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Transgenic male-sterile plant induced by an unedited *atp9* gene is restored to fertility by inhibiting its expression with antisense RNA

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ABSTRACT **We have previously shown that the expression of an unedited *atp9* chimeric gene correlated with male-sterile phenotype in transgenic tobacco plant. To study the relationship between the expression of chimeric gene and the male-sterile trait, hemizygous and homozygous transgenic tobacco lines expressing the antisense *atp9* RNA were constructed. The antisense producing plants were crossed with a homozygous male-sterile line, and the F1 progeny was analyzed. The offspring from crosses between homozygous lines produced only male-fertile plants, suggesting that the expression antisense *atp9* RNA abolishes the effect of the unedited chimeric gene. In fact, the plants restored to male fertility showed a dramatic reduction of the unedited *atp9* transcript levels, resulting in normal flower development and seed production. These results support our previous observation that the expression of unedited *atp9* gene can induce male sterility.**

The development of strategies to improve crop plants by the production of hybrid varieties is a major goal in plant breeding. Hybrid progeny often have a higher yield, increased resistance to disease, and an enhanced performance in different environments compared with the parental lines (1). Male sterility mutations that guarantee the outcrossing of naturally autogamic plant lines (2) have proven to be useful for the production of hybrid lines with increased crop productivity. However, hybrid production is limited to those species of plants in which male sterility mutations have been detected. Moreover, in certain plants, such as corn, wheat, rice, or tomato, where seeds and fruits are harvested products, a male fertility restorer system is required.

Many male sterility mutations interfere with tapetal cell differentiation and/or function, indicating that this tissue is essential for the production of functional pollen grains (3–7). The tapetal cells are the target to produce engineered male-sterile plants. In view of this, several authors have reported the production of transgenic male-sterile plants by the expression of degradative enzymes in tapetal cells (8–10) or by the inhibition of particular enzymes by antisense strategies (11).

One interesting, naturally-occurring phenotype is cytoplasmic male sterility. Molecular analysis of cytoplasmic male sterility plants has shown the presence of chimeric genes arising from rearrangement of the mtDNA. The production of proteins encoded by the chimeric genes has been correlated with male-sterile phenotype, and it has been proposed that it affects mitochondrial function (12–14).

The different nature of the genes involved in cytoplasmic male sterility plants reported so far suggests that mitochondrial impairment may be the common consequence of the different chimeric gene products. If this is true, then a mitochondrial dysfunction could also be produced by other means and male-sterile plants could be obtained. Recently, we have reported the production of male-sterile tobacco plants by expression of the unedited coding region of mitochondrial ATPase subunit 9 gene (*atp9*), fused to a mitochondrial transit sequence (15). RNA editing in plant mitochondria involves the substitution of some C residues present in the initial transcript for U residues. The mitochondrial protein sequences predicted from edited transcripts are more similar to homologous proteins from other organisms than are proteins predicted from unedited mRNAs (16, 17). This observation suggests that the editing process assures the synthesis of functional mitochondrial proteins. In transgenic tobacco plants, the expression of the unedited form of *atp9* (*u-atp9*) causes a mitochondrial dysfunction that affects normal anther development, specifically in the tapetal cell layer, and reduces pollen formation (unpublished results).

In this article, we report the restoration to male fertility of *u-atp9*-expressing male-sterile plants by using antisense RNA technology (18). We show that crossing male sterile plants containing *u-atp9* transgene with transgenic plants containing the same gene in antisense orientation (*as-atp9*) produces progeny that is restored to male fertility. Antisense RNA inhibits *u-atp9* gene expression and thus abolishes the deleterious effects of this gene, resulting in restoration to male fertility. The *as-atp9* gene acts as a dominant male fertility restorer gene.

MATERIALS AND METHODS

Materials. The transgenic plants used were constructed as described (15). They contained either plasmids pH2 or pH4 (see Fig. 1) carrying respectively the chimeric unedited *atp9* DNA in sense (*u-atp9*) or antisense orientation (*as-atp9*) under the control of the 35S cauliflower mosaic virus (CaMV) promoter. Experiments were performed using greenhouse-grown transgenic male-sterile, male-fertile, restored male-fertile, and untransformed SR1 tobacco plants.

