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A. Mouras · Suharsono · M. Hernould
E. Zabaleta · A. Araya

Inheritance of an induced male-sterile trait in transgenic plants expressing an engineered unedited atp9 mitochondrial gene

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Abstract Flowers of tobacco transformed with the genomic (unedited) coding sequence of the mitochondrial *atp9* gene (*u-atp9*), fused to the yeast *coxIV* mitochondrial targeting pre-sequence, exhibited either semi-fertile or male-sterile phenotypes. The inheritance of the induced male-sterile trait, was first investigated on a population of 25 plants of the R_0 generation and then on R_1, F_1 and R_2 progeny of the lines H2.11 and H2.16. The analyses showed that the male-sterile character was generally inherited as a Mendelian trait for one or two loci, and that *u-atp9* is involved in the male-sterile phenotype and is stably inherited in the progeny of the original transformants. Molecular investigations performed on the plants of the R_0, R_1 and R_2 generations confirmed the genetic analyses. The final proof of the involvement of *u-atp9* in the emergence of the male-sterile trait was provided from experiments dealing with the restoration of male fertility. By using antisense RNA technology, we showed that crossing male-sterile plants containing the *u-atp9* transgene with transgenic plants containing the same gene in antisense orientation (*as-atp9*) produces F_1 progeny restored to male fertility. The high expression of as-*atp9* causes a dramatic reduction of *u-atp9* transcript levels and suppresses their deleterious effects, resulting in normal flower development and seed production.

Key words Heredity · Gene segregation · Engineered male-sterility · Gynogenesis · Unedited *atp9*

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A. Mouras · Suharsono[1] · M. Hernould
Laboratoire de Biologie Cellulaire et Biotechnologie Végétale, Université Victor Segalen Bordeaux 2, Avenue des Facultés, 33405 Talence Cedex, France

E. Zabaleta · A. Araya (✉)
Laboratoire de Réplication et Expression des Gènes Eucaryotes et Rétroviraux, EP-630, C.N.R.S.-Université de Bordeaux II, 1, rue Camille Saint-Saëns, 33077 Bordeaux Cedex, France
Fax: +33-5-56 99 90 57
E-mail: alexandre.araya@ibgc.u-bordeaux2.fr

Present address:
[1] Lab. Genetika, PAU Biotecknologi, JI. Puspa, Kampus IPB Damarga, Bogor, Indonesia

Introduction

The production of hybrid seeds to increase crop productivity is a major goal in plant breeding (Kaul 1988). Breeders use male sterility mutations that guarantee the outcrossing of naturally autogamic plant lines to ensure this goal. 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 (Edwarson 1970; Warmke and Lee 1977; Bino 1985a, b; Grant 1986). Thus, tapetal cells are the target of choice to produce engineered male-sterile plants. Several authors have reported the production of transgenic male-sterile plants by the expression of degradative enzymes in tapetal cells (Mariani et al. 1990, 1992; Worral et al. 1992) or by the inhibition of particular enzymes by antisense strategies (van der Meer et al. 1992).

Recently, male-sterile tobacco plants were produced by expression of the unedited form of the subunit 9 mitochondrial ATPase gene (*u-atp9*) (Hernould et al. 1993). In transgenic tobacco plants, the import of *u-atp9* protein into mitochondria affects normal anther development, and especially the tapetal cell layer, reducing or abolishing pollen formation (Hernould et al. 1998). The restoration to fertility of male-sterile plants has been obtained by using the antisense RNA approach (Zabaleta et al. 1996). Antisense RNA inhibits *u-atp9* gene expression and thus abolishes the deleterious effects of this gene.

In the present study we have investigated the inheritance of the induced male-sterile trait in R_0, R_1 and

F_1 progeny of transgenic tobacco plants. The results show that *u-atp9* is involved in the male sterile phenotype and is stably inherited in the progeny of the original transformants.

Materials and methods

Origin of plant material

The transgenic plants used were constructed as previously described (Hernould et al. 1993). They contained either plasmids pH2 (H2 lines) or pH4 (H4 line) carrying respectively the chimeric "unedited" *atp9* DNA in sense (*u-atp9*) or antisense orientation (*as-atp9*) under the control of the 35S CaMV promoter (Fig. 1). Experiments were performed with transgenic male-sterile H2 lines, the antisense-producing homozygous H4 line and, untransformed SR1 tobacco plants.

