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The use of rootless mutants for the screening of spontaneous androgenetic and gynogenetic haploids in *Nicotiana tabacum*: evidence for the direct transfer of cytoplasm

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Summary. In crosses between a homozygous rootless mutant line of *Nicotiana tabacum* used as female and other *Nicotiana tabacum* lines, androgenetic haploids can be directly selected by their ability to form plantlets with a normal rooting system, whereas hybrid plants are killed few weeks after sowing. These androgenetic plants have the nucleus of the male parent into the cytoplasm of the female parent. In crosses where the homozygous rootless mutant line is used as a pollen donor, gynogenetic haploids can also be directly selected. Haploids can therefore be derived from male sterile plants using this approach. A generalization of this system for direct cytoplasm transfer and for the screening of spontaneous haploids in dicotyledons is proposed.

Key words: *Nicotiana tabacum* – Cytoplasm transfer – in situ androgenesis – Rootless mutants

Introduction

When a cytoplasm has been selected for its agronomical characteristics it would be of great interest, from a practical point of view, to rapidly transfer the nuclear information of new cultivars into this cytoplasm. This transfer by conventional methods (repeated backcrosses) is a time-consuming process.

It has already been suggested to use natural androgenesis for transferring cytoplasmic male sterility (cms) in maize (Goodsell 1961): androgenetic plants arise from cells containing the paternal nucleus into the cytoplasm of the female parent. This procedure is the simplest and most rapid way to transfer an entire-cytoplasm without altering its genetic properties. This is not always the case in cytoplasm transfer through protoplast fusion (another one-step procedure to transfer cytoplasmic traits) because of possibilities of chloroplast exchange and chloroplast and mitochondrial recombination (Belliard et al. 1978, 1979; Rothenberg et al. 1985; Medgyesy et al. 1985).

The most serious limitation to the practical use of natural androgenesis and related techniques for conversion of inbred lines to male sterility is the very low frequency of paternal haploids [from 10^{-3} in very favorable cases in tobacco (Burk 1962), 10^{-4} in Petunia (Singh and Cornu 1976), and 10^{-5} in maize (Chase 1963)], stressing the need for a suitable screening system at an early stage of development (seed or plantlet).

Naphthaleneacetic acid-tolerant mutants of *Nicotiana tabacum* selected from protoplast culture led to regenerated plants unable to form normal roots (Muller et al. 1985). These mutants possessed a single dominant new allele, Rac^-, which may be considered as a lethal mutation at the plantlet stage. These plants can be grown by grafting onto a normal plant until flowering and seed harvesting. Spontaneous haploids of a variety would be efficiently selected by their ability to grow normally after germination of seeds obtained from crosses between this variety and a homozygous Rac^- plant.

We report here the efficiency of this genetic system in tobacco for gynogenetic and androgenetic haploid recovery, and give molecular evidence of cytoplasm transfer without alteration in paternal haploids.

Material and methods

The 36AF25 (Xanthi cv) *N. tabacum* line, homozygous for the Rac^- gene, was grafted onto Wisconsin 38 variety stock as previously described by Muller et al. (1985).

In androgenesis experiments it was used as the female parent. The Wisconsin 38 variety was used in one experiment as the male parent, and the T47 line of Techné variety was used

in another experiment as the male parent. The latter, obtained from protoplast fusion experiment, contains *N. debneyi* chloroplasts and recombined mitochondria between *N. debneyi* and *N. tabacum* (Belliard et al. 1978, 1979). This line, maintained by repeated backcrosses with *N. tabacum* c.v. Techné, is relatively male fertile although having a specific morphology of blossom and stamens reminiscent of male sterile plants with a pure *N. debneyi* cytoplasm (Pelletier et al. 1985). The T47 floral morphology was correlated with a specific mitochondrial DNA pattern.

In gynogenesis experiments, the 36AF25 line was used as the male parent to pollinate a *N. tabacum* (Xanthi cv.) genotype heterozygote for two recessive mutations conferring valine resistance (Vr2, Vr3) and bearing a single kanamycine resistance gene (Ka2). This clone was obtained by crossing a kanamycin resistant transformant regenerated from liposome-mediated transfection (Deshayes et al. 1985) with the valine-resistant mutant Val®2 isolated by Bourgin (1978).