RNA Isolation and Blotting. Total RNA was extracted from leaves of untransformed and transgenic plants as described (15). Poly(A)+ RNAs were isolated by oligo(dT) magnetic beads (Dynal, Oslo) according to the manufacturer's protocols. Poly(A)+ RNAs (1 μg) were size-fractionated in 2.2 M formaldehyde/1.5% agarose gels and blotted onto Hybond N membranes (Amersham). Blots were prehybridized for 5 hr at 65°C in 6× SSC, 8× Denhardt's solution, 0.5% SDS, and 10 mg of denatured calf thymus DNA per ml. Hybridizations were carried out overnight in the same solution containing nick-

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Abbreviation: CaMV, cauliflower mosaic virus.

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translated DNA *atp9* probe. Strand-specific hybridization was carried out with *in vitro*-transcribed sense or antisense RNA *atp 9* probes. Blots were washed in 2× SSC/0.1% SDS at room temperature for 10 min and twice for 30 min at 65°C in 0.1× SSC/0.1% SDS. Blots were exposed to x-ray films.

PCR Analysis. Total plant genomic DNA from untransformed and transgenic plants was purified as described (15). PCR amplifications were performed with 1 μg of total DNA in a reaction mixture of 50 μl, containing 1 unit of Super *Taq*I polymerase (Stehelin, Basel, Switzerland), using conditions suggested by the manufacturer: 0.2 mM of each dNTP and 100 pmol of each primer. To detect the *u-atp9* gene, two specific primers were used: (*i*) 5′-CACTACGTCAATCTATAAG-3′, spanning codons 3–9 of the *cox IV* presequence; and (*ii*) 5′-TATGCTCAACACATGAGCG-3′ (oligoterm VI) located at the CaMV terminator gene VI, 45 bp upstream of the polyadenylylation signal (15). To detect the *as-atp9* antisense gene, the primers used were 5′-TAGCACCTTCTAACATCTCG-3′, located at the 3′ end of *atp9* antisense gene, and the primer oligoterm VI (see Fig. 1). Twenty-five PCR cycles were performed: the denaturation step was at 95°C for 1 min, the annealing step at 52°C for 2 min, and the polymerization reaction was performed at 72°C for 1 min. Samples were fractionated by electrophoresis in 1% agarose gels and blotted onto Hybond N membranes (Amersham). Hybridizations with the *atp9* probe were carried out as described above.

Reverse Transcription-PCR Amplification. Total RNA (4 μg) from untransformed and transgenic plants was treated with RNase-free DNase I (BRL). cDNA was synthesized with oligo(dT) primer using SuperScript Reverse transcriptase (BRL). PCR amplifications were performed with 50 ng of cDNAs and orientation-specific primers in a reaction mixture of 50 μl, as described above.

Fruit Capsules and Pollen Germination. Fruit capsules (20–30 capsules/plant) were collected from the apical inflorescence, and the weight of seeds produced was recorded. Pollen viability was evaluated by the ability of pollen grains to germinate in a medium containing 5% sucrose and 3.75 ppm (wt/vol) H_3BO_3 (pH 5.8) at 25°C for 3–5 hr. The germination percentage was calculated on samples of 10^3 pollen grains.

Histologic Analysis of Anthers. For ultrastructural analysis, anthers were fixed with 2.5% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 4 hr at 4°C, washed in the buffer, and fixed with 1% osmium tetroxide in the same buffer. The samples were then treated with 1% (wt/vol) tannic acid (BDH) in water for 30 min at 20°C, dehydrated, and embedded in epon (19). The structure of anther tissue was analyzed under the light microscope from 1- to 2-μm thick sections cut from epon-embedded anthers and stained with toluidine blue.