In vitro induced gynogenesis

Flowers of the SR1 tobacco plant containing dehiscent anthers were irradiated with 60, 100 and 200 krad in a γ-ray sterilization chamber (^{137}Cs). Irradiated pollen was used to pollinate transgenic male-sterile plants. After 9–30 days, the sterilized capsules were longitudinally cut into two pieces and disposed in such a way that the placenta and ovules were in contact with MS culture medium without hormones (Murashige and Skoog 1962). Regenerated plants were transferred onto the same medium supplemented with 40 mg/l of hygromycin B. The plants resistant to the antibiotic were transferred to the greenhouse and examined for the male fertility trait.

Analysis of resistance to hygromycin B

An average of 1000 seeds were sown on MS medium containing 40 mg/l of hygromycin B and cultivated in a culture chamber at 25°C. After 2 weeks germination frequency was estimated, and after 4 weeks the ratio between greenish growing (resistant) and pale-white undeveloped (sensitive) seedlings was scored.

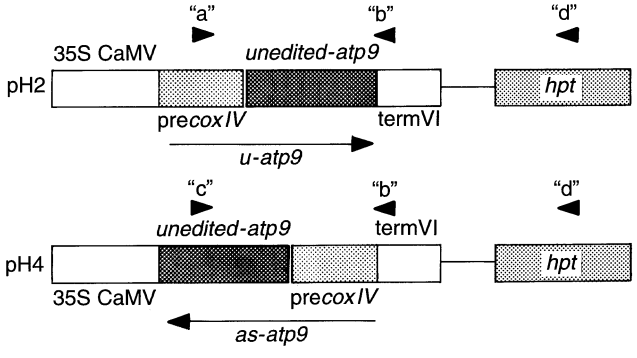

Fig. 1 Chimeric vectors pH2 and pH4. The expression vectors contain the yeast *coxIV* pre-sequence fused to the 5′ end of the unedited *atp9* cDNA. The resulting fragment is inserted in sense (*u-atp9*) and antisense (*as-atp9*) orientation (*arrows*) in the pDH51 plasmid (Pietrzak et al. 1986). The hygromycin phosphotransferase gene (*hpt* cassette) represents the selectable marker. *Arrow heads* indicate the positions of the primers coxIV "a", termVI "b", as-atp9/3' "c" and *hpt* "d" used in the PCR experiments

Fruit capsules and pollen germination

Self-pollination, hand pollination when the plants were unable to self-pollinate, and back-crosses were performed. Fruit capsules were collected from the apical inflorescence and surface sterilised in 0.5% (w/v) $CaHClO_3$ solution containing two drops of Tween 20. All the capsules from the first inflorescence were harvested, except for the H2 primary transformants (R_0 plants) where only 5–6 capsules were collected, and the seed weight was recorded.

Pollen viability was evaluated by the ability of pollen grains to germinate in a medium containing 5% sucrose and 3.75 ppm (w/v) H_3BO_3, pH 5.8, at 25°C for 3–5 h. The germination percentage was calculated on samples of 10^3 pollen grains.

Karyotype analysis

Chromosome preparations were obtained from seedling root protoplasts as described (Mouras et al. 1987; Hernould et al. 1997).

RNA isolation and blotting

Total RNA was extracted from leaves of untransformed and transgenic plants as described by Hernould et al. (1993); 50 µg were spotted on a Hybond-N^+ nylon membrane as described by Sambrook et al. (1989). Hybridization was carried out with the end-labelled primer "term VI" (5′-TATGCTCAACACATGAGCG-3′) located at the CaMV terminator gene VI, 45 bp upstream of the polyadenylation signal (Hernould et al. 1993) in 5 × SSC, 8 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml of calf thymus DNA at 50°C. Final washing was performed at 50°C in 0.1 × SSC and 0.1% SDS.

Poly(A^+) RNAs were isolated by oligo(dT) magnetic beads (DYNAL) according to the manufacturer's protocol. 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 pre-hybridized for 5 h at 65°C in 6 × SSC, 8% Denhardt's solution, 0.5% SDS and 10 mg/ml of denatured calf thymus DNA. Hybridizations were carried out overnight in the same solution containing nick-translated *atp9* cDNA as a probe. Strand-specific hybridization was carried out with in vitro transcribed sense or antisense *atp9* ^{32}P-labelled riboprobes. 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 and then exposed to X-ray films.

