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A new CMS source in *Nicotiana* developed via somatic cybridization between *N. tabacum* and *N. alata*

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Abstract Cytoplasmic somatic hybrids (cybrids) between the two sexually incompatible species *Nicotiana tabacum* and *Nicotiana alata* were constructed. A total of 33 green regenerants were obtained after fusion of protoplasts from a tobacco cytoplasmic chlorophyll-deficient mutant and gamma irradiation-inactivated leaf protoplasts of *N. alata*. Twenty nine of them were male sterile and displayed an altered stamen morphology (formation of petaloid and stigmoid structures instead of stamens). Southern-blot analyses of eight CMS plants using *N. alata*-specific nuclear repetitive DNA and cpDNA probes revealed that they contained nuclear genetic material of *N. tabacum* and chloroplasts from *N. alata*. Restriction-enzyme analysis of mitochondrial DNAs of the cybrids in question showed different patterns consisting of an incomplete mix of mtDNA fragments from both parents, as well as new fragments. Southern-blot analysis of mtDNAs with a sunflower *atpA* probe gave the same recombinant hybridization pattern for all analyzed cybrids, indicating that high-frequency specific recombination occurs in the *atpA* region. Analysis of the progeny from three successive backcrosses of the studied cybrids with *N. tabacum* demonstrated a strict cytoplasmic inheritance of the male-sterile phenotype.

Key words *N. alata* · *N. tabacum* · Cybrids · Cytoplasmic male sterility · Mitochondrial recombination

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that has been associated with incompatibility between nuclear and mitochondrial gene products and/or modifications of the mitochondrial genome in higher plants (Breiman and Galun 1990; Hanson 1991). In tobacco, CMS lines have been obtained from a number of inter-specific sexual crosses and through protoplast fusion experiments. Flower development of most of these lines is related to different homeotic-like developmental abnormalities determined by the cytoplasmic source (Gerstel 1980;Bonnett et al. 1991; Zubko et al. 1996). Molecular analyses of CMS lines from different cytoplasmic sources and lines with restored fertility made possible the identification of mitochondrial DNA-fragments and transcripts correlating with the CMS phenotypes (Bonnett et al. 1991; Hakansson and Glimelius 1991; Bergman et al. 1995).

Nicotiana alata and *Nicotiana tabacum* are unilaterally sexually incompatible species. The seed formation from *N. alata* × *N. tabacum* crosses is prevented through the inhibition of *N. tabacum* pollen-tube growth in *N. alata* styles (McClure et al. 1989). As yet there is no report on the production of alloplasmic tobacco carrying *N. alata* cytoplasm.

In the present study, we describe the flower morphology and genetic constitution of CMS cybrids obtained after fusion of protoplasts from the cytoplasmic chlorophyll-deficient mutant *N. tabacum* 1146-D5 and gamma-irradiated protoplasts from *N. alata*.

Materials and methods

Protoplast isolation, fusion and somatic hybrid regeneration

One-month-old in vitro grown plants from the cytoplasmic albino mutant *N. tabacum* 1146-D5 (Atanassov et al. 1993) and from *N. alata* were used as explant material for protoplast isolation. Leaf protoplasts from both parents were isolated, fused, and cultured as described previously (Atanassov et al. 1993). Prior to fusion, *N . alata* protoplasts were irradiated with a dose of 450 Gy (Cs137 gamma rays, 0.166 Gy/s dose rate). One-month later, cell colonies were plated on MS solid medium supplied with 2 mg/l of NAA, 0.5 mg/l of BAP and 30 g/l of sucrose. The green calli obtained were transferred onto shoot–induction medium (MS with 0.1 mg/l of NAA and 1 mg/l of BAP) and regenerated shoots were subsequently transferred onto MS basal medium for rooting.

Chromosome counting

Colchicine-treated root tips from in vitro plants were used to determine the number of chromosomes. The squash preparations were Feulgen stained and chromosomes were counted in about 20 cells.

Analysis of nuclear DNA

Plant DNAs were isolated from leaves of *N. tabacum*, *N. alata*, and putative hybrids by the method of Dellaporta et al. (1983). *Hin*dIII-digested DNAs were slot-blot transferred (0.4 μg DNA/slot) onto Gene Screen Plus nylon membranes (DuPont, USA) and hybridized with a [γ-P^{32}]dCTP-labelled probe from an *N. alata*-specific nuclear repetitive DNA fragment pNP21 (Piastuch and Bates 1990, courtesy of Dr. G.Bates, Institute of Molecular Biophysics, Florida State University, USA). Radiolabelling of the DNA probe, hybridization and high-stringency washing were as described by Sambrook et al. (1989).

Analysis of organelle DNA

Chloroplast and mitochondrial DNAs were isolated from in vitro grown plants as described by Bookjans et al. (1984) and Hakansson et al. (1988). These DNAs were digested with *Hin*dIII, separated by electrophoresis on an 0.8% agarose gel and transferred to Gene Screen Plus membrane filters (DuPont) according to the manufacturer. Southern-blot hybridizations with probes derived from rice chloroplast 16S rDNA (courtesy of Dr. V. S. Reddy, ICGEB, New Delhi, India) and the sunflower mitochondrial *atpA* gene (Spassova 1993) were performed according to Sambrook et al. (1989). The restriction pattern of mitochondrial DNAs digested with *Pvu*II was analyzed through electrophoresis on an 0.6% agarose gel with staining by ethidium bromide (EtBr) according to Hakansson et al. (1988).