RESULTS

Transgenic Tobacco Plants Expressing Antisense *u-atp9* Develop Normally. In a previous report, we described the production of male-sterile plants when tobacco protoplasts were transformed with the unedited form of mitochondrial *atp9*. The *atp9* coding region, fused to the yeast *cox IV* mitochondrial targeting sequence, was expressed and addressed into mitochondria in regenerated tobacco plants. The expression of unedited *atp9* transgene correlated with the emergence of male sterility, while the expression of the edited form of *atp9* did not affect male fertility (15).

To establish a clear relationship between the function of the unedited transgene and male sterility, we decided to effect its expression by introducing the *u-atp9* gene in an antisense orientation in male-sterile transgenic plants by means of crossing. For this purpose, a construct analogous to the pH2 plasmid used for male-sterile induction (15) was performed. The chimeric *u-atp9* gene was ligated in inverted orientation relative to the 35S CaMV promoter and the terminator sequence of the CaMV gene VI, resulting in plasmid pH4 (Fig. 1). The hygromycin-resistant gene was used as selectable marker.

The *as-atp9*-containing plasmid was introduced into tobacco protoplasts by polyethylene glycol-mediated transformation (20). All the transformants were male-fertile and developed normal anthers, compared with those of wild-type tobacco plants. The transformants were analyzed by PCR for the presence of the antisense transgene using the specific primers shown in Fig. 1. Seven transformants containing the *as-atp9* chimeric gene were selected for further analysis, and the expression of the antisense transcripts was determined by Northern blot analysis of total RNA using *in vitro*-labeled sense RNA probes. A 0.95-kb *as-atp9* transcript indicates that the chimeric gene was expressed as a stable RNA. The expression levels, which varied between individuals, was probably due to a position effect resulting from the random insertion of the transgene in the host genome of each individual (data not shown). From *as-atp9*-expressing plants, the H4.4 line showing the higher antisense RNA expression level was chosen as male parent to guarantee the antisense effect in the crosses.

The phenotype of the *as-atp9*-expressing plants was identical to wild-type SR1 plant. Female fertility was tested by backcross with pollen from wild-type plants; the progeny segregated in a 1:1 ratio of hygromycin-resistant to -sensitive plants. To produce an homozygous line, we selfed the H4.4 plant and chose a plant segregating 1:0 hygromycin-resistant to -sensitive (H4.4R1).

***as-atp9* Gene Restores Male Fertility.** To determine whether the high expression of *as-atp9* could inhibit translation or transcription of *u-atp9* gene, the male-sterile H2.11 line (15) was crossed with the hemizygous (H4.4) and homozygous (H4.4R1) restorer lines. To obtain more reliable results in the offspring, an homozygous H2.11R1 line was constructed. If the antisense transcripts of *atp9* inhibit the expression of *u-atp9* that induce the male-sterile phenotype, then all the offspring of the cross between male-sterile H2.11R1 and H4.4R1 should be restored to male fertility.

The H2.11 line produces <2% of viable pollen (15), but it was possible to obtain some seeds by forced pollination, thus making it possible to generate the homozygous line H2.11R1. This line showed reduced capacity to produce viable pollen (<0.2%) relative to the parent. It should be mentioned that the homozygous line produced fewer seeds than the H2.11 hemizygous line when crossed with wild-type pollen. The analysis of the R1 and R2 generations and F1 backcross showed that the male-sterile phenotype correlated with the expression of *u-atp9* gene and that this trait was stably inherited (unpublished data).

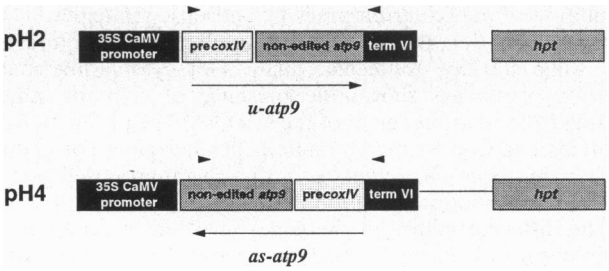

FIG. 1. Structure of chimeric genes used in this work. A *Hin*cII fragment containing the entire coding sequence of the unedited coding sequence of *atp9*, fused to the *coxIV* presequence from yeast (15), was placed in sense (*u-atp9*) or antisense (*as-atp9*) orientation relative to the 35S promoter from CaMV. Hygromycin (hpt) gene was included as a selectable marker. The arrowheads indicate the position of the primers used to detect the sense and antisense genes.