Polymerase chain reaction analysis

Total plant genomic DNA from untransformed and transgenic plants was purified as described (Hernould et al. 1993). 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), 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 employed: 5′-CACTACGTCAATCTATAAG-3′ ("a"), spanning codons 3–9 of the *cox IV* pre-sequence, and term VI ("b"). To detect the *as-atp9* antisense gene, the primers employed were: 5′-TAGCACCTTCTAACATCTCG-3′ ("c"), located at the 3′ end of the *atp9* antisense gene, combined with the primer term VI (Fig. 1). The expected fragment size was approximately 0.75 kb. To confirm the co-segregation of the *u-atp9* and the selection marker (*hpt*) in the progeny of transformants, the primer corresponding to *coxIV* and the primer 5′-ATTCGCCATTCAGGCTGC-3′ ("d") located within *hpt* were used. The expected fragment size was approximately 2 kb. 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 polymerisation 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 were treated with RNAse-free DNase I (GIBCO-BRL). cDNA was synthesised with oligo(dT) primer using SuperScript Reverse transcriptase (GIBCO-BRL). PCR amplifications were performed with 50 ng of cDNAs and orientation-specific primers in a reaction mixture of 50 μl, as described above.

Results

Genetic analysis of transgenic H2 plants

Analysis of the R_0 generation

The seed set was evaluated from 5 to 6 capsules per plant (Fig. 2). Of 25 plants investigated, 12 were fertile (F) and produced 103 ± 32 mg/capsule of seeds, similar to wild-type plants (102 ± 15 mg/capsule). The semi-fertile (SF) plants produced a significantly lower amount of seeds (29 ± 17 mg/capsule). The sterile (S) plants (five plants) failed to produce seeds. However, when these plants were back-crossed with wild-type pollen, seeds were obtained indicating that the plants were female-fertile. Self-pollination, by hand, of S plants was performed. Only plant H2.11, producing a very low amount of viable pollen (≤ 5%), set some seeds.

The inheritance of the transgene was analysed through hygromycin B (*hpt*) segregation in the offspring. After selfing F and SF plants, the *hpt* gene segregated in a ratio of 3 : 1, i.e. as a single Mendelian trait. For S plants three different results were found: in the H2.11 plant a segregation ratio of 3 : 1 was obtained after self-pollination by hand, and a segregation ratio of 1 : 1 after back-crossing, indicating that the plant contained one *hpt* locus. The other male-sterile plants segregated either in a ratio of 3 : 1 or 4 : 1 after back-crossing, indicating that these plants contained at least two loci for hygromycin B resistance.

Both, SF and S plants were amphidiploid and showed a chromosome pairing similar to that of wild-type plants. However, the S plants segregating in the ratio 3 : 1 or 4 : 1 were amphitetraploid, with regular chromosome pairing during meiosis (Fig. 3 a). Two plants, H2.11 (amphidiploid) and H2.16 (amphitetraploid), were further analysed for inheritance of the sterility character in the offspring.

Analysis of F_1, R_1 and R_2 generations of the H2.11 male-sterile plant

The seeds obtained from back-crosses of the sterile H2.11 with a wild-type plant (F_1 generation) or from

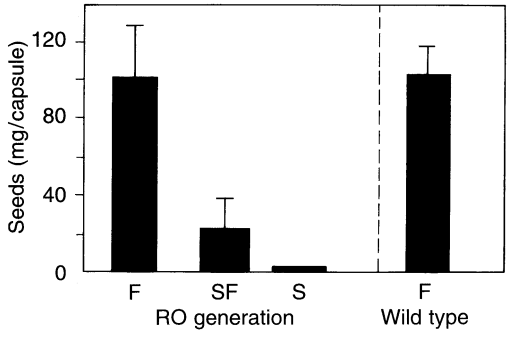

Fig. 2 Evaluation of the fertility in H2 transgenic (R_0 lines) and untransformed (wild-type) tobacco plants after self-pollination. *F* fertile; *SF* semi-fertile, and *S* sterile H2 lines. Seed production was evaluated in mg/capsule from five to six fruits. Values are means ± SD