Results

Morphology and nuclear constitution of the somatic hybrids

Thirty three green colonies were obtained after fusion of protoplasts from a cytoplasmic chlorophyll-deficient mutant of *N. tabacum* 1146-D5 and gamma radiation-inactivated leaf protoplasts of *N. alata*. No pigment

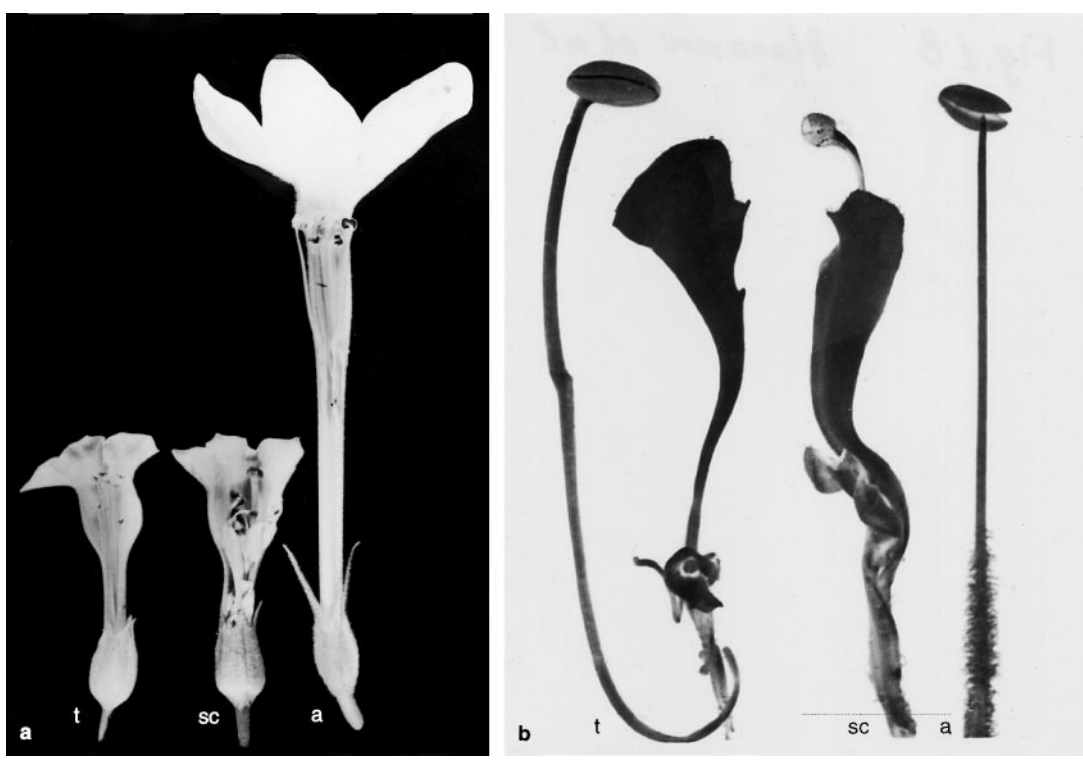

Fig. 1 **a** Flower and **b** stamen morphology of parental and CMS cybrid plants. *t N. tabacum*; *a N. alata*; *sc* somatic cybrids

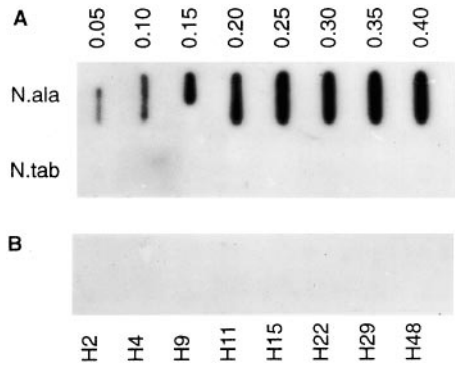

Fig. 2 Slot-blot hybridization analysis with an *N. alata*-specific nuclear repetitive DNA probe. **A** Concentration series of *N. alata* and *N. tabacum* DNAs (0.05–0.4 μg/slot); **B** DNAs (0.4μg/slot) from somatic cybrids H2–H48

variegation was observed among regenerated shoots. Leaf, whole-flower (Fig. 1 a), and plant morphology of the regenerants was identical to the wild-type *N. tabacum* parent. Twenty nine plants were male-sterile and exhibited altered stamen morphology (formation of secondary petaloids and stigmoids instead of stamens, Fig. 1 b). Eight of the male-sterile plants obtained were further analyzed. All of them had the *N. tabacum* (2n = 48) chromosome number. No signal was detected after slot-blot hybridization of total DNA from these plants with an *N. alata*-specific nuclear repetitive DNA probe (Fig. 2).