The genotype of the progeny produced from crosses between H2.11R1 and H4.4 and H4.4R1 was determined by PCR analysis using specific primers to distinguish sense and antisense genes (see Fig. 1). The male fertility phenotype was examined by the ability to self-pollinate. The production of fruit capsules, the weight of seeds produced by each plant, and pollen viability at anther dehiscence were recorded. The data were taken from 20–30 flowers produced in the apical inflorescence of each tobacco plant.

The male-sterile plant H2.11R1 was unable itself to produce fruit capsules or seeds (Fig. 2 *a–d*). When this plant was crossed with pollen from plants bearing the *as-atp9* gene (H4.4 or H4.4R1), it produced fruit capsules and seed sets. The F1 progeny of the crosses H2.11R1 × H4.4 and H2.11R1 × H4.4R1 produced 100% hygromycin-resistant plants, as expected, when seeds were germinated in the presence of the antibiotic.

The F1 plants were grown in a greenhouse. After flowering, the anthers were able to produce pollen grains, thus indicating that male fertility restoration had occurred. In crosses where homozygous parents were used, all the F1 plants (six plants) showed a male-fertile phenotype, as measured by the ability to produce seeds by selfing with a mean value of 2.4 g of seeds per plant, compared with 3 g of seeds per plant produced on average by the H4.4R1 male parent (Fig. 3*A*) or wild-type plants. Flowers of restored plants were indistinguishable from those of wild-type or restorer plants. They produced normal fruit capsules (Fig. 2*h*) and had pollen viability values between 20% and 80%. All F1 plants showed the presence of sense and antisense genes when analyzed by PCR with specific primers.

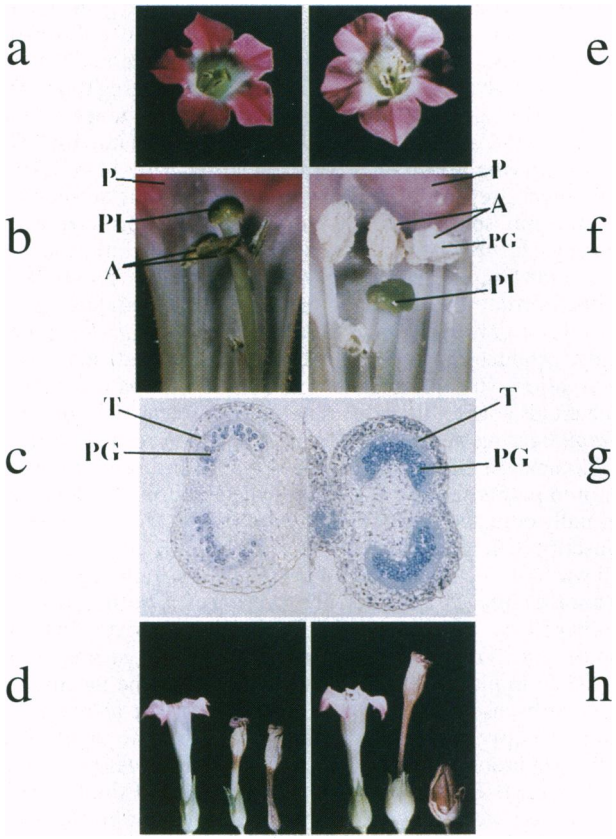

FIG. 2. Phenotypic analysis transgenic plants. (*a*) Flower; (*b*) anthers; (*c*) bright-field photograph of anther cross sections; and (*d*) fruit formation from *u-atp9* transformed male sterile plant. (*e*) Flower; (*f*) anthers; (*g*) bright-field photograph of anther cross-sections; and (*h*) fruit formation from plant restored to fertility containing both *u-atp9* and *as-atp9* genes. A, anthers; PI, pistils; P, petals; T, tapetum; and PG, pollen grains.