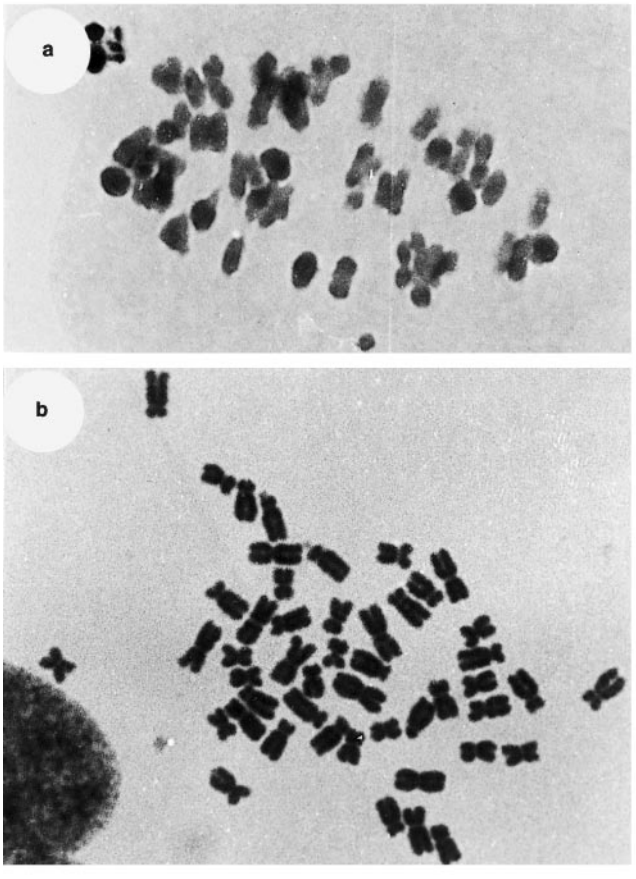

Fig. 3a, b Cytogenetic analysis. **a** Metaphase-I with 48 bivalents in a microsporocyte of the amphitetraploid (2n = 8*x* = 96 chromosomes) H2.16 male-sterile transgenic plant; **b** typical metaphase plate with 2n = 4*x* = 48 chromosomes in a gynogenetic plant derived from the H2.16 male-sterile plant

hand pollination (R_1 and R_2 generations) were sown and cultivated to allow flowering. Seed production was determined from flowers of the first inflorescence (Table 1).

The seed production of F_1 progeny is reported in Table 1: from 35 plants 17 were fertile (F) and 18 were severely modified in fertility (SF plants). All seeds from F plants, taken at random and sown on hygromycin B selective medium, were sensitive. In contrast, seeds from semi-fertile plants segregated in a 3:1 (resistant:sensitive) ratio.

The R_1 progeny constituted 22 plants of which 12 were fertile (F), six showed reduced fertility (SF) and four plants (S) produced a very low amount of seed (Table 1). The SF plants were heterozygous (segregation ratio of 3:1) while the S plants were homozygous (segregation ratio of 4:0) for the transgene.

The homozygous H2.11/S1 plant, was selected to analyse the R_2 generation. This plant was able to produce a few seeds after self-pollination by hand. From the R_2 population, six out of ten plants did not produce any capsules, and four produced one or two very small capsules mainly with aborted seeds. Crosses of H2.11/S1 with homozygous or heterozygous H4 antisense-producing plants gave either 100% fertile plants or fertility restored and male-sterile plants in the ratio of 1:1 respectively (see Zabaleta et al. 1996).

Analysis of R_1 and R_2 generations of H2.16 male-sterile plants

Self pollination by hand of the H2.16 male-sterile plants failed to produce any seeds. When back-crossed with a wild-type plant the resistance/sensitivity trait segregated 4:0 revealing that this plant was female-fertile. As an alternative to produce R_1 progeny, induced gynogenesis was performed. By this strategy, a progeny of 25 plants were obtained. Seventeen were resistant to hygromycin B.

The ploidy level of the hygromycin B-resistant gynogenetic plants was analysed as described in Materials and methods. Ten out of the seventeen plants proved to be amphidiploid with $2n = 4x = 48$ chromosomes (Fig. 3 b). The analysis of chromosome behaviour during meiosis showed normal chromosome pairing (24 bivalents) like that found in wild-type plants (data not shown).

The R_1 hygromycin B-resistant plants were transferred to the greenhouse and allowed to flower and self-pollinate. According to seed production, the progeny were divided into two groups (Table 1): F plants (eight individuals) and SF plants (nine individuals).

Selfed R_1 plants (spontaneous or self-pollinated by hand) segregated the resistance/sensitive trait in a ratio of 3:1 (11 plants) or 4:0 (six plants). Interestingly, only the F plants segregated in a ratio of either 3:1 or 4:0; none of the S plants were homozygous for *hpt*.