Seeds resulting from these crosses were sown on sand in rectangular earthenware vessel (20×25 cm), each containing seeds from one capsule. After sowing, water was regularly dispensed during the first two to three weeks. When plantlets attained the stage of the second leaf emergence, watering was reduced to arrest the growth of rootless plantlets.

Chromosome counting was performed on root tips of assumed haploid plants by staining with pararosaniline (Merck, Darmstadt) after fixation in Carnoy solution (1: acetic acid, 3: chloroform, 6: ethanol) and hydrolysis by 5N HCl during 30 mn at room temperature.

Organelle DNA analysis was performed following previously published procedure (Belliard et al. 1978, 1979). For chloroplast and mitochondrial DNA digestion, EcoR1 and Sal1 restriction enzymes were used respectively.

Results

Androgenesis

One hundred flowers of the 36AF25 line were pollinated with Wisconsin 38 pollen after emasculation. After sowing, in the described conditions, hybrid plants without roots developed only until the second leaf stage and were easily killed by the decrease of water supply, whereas some plants having a developed root system were able to continue to grow. Thirty-nine plants were so selected and individually grown. Among them 30 were morphologically of the hybrid type (Fig. 1) and were considered to be the result of reverse or suppressive mutations of the Rac^- allele (Muller and Caboche, in preparation). Nine plants were of the paternal type considering the leaf morphology (Fig. 1), and were supposed to be haploids. Their haploid structure was confirmed by chromosome counting (Fig. 2). From this experiment the rate of paternal haploid was estimated as one plant from about ten fruits which could correspond roughly to 10^{-4} per seed.

Fifty flowers from the 36AF25 line were pollinated by the T47 line. The seed set per fruit was generally lower compared to a cross with a normal fertile line because of the reduced fertility of T47 pollen. From this experiment two haploid plants were selected by the same procedure. Chloroplast and mitochondrial DNAS were analysed and proved to be of the maternal type (Fig. 3).

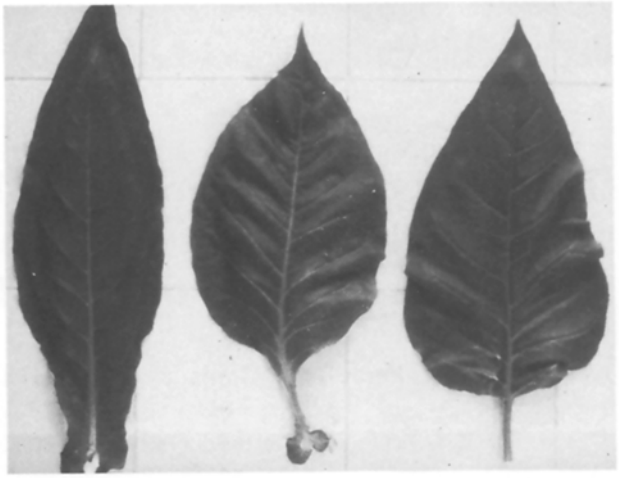

Fig. 1. Leaf morphologies *from left to right* of: Wisconsin 38, variety hybrid between Wisconsin 38 and 36AF25 line, and 36AF25 line

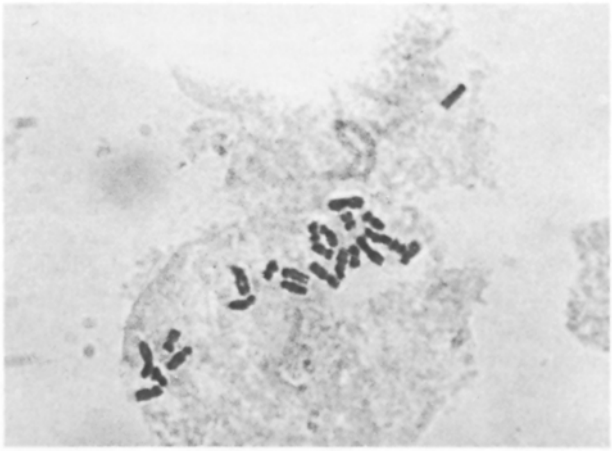

Fig. 2. Metaphase in root tip cells showing the haploid (24) chromosome number

Gynogenesis

The kanamycine resistant and valine resistant clone described in "Material and methods" does not transmit the kanamycin resistant trait when used as a pollen donor, although it does transmit this trait when used as a female parent as a single mendelian gene. It is therefore impossible to obtain homozygotes for the kanamycin resistance marker in the progeny of this transformant after self pollination. Twelve flowers from this clone were emasculated and cross pollinated with pollen from the 36AF25 line, and the corresponding seeds were sown as previously described. Four plants able to grow normally on their roots were obtained,