In crosses where the hemizygous H4.4 was used as male parent, ≈50% of the progeny (10 of 18 plants) contained both *u-atp9* and *as-atp9* genes, as determined by PCR analysis, and were male-fertile, producing an average of 2.4 g of seed per plant (Fig. 3*B*). The restored plants produced normal fruit capsules and had pollen viability values between 20% and 70%. Fifty percent of the progeny (8 of 18 plants) showed sterile phenotypes and did not carry the antisense gene. All restored male-fertile plants produced normal anthers (Fig. 2 *e* and *f*). Microscopic analysis of transverse anther sections showed well-developed tapetal cell layers and the presence of normal pollen tetrads (Fig. 2*g*).

Restored plants produced from crosses between homozygous male-sterile and restorer lines were selfed. In two independent crosses, the F2 progeny (28 plants) segregated consistently in a 13:3 ratio ($\chi^2 = 6.25, P > 0.05$) of male-fertile to male-sterile (Fig. 3*A*). Twenty-one plants (75%) were fertile and seven were male-steriles. In this cross, three kinds of male-fertile plants are expected: plants containing both sense and antisense genes, plants containing only the antisense gene, and wild-type individuals lacking both genes. The genotypes of these plants were analyzed by PCR with sense and antisense specific primers. Of the male-fertile plants, 34% contained both genes, 33% contained only the antisense gene, and 7% did not contain either the sense or the antisense genes. Interestingly, the seven F2 male-sterile plants contained only the sense (*u-atp9*) gene.

These results strongly suggest that the *as-atp9* gene was responsible for male-fertile restoration and that it functioned as a dominant gene.

Antisense Gene Inhibits the Expression of Unedited *atp9* Gene. The expression of sense and antisense genes in male-sterile and restorer parent plants was determined by Northern blot analysis of poly(A)$^+$ mRNA with an *atp9* double-stranded DNA as probe (Fig. 4*a*). The use of poly(A)$^+$ RNA fraction makes it possible to eliminate the signal generated by the endogenous mitochondrial *atp9* message and to detect specifically the transgene product. As expected, the nick-translated probe detected the transgenic mRNAs in male-sterile and restorer plants transformed with either *u-atp9* or *as-atp9*, respectively. The *as-atp9* mRNA in the restorer plant had ≈10 times higher steady-state levels than the sense transcript in the male-sterile plant. It is interesting to note that the homozygous male-sterile H2.11R1 plant consistently showed higher level expression than the hemizygous H2.11 plant (Fig. 4*a*, lanes S1 and S2).

To determine the effect of the antisense gene on the expression of *u-atp9*, the steady state level of the transgene mRNAs in plants restored to fertility was determined. Poly(A)$^+$ RNAs were purified from F1 plants produced from the cross between H2.11 R1 × H4.4 R1 and after electrophoresis, transferred into membranes, and hybridized with either sense or antisense *atp9* RNA probes. The *as-atp9* transcript was detected with sense riboprobe in restorer parent H4.4 R1 (Fig. 4*b*, lane A) and also in male-fertile restored plants (Fig. 4*b*, lanes 1–6). No signal was detected with mRNAs obtained from the male-sterile plant (Fig. 4*b*, lane S2), indicating that the probe used was indeed strand-specific. The *u-atp9* transcript was detected with antisense riboprobe only in RNA from the male-sterile H2.11R1 plant (Fig. 4*c*, lane S2). Interestingly, all restored plants showed a steady state level of *u-atp9* mRNA that was below the detection limits of this technique (Fig. 4*C*, lanes 1–6). The same negative response was obtained by using reverse transcription-PCR analysis (data not shown). These results clearly demonstrate that the inhibition of *u-atp9* gene expression by antisense RNA leads to the recovery of male fertility.