The male-sterile plant H2.16/2S1, heterozygous for hygromycin (Hh), was selected to analyse the R_2 generation. This plant produced in the range of $0.3 \pm 0.4\%$ viable pollen (data not shown) and was still able to produce a few seeds by forced selfing. From the R_2 population, seven plants were recovered (Table 1). Two were fully fertile, two were semi-fertile and the other three were fully male-sterile even after hand pollination. In fact, in the latter plants growth was reduced compared to the fertile and even to male-sterile plants; the first inflorescence senesced early, and only the lateral inflorescences produced some flowers which fell before blooming.

Molecular analysis of transgenic H2 plants

Analysis of the R_0 generation

The relationship between the semi-fertile/male-sterile trait and the expression level of the transgene was evaluated. Total RNA from some SF and S plants was

Table 1 Seed production of the offspring from H2.11 and H2.16 male-sterile plants. Seeds of R_1, R_2 and F_1 generations were obtained either by self-pollination by hand or back-crosses with a wild-type plant (WT). The seed production was evaluated independently for each plant, from each capsule and from the whole capsules of the first inflorescence. The data were pooled according to type, F fertile, SF semi-fertile or S male-sterile, to evaluate the average seed production ($\pm$SD) in g/plant and in mg/capsule. ( ): number of plants

Plant		F_1		R_1		R_2	
		g/plant	mg/capsule	g/plant	mg/capsule	g/plant	mg/capsule
H2.11	F	1.08 ± 0.3 (17)	99.4 ± 19.3	1.0 ± 0.2 (12)	84.6 ± 11	0	–
	SF	0.26 ± 0.19 (18)	63.6 ± 42.2	0.42 ± 0.25 (6)	37.9 ± 24.4	0	–
	S	–	–	0.007 ± 0.005 (4)	3 ± 6	0 (10)	–
H2.16	F	–	–	1.19 ± 0.27 (8)	125.5 ± 21.7	1.1 ± 0.03 (2)	68 ± 2
	SF	–	–	0.16 ± 0.18 (9)	19.2 ± 20.7	0.09 ± 0.03 (2)	10 ± 1
	S	–	–	–	–	0 (3)	0
WT		–	–	1.25 ± 0.66 (10)	102 ± 15	–	–

isolated and hybridized with "term VI" as a probe (Fig. 4). The radioactivity associated with each slot was determined by the Cerenkov method. Taking the fully male-sterile plant H2.2 as a reference (100%): (1) the expression level of the transgene was lower in SF plants compared to S plants, and (2) the amount of mRNA was proportional to the degree of fertility. The expression level of the transgene in H2.2 compared to the production of the endogenous *atp9* transcript is similar (Hernould et al. 1993).

Analysis of the H2.11 male-sterile plant and progeny

The presence of the whole transgene, *u-atp9*, in the H2.11 and H2.16 male-sterile plants and their linkage with the selection marker hpt was evaluated by PCR using primers "a", "b' and "d" (Fig. 5). Two amplification products were obtained: a DNA fragment of 2000 bp corresponding to the fragment from pre-*coxIV* to *hpt*, and another of 750 bp corresponding to the expected size of the *u-atp9* construct.

Poly(A^+) RNAs were isolated from H2.11 and H2.16 male-sterile plants and hybridized with *atp9* as a probe. A positive signal was obtained with H2.11 and H2.16 and also with an antisense transformed plant (H4), while an untransformed wild-type plant gave a negative result (Fig. 6).

Analysis of the H2.16 male-sterile plant and progeny

The presence of the whole transgene and the *u-atp9* transcript in the male-sterile plant (Figs. 5, 6) indicated that the H2.16 plant contained at least one whole copy of the transgene.

Surprisingly, we observed that approximately half of the R_1 generation, obtained by induced gynogenesis and selected on hygromycin B medium, was as fertile as the control; the other half was sterile. To elucidate this situation, the transgene and the respective transcript were analyzed. The results are shown in Fig. 7a and b. The transgene was found in all the R_1 plants tested, as in the H2.16 primary transformant, while the transcript was detected only in the S progeny, as in the H2.16 parent line. None of the fertile plants analysed contained the *u-atp9* transcript. The progeny of the heterozygous (Hh) H2.16/2S1 plant back-crossed with the wild-type plant, revealed a co-segregation of hygromycin B resistance and male sterility, while hygromycin B-sensitive fertile plants did not (Fig. 7 c).

