** The sizes of 14-week-old plants are reported in Table 1 for H1 (control), H2, and H5 lines; the plant sizes are not significantly different from untransformed SR1. The average node number is similar in the three different transgenic lines (19–24 nodes per plant). Apparently there is no change in functioning of the vegetative meristems in differentiating nodes and leaves of the transgenic plants.

Flowering in H1, H2, and H5 lines was induced 7–14 weeks after transplanting. The flowers of transgenic plants were similar in shape and color to those of SR1 plants, with five reddish-pink petals and five anthers in each flower. Male-sterile plants had white anthers, whereas fertile plants had yellowish-white anthers with normal pollen grains (Fig. 2A). There was no difference in form and color of the pistil between male-sterile and male-fertile plants.

**Fertility Analysis of Transgenic Plants.** H1 and H5 transformants produced fertile plants, whereas H2 transformants exhibit sterile, semi-sterile, or sterile traits, defined on the basis of pollen germination or by the fluorescein diacetate reaction (31). In fertile transgenic plants, pollen viability ranged between 31% and 75%, close to the values found in the SR1 control; in semi-sterile plants, pollen viability was around 10% to 20%; in male-sterile plants, the viability was generally <2%.

The fertility of plants was also determined by seed production after self pollination or back-crossing. Results are reported in Table 1. H1 and H5 lines have an average seed production of 100 mg per capsule, comparable to SR1 controls (110 mg per capsule). In H2 lines, sterile plants produced no seeds, semi-sterile plants produced between 12 and 50 mg per capsule; and fertile plants produced on average 100 mg per capsule. These values correlate with pollen viability.

The female fertility trait for sterile and semi-sterile plants was determined by back-crossing with SR1 as the male parent. All male-sterile plants were female-fertile and produced a normal quantity of viable seeds (63–92 mg per capsule) with a seed viability value >77%. Thus, the sterile and semi-sterile trait in 50% of H2 lines was due to the absence or very low production of viable pollen.

The inheritance of the transgenes was analyzed through hpt segregation in offspring. After selfing, fertile and semi-sterile plants inherited hpt as a single Mendelian trait (ratio 3:1).

**Table 1. Phenotype characterization of transgenic plants**

<table>
<thead>
<tr>
<th>Lines*</th>
<th>Plants tested, no.</th>
<th>Plant fertility, †%</th>
<th>Growth, cm</th>
<th>Node, no.</th>
<th>Seeds, ‡ mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR1</td>
<td>100</td>
<td>0</td>
<td>87.0</td>
<td>24</td>
<td>109 ± 36</td>
</tr>
<tr>
<td>H1</td>
<td>100</td>
<td>0</td>
<td>120 ± 6</td>
<td>19 ± 1</td>
<td>108 ± 14</td>
</tr>
<tr>
<td>H2</td>
<td>16</td>
<td>50</td>
<td>103 ± 26</td>
<td>19 ± 2</td>
<td>25 ± 17</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5</td>
<td>100</td>
<td>0</td>
<td>92 ± 23</td>
<td>23 ± 5</td>
<td>94 ± 28</td>
</tr>
</tbody>
</table>

*Lines H1, H2, and H5 are transgenic plants obtained with plasmids pH1, pH2, and pH5, respectively. Control SR1 is the untransformed plant.
†F, fertile; F/S, semi-fertile; S, male sterile.
‡Mean value of seed production per capsule after self-pollination.

**FIG. 2.** Morphology of pollen grains from the male-sterile H2 plant (A) and the fertile H5 plant (B) under identical magnification.

After back-crossing, four of five male-sterile plants inherited hpt as a digenic trait (ratio 3:1 instead of 1:1 as expected for a single locus), thus expressing two active loci; one of them showed a monogenic inheritance.

**Molecular Analysis of Transforms.** To ascertain the presence and the transcription of the atp9 transgene, Southern and Northern blotting experiments were performed. Total DNA was isolated from H2 (sterile, semi-fertile, and fertile) and H5 lines. The chimeric gene was analyzed by polymerase chain reaction (PCR) amplification. A primer located at the coxIV presequence and another at the terminator region of the gene Vf from CaMV were used. The use of these primers excludes the amplification of the endogenous atp9. A band of 700 bp was observed in most H2 and H5 lines as expected. Fig. 3A shows the results obtained with H2.2 and H2.16 DNA from male-sterile plants (lanes 1 and 2) and H5.6 and H5.15 DNA from fertile plants (lanes 3 and 4). DNA from an untransformed SR1 plant gave no signal (lane 5).