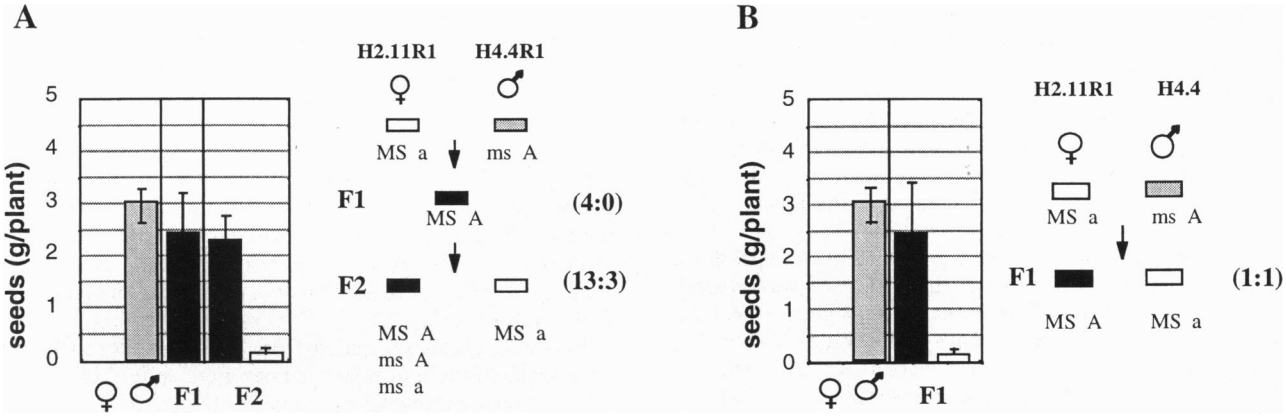

FIG. 3. Restoration of male fertility in crosses with sense and antisense gene-bearing plants. Plants containing sense *u-atp9* or antisense *as-atp9* genes were crossed as shown in *A* and *B*, respectively. Seeds were collected from mature capsules obtained from the apical inflorescence. The genotypes of parent plants or progeny were determined by PCR analysis and phenotypes were scored by the presence or absence of viable pollen and by the amounts of seeds formed by the plant. (*A*) Crosses between homozygous *u-atp9* male-sterile plants and homozygous *as-atp9* restorer plants and their progeny. (*B*) Crosses between homozygous *u-atp9* male-sterile plant and hemizygous *as-atp9* pollen donor plant and their progeny. MS, presence of *u-atp9*; A, active *as-atp9* gene; and ms and a, hemizygous chromosomal loci lacking the sense or the antisense transgenes, respectively. Numbers in parenthesis refer to the fertile to male sterile ratio obtained in each cross. The histograms show the mean production of seeds in the parents and the progeny expressed in g per plant. Values are means ± SEM. From each plant, the total seed production from 20–30 flowers produced at the apical inflorescence was recorded.

DISCUSSION

Previously, we showed that *u-atp9* expression in transgenic tobacco plants correlates with the appearance of male sterility (15). The male-sterile phenotype observed seems to be the consequence of mitochondrial dysfunctions affecting normal anther development and thus reducing the formation of pollen grains (unpublished results). We hypothesized that the presence of unedited proteins in mitochondria could affect mitochondrial function at such levels that might impair the sporogenic process without affecting vegetative development.

The antisense strategy has been successfully used for *in vivo* studies on the role of several proteins by reduction of the steady state level of the corresponding transcripts (21–23). We adopted this strategy to block the expression of the unedited transgene and thus to test our working hypothesis. Both the sense *u-atp9* and antisense *as-atp9* genes were put under the control of the same regulatory sequences to ensure that the transcription activity of sense and antisense genes occurs in the same conditions in every tissue of the plant.