Experiments to investigate the restoration of fertility using an antisense strategy were performed as described (Zabaleta et al. 1996). The homozygous H4 plant producing an antisense transcript (*as-atp9*) was used as the male parent in crosses with the male-sterile H2.16/2S1 plant as female parent. The progeny was constituted only of fertile plants. RT-PCR experiments were performed with specific primers to detect sense or antisense transcripts in the male-sterile H2.16/2S1 plant, the antisense homozygous H4 plant, and in six progeny plants. The sense mRNA was amplified in the male-sterile plant, but no amplification product could be obtained in the progeny (Fig. 7 d1). In contrast,

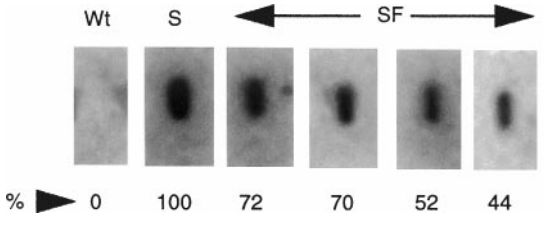

Fig. 4 Slot-blot analysis of total RNAs of sterile (*S*), semi-fertile (*SF*) R_0 transgenic H2 lines and the untransformed tobacco plant (*Wt*). Fifty micrograms of RNA were loaded in each slot. Hybridization was carried out with a ^{32}P-end labelled primer term VI as the probe. The radioactivity associated with each slot was estimated by Cerenkov counting in a Wallac 1409 Scintillation Counter. The result obtained with the male-sterile H2.2 plant was considered as 100%. For the semi-fertile transgenic plants, the percentage of hybridization was evaluated by comparison with the male-sterile H2.2 transgenic plant

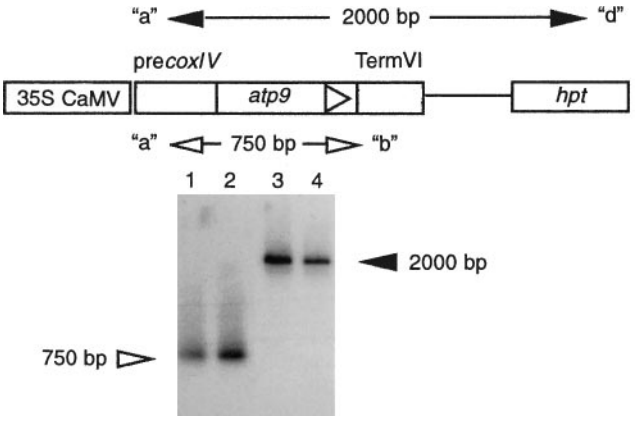

Fig. 5 PCR amplification of the chimeric transgene in H2.11 (*lanes 1*, *3*) and H2.16 (*lanes 2*, *4*) male-sterile plants. *Lanes 1 and 2* amplified DNA with primers "a" and "b"; *lanes 3 and 4*: amplified DNA with primers "a" and "d" (see legend to Fig. 1). The *arrows* indicate the 750-bp and the 2000-bp amplified fragments

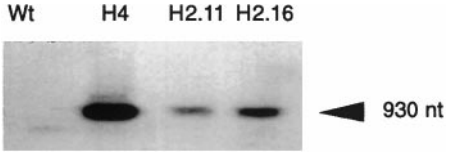

Fig. 6 Northern blot of polyA$^+$ RNA samples extracted from untransformed (*Wt*), H4, H2.11 and H2.16 tobacco plants. The blot was hybridized to ^{32}P-labelled *atp9* cDNA. The *arrow* indicates the 930-nt transcripts in transgenic plants