Total RNA from H2, H5, and untransformed SR1 lines was extracted and probed with the atp9 coding sequence. A band of 480 nt obtained with control SR1 is shown in lane 1 of Fig. 3B. This band was present in all lines (not shown) and corresponds to endogenous mitochondrial mRNA. An additional transcript of 980 nt was present only in transformed plants. These molecules can be separated from endogenous mRNA by oligo(dT)-cellulose chromatography, confirming its cytoplasmic origin (Fig. 3B). Lanes 3 and 4 of Fig. 3B show the results obtained for H2.2 and H2.16 male-sterile plants, and lanes 5 and 6 show results for H5.6 and H5.15 fertile plants. The 980-nt transcript is absent from the untransformed control (lane 2). PCR experiments on cDNA with the primers described for genomic analysis confirm that the poly(A)‘ atp9 transcripts originate from the chimeric genes. Moreover, only the 980-nt transcript hybridized with a probe from the yeast coxIV sequence fused to atp9 (not shown).

To understand whether the transgene affects the expression of the endogenous atp9 mitochondrial gene, total RNA from transformed H2, H5, and control plants was blotted and hybridized with a mitochondrial-specific probe (see Materials and Methods). No substantial differences were observed whether the transgene was edited or unedited, and the labeling was similar to the control (Fig. 3C).

The production of transgenic protein was analyzed by immunoblotting of cytosolic and mitochondrial extracts. Antibodies directed against residues 21–54 from yeast COXIV were used. No signal was observed either in the mitochondrial fraction (Fig. 4, lane 1) or in the cytosolic fraction (not shown) of the untransformed SR1 line. The mitochondrial
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Fig. 3. (A) PCR amplification analysis of total DNA from transgenic tobacco plants. Total DNA from tobacco plants was prepared as described and subjected to PCR amplification with amplification primers located at the coxIV presequence and CaMV gene VI terminator. Amplification products were electrophoresed on agarose gel, transferred into nylon membrane, and hybridized with an atp9 coding probe. Lanes: 1 and 2, DNA from sterile lines H2.2 (lane 1) and H2.16 (lane 2); 3 and 4, DNA from fertile lines H5.6 (lane 3) and H5.15 (lane 4); 5 and 6, controls with DNA from untransformed SR1 plants (lane 5) and plasmid pH2 (lane 6). (B) RNA gel blot analysis. Lanes: 1, control with total RNA from an untransformed SR1 plant; 2, poly(A)+ RNA fraction of a SR1 plant; 3 and 4, poly(A)+ RNA from sterile lines H2.2 (lane 3) and H2.16 (lane 4); 5 and 6, fertile lines H5.6 (lane 5) and H5.15 (lane 6). The atp9 coding sequence was used as probe. nt, Nucleotides. (C) RNA slot-blot analysis. Total RNA from H2, H5, and SR1 lines was blotted on a nylon membrane and probed with the untranslated 3' region of tobacco mitochondrial atp9 mRNA. Slots: 1, control SR1; 2 and 3, male-sterile H2 lines; 4, semisterile H2 line; 5 and 6, fertile H5 lines.

fraction from the male-sterile H2.2 and male-fertile H5.15 lines (lanes 2 and 4, respectively) showed a 12-kDa band consistent with the size expected for a processed protein. The cytosolic proteins from these lines (lanes 3 and 5) showed two bands, one being at 15 kDa, the size expected for the chimeric precursor polypeptide, and the other being at 14 kDa. The nature of this polypeptide remains to be determined; it is probably a degradation product from the 15-kDa precursor. The protein associated with the mitochondrial fraction from H5.15 (lane 4) migrated slightly ahead of the H2.2 mitochondrial protein. This difference can be due to the fact that the chimeric genes differ in the position of the stop codon. The edited protein is six residues shorter than the unedited one due to a codon stop generated by RNA editing (11).