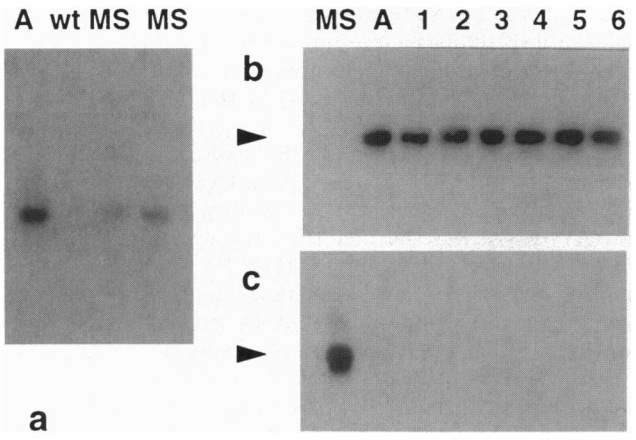

FIG. 4. Northern blot analysis of trangenic restored plants. Poly(A)$^+$ RNA was extracted from leaves of male-sterile and antisense-producing parent plants (*a*) or restored plants (*b* and *c*), size-fractionated on agarose denaturing gel, and transferred to a nylon membrane. The transcripts of *u-atp9* were detected with a nick-translated *atp9* probe (*a*). The antisense *as-atp9* (*b*) or the sense *u-atp9* RNAs (*c*) were detected with ^{32}P-labeled strand-specific riboprobes. Membranes were exposed to x-ray films for 24–96 hr. wt, Wild-type SR1 tobacco plant used as a control; S1, hemizygous H2.11 plant; S2, male-sterile homozygous H2.11R1 plant; and A, restorer antisense plant. Lanes 1–6, restored male-fertile plants.

Genetic analysis showed that the restoration effect was correlated with the presence of the *as-atp9* transgene. Indeed, when the homozygous male-sterile plant was crossed with the homozygous male parent expressing the antisense gene, the F1 showed a male-fertile phenotype segregating in a 4:0 ratio. Moreover, the crosses between the homozygous male-sterile and the hemizygous antisense line gave a 1:1 segregation. The analysis of F2 progeny of fertile-restored plants indicated that the sense and antisense genes segregate independently. The plants carrying both genes were phenotypically male-fertile, while the segregants containing *u-atp9* were male-sterile. The flowers and specifically the anthers of the fertile-restored progeny showed normal development and dehiscence and were indistinguishable from those produced in wild-type plants. Fertile restored plants had well-differentiated tapetal cell layers, gave functional pollen grains, and, more importantly, produced normal fruit capsules and seed sets. These observations strongly suggest that *as-atp9* acts as a dominant gene and is able to block the deleterious effects of *u-atp9*. The *as-atp9* bearing plant can be considered as a restorer line.

The analysis of the transgenic *u-atp9* transcripts in fertile-restored plants showed the lack of the sense mRNA that was normally detected in the female parent. The absence of sense transcripts can be explained by the effect of the antisense RNA and was not due to the loss of *u-atp9* by aberrant segregation or another process. In fact, the presence of both genes was confirmed by PCR in all the F1 progeny derived fom the homozygote cross. The expression of *u-atp9* seems to be abolished in plants restored to male fertility. The mechanism of the antisense effect presumably occurs by the formation of a duplex between complementary RNAs and the rapid selective degradation of the double-stranded molecules by cellular RNases (23). To maximize the probability that duplex RNA was formed, we chose a transgenic tobacco plant producing at least ten times the steady state levels of antisense transcript relative to the sense one. The fact that fertile restored plants presented an excess of antisense RNA and lacked a sense transcript argues in favor of a mechanism involving a double-stranded target. However, other mechanisms involving interference with transcription, processing, or transport of transcripts may explain the antisense effect (24, 25).

The results presented in this article and our previous report (15) demonstrate that the expression of the unedited *atp9* gene in tobacco plant can induce male sterility and that male fertility can be restored by suppressing the expression of the unedited transgene. From a fundamental point of view, these results reinforce the idea that RNA editing is required for the synthesis of functionally competent proteins (26, 27). Although the experimental model used in this study seems attractive to test the functionality of unedited protein products, it should be remembered that all the proteins encoded in mitochondria do not have the same importance in the physiology of the organelle. Thus, the effect observed with *u-atp9* may be attributed to the key role played by ATPase subunit 9 in the energy transduction pathway in mitochondria.