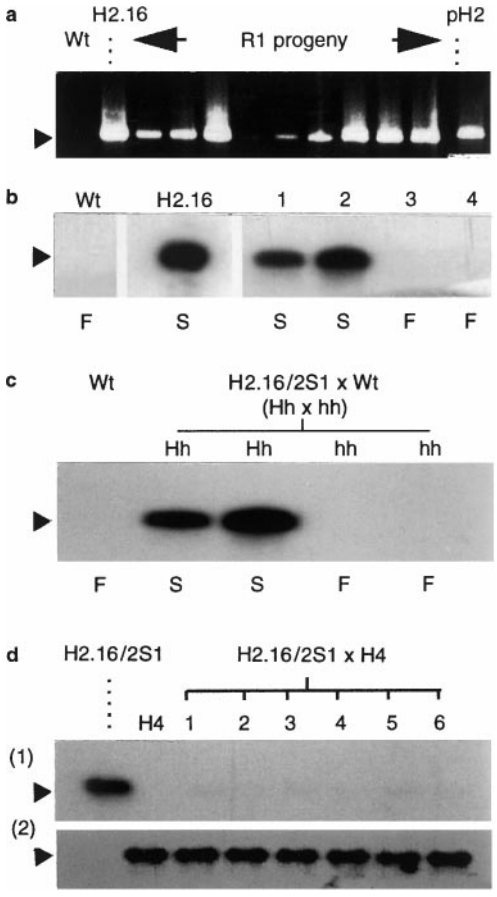

Fig. 7a–d Molecular analysis of the H2.16 line and its gynogenetic R_1, F_1 and restored progenies. **a** PCR amplification of the chimeric transgene with primers "a" and "b" in the H2.16 line and some of its gynogenetic R_1 progeny. Amplified DNA fragments were stained with ethidium bromide. **b** RT-PCR reactions with primers "a" and "b" in the H2.16 line and some of its gynogenetic R_1 progeny. Sterile plants (*lanes 1 and 2*), fertile plants (*lanes 3 and 4*). Amplified cDNA fragments were hybridized to ^{32}P-labelled *atp9* cDNA. **c** Analysis of the co-segregation of the male-sterile transgene (transcript) and resistance to hygromycin B in the F_1 progeny of the H2.16/S1 plant. RT-PCR was performed with primers "a" and "b" and amplified cDNA fragments were hybridized to ^{32}P-labelled *atp9* cDNA. *H* hygromycin-resistant; *h* hygromycin-sensitive. **d** Analysis of the inhibition of *u-atp9* expression in the fertile progeny resulting from the cross between the gynogenetic H2.16/2S1 male-sterile line and the homozygous H4 restorer line. The *u-atp9* and *as-atp9* transcripts in the parents and the progeny were identified by RT-PCR with, respectively, the primers "a" and "b" **d1** and "c" and "b" **d2**. *S* male-sterile, *F* fertile. *Arrows* indicate the 750-bp amplified fragments

antisense mRNA was amplified in the antisense H4 plant and in the progeny (Fig. 7 d2).

Discussion

Transgenic male-sterile plants were constructed with the unedited *atp9* coding sequence fused to the yeast *cox IV* mitochondrial signal sequence. The hybrid transgene was under the control of the 35S cauliflower mosaic virus (CaMV) promoter. It was previously proven that in male-sterile plants derived from transformation experiments, the chimeric transgene was integrated into the nucleus of the host plant and expressed (Hernould et al. 1993). Genetic and molecular analyses were performed to show the inheritance of the male-sterile trait.

Genetic segregation of the *hpt* resistance marker

Genetic analyses performed from 25 independent H2 trangenic lines (R_0 population) showed that *hpt* generally segregated as a Mendelian trait for one or two loci. We focused our investigation on two male-sterile plants, H2.11 and H2.16, as being respectively amphidiploid and amphitetraploid and segregating with a classical Mendelian pattern.

The inheritance of the transgene in the progeny of the male-sterile H2.11 primary transformant plant showed that resistance segregated in the F_1, R_1 and R_2 generations as a single Mendelian trait (one locus). However, the copy number was not evaluated. It was also shown that the male-sterile phenotype co-segregated with the resistance character and that *u-atp9* and *hpt* were linked.

Plant H2.16 was particularly investigated as being fully male-sterile and not able to produce seeds even though self-pollination by hand was performed. Gynogenesis induced by pollination with irradiated wild-type pollen was used as an alternative to generate an R_1 generation directly from the female gametes. Twenty five individuals were regenerated, of which 17 were resistant to the antibiotic. By classical cytogenetic analyses (mitosis and meiosis) we verified the ploidy level of 11 plants from the R_1 progeny compared with the 17 selected on medium containing hygromycin B. All the plants investigated were amphidiploid with $2n = 4x = 48$ chromosomes, like the wild-type plant. The segregation ratio of 3:1 or 4:0 for *hpt* in these amphidiploid R_1 plants indicates that the H2.16 primary transformant presumably contains two independent *hpt* loci. Regarding the F or S phenotype of the resistant R_1 plants, two hypotheses may explain the discrepancy in the segregation between *hpt* and male-sterility: (1) the H2.16 primary transformant could derive from an endoreduplicated transformed protoplast containing two independent loci; a functional *u-atp9/hpt* transgene and a truncated transgene expressing only *hpt*; (2) the H2.16 primary transformant could derive from the fusion between two independently transformed protoplasts, one containing a functional *u-atp9/hpt* transgene, and the other a truncated transgene expressing only *hpt*.