**DISCUSSION**

The expression in transgenic tobacco plants of an exogenous wheat mitochondrial atp9 DNA sequence seems to have no effect on most phenotypic characteristics. The size, growth rate, node number, shape, and size of leaves and flowers are similar in transgenic and control plants. However, dramatic effects were observed at the level of male reproductive organs when unedited wheat atp9 sequence was expressed in tobacco plants. Indeed, transformation experiments performed with plasmid pH2 lead to the production of many plants (50%) severely modified in their fertility. Approximately 19% were semisterile, and 31% were entirely sterile. All semisterile and sterile H2 lines expressed the transgene as a polyadenylated mRNA. Fertile H2 lines did not exhibit the 980-nt transcript even when the transgene was detected after PCR amplification (not shown), thus indicating that the transgene was inactive.

In plant mitochondria, the atp9 mRNA is post transcriptionally edited before translation (11). This fact suggests that the editing process is necessary to assure the synthesis of a functional polypeptide. Thus, one can assume that the translation product from an unedited mRNA may be partially or totally nonfunctional. In tobacco plants the nuclear-located transgene should be translated in cytoplasm without codon modifications, since RNA editing in plants has been reported only in mitochondria (12) and chloroplasts (32).

The chimeric protein is addressed into the mitochondrial compartment by the yeast COXIV presequence. Precursor forms (15 kDa) were detected in the cytoplasm. The protein associated with the mitochondrial fraction, either in edited or unedited form, was probably processed, since the transgenic protein migrates at the position of 12 kDa—the size expected if the protein processing occurs as described in yeast (22).

The transgene protein differs in the amino-terminal portion from the native ATP9 (Fig. 1A) by 37 additional residues derived from the yeast COXIV and by the presence of 8 amino acids corresponding to codons where cytidine residues remain unedited. The amino-terminal extension has no effect on the transformants, since this sequence is present in both H2 and H5 transformants. Our results indicate that the male-sterile phenotype correlates only with the presence of unedited sequences of atp9, while transformants obtained with the edited form of atp9 were all fertile. In all cases, sterile plants are solely male sterile; they can be pollinated with alien pollen, thus reflecting a normal female fertility.

Our working hypothesis was that a modified ATP9 should compete with the endogenous protein in constituting the ATP synthase complex to create a mixed population of functional and nonfunctional complexes. According to the ratio of endogenous and exogenous protein, the number of "unedited" ATP9 proteins in the proton channel may affect the functioning of ATP synthase and consequently reduce
ATP production. As the energy requirement is maximal in tapetal cells during microsporogenesis, the low percentage of viable pollen in semisterile plants or the total pollen abortion in some male-sterile plants could be the consequence of the reduced mitochondrial function in transgenic tobacco plants.

Although protein processing seems to occur properly in the mitochondrial compartment, suggesting that the transgenic protein is correctly imported into the organelle, other explanations are possible. For example, the insertion of modified ATP9 (with its hydrophobic character) in the inner membrane could affect the membrane potential necessary for ATP synthesis. This is improbable because the "edited" form of the protein is more hydrophobic than its "unedited" counterpart, as revealed by hydrophatic analysis (not shown).

The reports concerning altered electron transport in Petunia (2) and the toxin-dependent effect of the *urf 13* gene product on mitochondrial membrane permeability (33, 34) suggest that mitochondrial dysfunction could be the first symptom of the male-sterile phenotype. Although different in molecular terms, this situation could be compared to that observed in mammals where single-base changes or deletions in mtDNA can result in tissue-specific diseases (35).

In conclusion, the transformation experiments performed with unedited and edited forms of wheat *atp9* coding sequences have shown that the chimeric genes are integrated into the nucleus of the host plant and expressed, as shown by Northern and immunoblot analyses. The plasmid construct bearing the unedited form of *atp9* severely affects male fertility in tobacco plants. This important observation implies that the introduction of modified mitochondrial information, engineered to be addressed to the organelle, may induce male sterility through pollen abortion. This is an innovative way to introduce such a phenotype. Moreover, this approach constitutes an exciting model for studies concerning the function of the RNA editing process in plants.

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