The engineered genes *u-atp9* and *as-atp9* could be useful as nuclear male-sterile inducer and restorer genes, respectively, to be used in breeding programs for the improvement of crop plants, if these genes do not affect the yield of the transgenic plants.

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1. Mayo, H. (1980) *The Theory of Plant Breeding* (Clarendon, Oxford).
2. Kaul, M. L. (1988) *Monographs on Theoretical and Applied Genetics* (Springer, Berlin), Vol. 10,.
3. Edwarson, J. R. (1970) *Bot. Rev.* **36,** 341–420.
4. Warmke, H. E. & Lee, S. L. J. (1977) *J. Hered.* **68,** 213–222.
5. Bino, R. J. (1985) *Theor. Appl. Genet.* **69,** 423–428.
6. Bino, R. J. (1985) *Protoplasma* **127,** 230–240.
7. Grant, I. (1986) *Can. J. Bot.* **94,** 1055–1068.
8. Mariani, C., de Beuckeleer, M., Truttner, J., Leemans, J. & Goldberg, R. B. (1990) *Nature (London)* **347,** 737–741.
9. Mariani, C., Gossele, V., de Beuckeleer, M., de Block, M., Goldberg, R. B., de Greef, W. & Leemans, J. (1992) *Nature (London)* **357,** 384–387.
10. Worrall, D., Hird, D. L., Hodge, R., Paul, W., Draper, J. & Scott, R. (1992) *Plant Cell* **4,** 759–771.
11. van der Meer, I. M., Stam, M. E., van Tunen, A. T., Mol, J. N. M. & Stuitje, A. R. (1992) *Plant Cell* **4,** 253–262.
12. Dewey, R. E., Levings, C. S., III, & Timothy, D. H. (1986) *Cell* **44,** 439–449.
13. Levings, C. S., III (1990) *Science* **250,** 942–947.
14. Hanson, M. R. (1991) *Annu. Rev. Genet.* **25,** 461–486.
15. Hernould, M., Suharsono, S., Litvak, S., Araya, A. & Mouras, A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2370–2374.
16. Araya, A., Bégu, D. & Litvak, S. (1994) *Physiol. Plant.* **91,** 543–550.
17. Schuster, W. & Brennicke, A. (1994) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45,** 61–78.
18. Izant, J. G. & Weintraub, H. (1984) *Cell* **36,** 1007–1015.
19. de Vries, A. P. H. & Ie, T. S. (1970) *Euphytica* **19,** 103–120.
20. Paszkowski, J., Shillito, R. D., Saul, M., Mandak, V., Hohn, T., Hohn, B. & Potrykus, I. (1984) *EMBO J.* **3,** 2717–2722.
21. Hamilton, A., Lycett, G. & Grierson, D. (1990) *Nature (London)* **346,** 284–287.
22. Oeller, P., Min-Wong, L., Taylor, L., Pike, D. & Theologis, A. (1991) *Science* **254,** 437–439.
23. Zabaleta, E., Oropeza, A., Assad, N., Mandel, A., Salerno, G. & Herrera-Estrella, L. (1994) *Plant J.* **6,** 425–432.
24. Smith, C., Watson, C., Ray, J., Bird, C., Morris, P., Schuch, W. & Grierson, D. (1988) *Nature (London)* **334,** 724–726.
25. van der Krol, A., Lenting, P., Veenstra, J., van der Meer, I., Koes, R., Gerats, A., Mol, J. & Stuitje, A. (1988) *Nature (London)* **333,** 866–869.
26. Bégu, D., Graves, P. V., Domec, C., Arselin, G., Litvak, S. & Araya, A. (1990) *Plant Cell* **2,** 1238–1290.
27. Lu, B. & Hanson, M. R. (1994) *Plant Cell* **6,** 1955–1968.