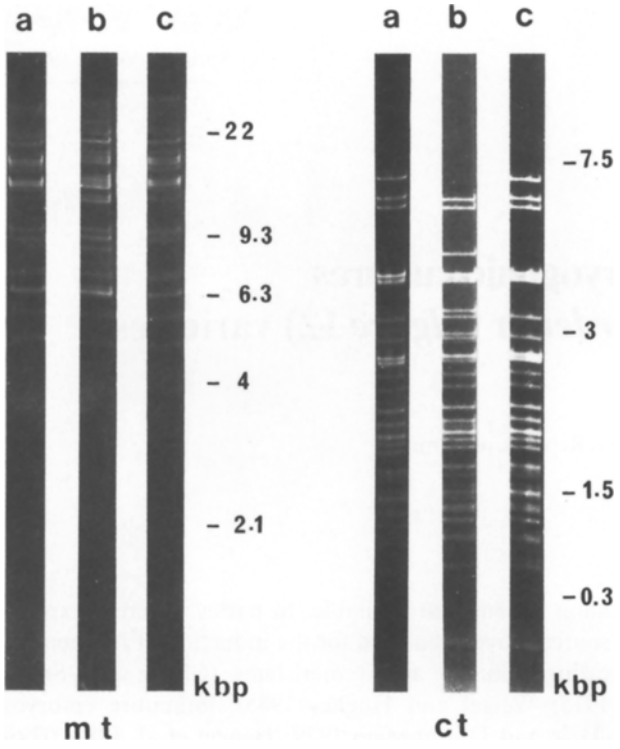

Fig. 3. Agarose gel electrophoresis of mitochondrial (mt) and chloroplastic (ct) DNAs digested respectively by SalI and EcoR1. *a* 36AF25 line (*N. tabacum* cytoplasm), *b* T47 line (*N. debneyi* ctDNA and recombined mtDNA), *c* androgenetic plants obtained using 36AF25 as the female parent and T47 as the pollen donor

among which two proved to be haploids by morphology, flower structure, sterility and chloroplast number in stomata guard cells (Butterfass 1973). These two plants arose through in situ gynogenesis; one of them carried the kanamycin resistance marker and will be used to obtain a diploid homozygote for this marker.

Discussion

Rootless mutation, a new dominant trait in a species and lethal at the plantlet stage, is a suitable genetic marker which permits the use of all tobacco varieties and provides an automatic screening system in normal sowing conditions.

We report here the efficiency of this genetic system in tobacco, and give molecular evidence of cytoplasm transfer without alteration in androgenetic haploids.

Maternal haploids can also be obtained by using this technique. This approach is a useful substitute to in vitro gynogenesis by culture of unpollinated ovaries (Zhu and Wu 1979), a technique which gives variable results. The use of the rootless mutant allows for the isolation of haploids from male sterile plants or genotype bearing genes which are not transmitted by the pollen. Rootless dominant mutants are (to our knowledge) up to now only available in tobacco. They could be induced in other plants by the same procedure previously described (Muller et al. 1985). Such a system is usable only if the species can be grafted (excluding monocots), and if the number of seeds per plant is sufficiently high to allow for the detection of a very rare event (10^{-4} to 10^{-6}).

Two ways of research are possible to generalize the procedure: (1) inducing a similar lethal conditional dominant character in different species, and (2) enhancing the frequency of the phenomenon by acting at the gametophytic level before fertilization.

Work is being developed to generalize the procedure by using the tms2 gene of the T-DNA of *Agrobacterium tumefaciens* as a conditional dominant marker. The expression of this gene in plantlets confers sensitivity to naphthaleneacetamide, an auxine analog used as an herbicide (Budar et al. 1986), preventing a normal root system to develop.

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