Organelle composition of the somatic hybrids

Only an *N. alata*-specific hybridization pattern was observed for all studied cybrids after RFLP analysis of chloroplast DNA with the rice 16S rDNA probe (data not shown). This result, together with the lack of pigment variegation in the regenerants obtained, suggests that complete elimination of *N. tabacum* chloroplasts took place prior to shoot regeneration.

Restriction analysis of mtDNAs from the studied plants with *Pvu*II revealed that all of them had rearranged mitochondrial genomes with specific restriction patterns containing an incomplete mix of fragments from both parents, as well as new fragments (Fig. 3). Southern hybridization of *Hin*dIII-digested mtDNAs with an *atpA* mitochondrial probe showed one and the same hybridization pattern in all these plants which contained the *atpA*-hybridizing fragments from both parents together with the new fragment (Fig. 4).

Inheritance of male sterility and altered stamen morphology

The inheritance of male sterility and altered stamen morphology was analyzed through sexual backcrossing

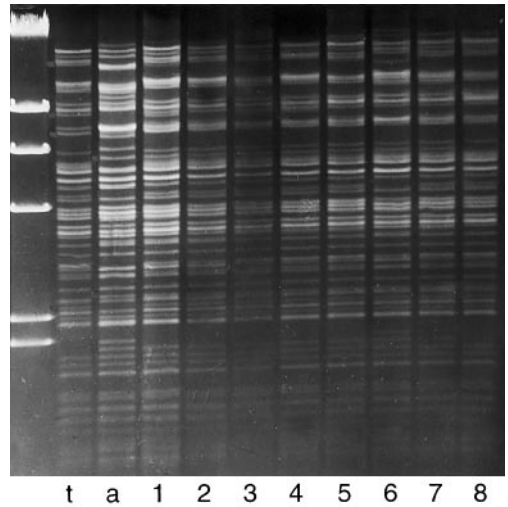

Fig. 3 RFLP analysis of mtDNA digested with *Pvu*II. *t N. tabacum*; *a N. alata*; (1–8) somatic cybrids H2–H48. *Square marks* indicate parent-specific fragments present on some of the cybrid restriction patterns

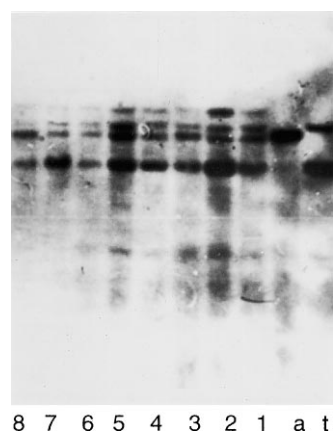

Fig. 4 DNA gel-blot hybridization of *Hin*dIII-digested mtDNA with an *atpA* probe. *t N. tabacum*; *a N. alata*; (1–8) somatic cybrids H2–H48

with wild-type *N. tabacum*. Strict maternal inheritance of the male-sterile phenotype with no revertants was observed after the analysis of seed progeny from three successive backcrosses.

Discussion

Cytoplasmic somatic hybrids between the sexually incompatible species *N. tabacum* and *N. alata* have been constructed through the fusion of protoplasts from a cytoplasmic chlorophyll-deficient tobacco mutant with gamma irradiation-inactivated protoplasts of *N. alata*. The hybrid selection was based on the complementation of chloroplast function in the chlorophyll-deficient tobacco mutant. This system for direct

cytoplasm transfer was very efficient in terms of obtaining CMS cybrids; 29 of the 33 regenerated plants were male-sterile. The morphology and chromosome number of the plants in question, as well as the Southern-blot analyses using *N. alata*-specific nuclear repetitive DNA and cpDNA probes, revealed that they contained nuclear genetic material of *N. tabacum* and chloroplasts from *N. alata*.

The restriction analysis of mitochondrial DNAs suggests that the mitochondrial genomes of the cybrids originate from different recombination events between parental mitochondrial genomes. Surprisingly, Southern-blot analysis of cybrid mtDNAs with a sunflower *atpA* probe showed an identical recombinant hybridization pattern for all the analyzed cybrids. The appearance of CMS in different plant species has often been associated with structural changes involving the *atpA* gene region (Chase and Ortega 1992; Spassova 1993; Bergman et al. 1995). It has been suggested that the relatively high frequency of these rearrangements is due to recombination-generating DNA-repeats lying close to the *atpA* gene (Small et al. 1987; Chanut et al. 1993; Bergman et al. 1995). A similar type of mtDNA recombination provides a possible explanation for the differences between the results from the restriction and Southern-blot analyses of cybrid mtDNAs. The mitochondrial genomes of the cybrids could result from higher-frequency specific (repeat-generated) recombination within the *atpA* gene region and lower-frequency recombination in other parts of the mitochondrial genome distant from the *atpA* gene. We have now initiated a more detailed analysis of regions flanking the different copies of the *atpA* gene in the mitochondrial genome of the CMS cybrids obtained.

The strict cytoplasmic inheritance of the male-sterile phenotype demonstrates that the mitochondrial DNA rearrangements related to CMS-expression are stably maternally transmitted. Consequently, the CMS lines obtained can be used for hybrid seed production in tobacco.

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