Within the R_1 population, all the male-sterile plants segregated for the *hpt* gene with a ratio of 3:1. This might be due to the fact that homozygous plants are

not viable. This hypothesis was supported by the data obtained from the R_2 progeny derived from hand pollination of the H2.16/2S1 male-sterile plant. Indeed, the presumably homozygous plants grew very slowly and showed an incomplete flower development. The plants flowered 6 months after transplanting in the greenhouse, twice as long a time compared to the other plants. Thus, it may be possible that gametes homozygous for the complete transgene are not viable and, thus, do not give rise to plants in a gynogenetic-induced R_1 population.

Inheritance of the male-sterile trait

The inheritance of the male-sterile trait was monitored according to the capability of plants to produce viable pollen and seeds. Pollen viability proved not to be a good marker since the presence of a range of 5–10% of viable pollen was sufficient to self-pollinate and to produce seeds. In contrast, the evaluation of the total amount of seeds produced in the first inflorescence, and to a lesser extent the number of seeds per capsule, was a more appropriate marker.

The H2.11 plant was unable to self-pollinate. Hand pollination gave rise to some seeds making it possible to analyse the inheritance of the male-sterile trait in the progeny. As expected, the R_1 generation gave rise to fertile and male-sterile plants, but the inheritance of the trait did not fit into a classical Mendelian pattern, probably because the number of plants analysed was low. However, the analysis of F_1 progeny showed that the inheritance of the male-sterile trait segregated 1:1 like a single Mendelian trait. This was confirmed by genetic and molecular analyses dealing with the restoration of fertility using an antisense strategy (Zabaleta et al. 1996).

Plant H2.16 in particular was investigated since it was amphitetraploid and fully male-sterile. We were interested to know if the male-sterile trait was due to genetic variability related with the in vitro culture (polyploidisation) or to the expression of the transgene. The R_1 plants derived directly from female gametes by induced gynogenesis showed that fertile plants produced as many seeds as control plants, while S plants had a significantly reduced amount of seed or none at all. Molecular analysis showed that only S plants contained the *u-atp9* transcript. The R_2 progeny from H2.16/2S1 produced fertile and male-sterile plants as expected. Of the latter, three were probably homozygous for the transgene since they were unable to flower properly.

The production of the *u-atp9* transcript was abolished in progeny obtained from crosses between H2.16/2S1 and homozygous H4 (antisense-producing plant). The progeny was also restored in male fertility. Therefore, the male-sterile trait is due to the expression of the *u-atp9* transgene, thus confirming observations by Zabaleta et al. (1996) with the H2.11 male-sterile line.

The results presented here, as well as our previous observations indicating that male sterility was due to the expression of the unedited *atp9* transgene in tobacco plants (Hernould et al. 1993; Zabaleta et al. 1996; Hernould et al. 1998), raise the question of the role of RNA editing in the production of the male-sterile phenotype. Some investigations performed, for example, on wheat failed to find a direct relationship between cytoplasmic male sterility and RNA editing (Laser et al. 1995; Kurek et al. 1997). However, Iwabuchi et al. (1993) showed that in the *Oryza sativa* CMS-Bo line, fertility restoration was correlated with the editing status and processing of an *atp6* transcript. Furthermore, one interesting case has been described by Howad and Kempken (1997) where *atp6* RNA editing is lost in anthers but normal in other tissues. This apparent contradiction is probably due to the fact that male sterility in plants can be generated by several different molecular events (Vedel at al 1994; Schnable and Wise 1998 and references therein) and RNA editing can be one of them in a particular system. Nevertheless, one common feature in different CMS systems sems to be mitochondrial dysfunction, which in turn generates tapetal impairment.

In conclusion, our previous reports have demonstrated a strong correlation between unedited *atp9* transgene expression and the development of a male-sterile phenotype in tobacco (Hernould et al. 1993). More convincing evidence was obtained when the fertility of male-sterile plants was restored by inhibiting *u-atp9* expression using an antisense approach (Zabaleta et al. 1996). However, even here the genetic segregation of the sterility determinant was lacking. We now demonstrate that the sterility transgene is transferred to the offspring as a Mendelian trait and is perfectly associated with male sterility.

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