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Spontaneous extensive chromosome elimination in somatic hybrids between somatically congruent species *Nicotiana tabacum* L. and *Atropa belladonna* L.

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Summary. Mesophyll protoplasts of the kanamycin-resistant nightshade, *Atropa belladonna*, were fused with mesophyll protoplasts of the phosphinothricin resistant-tobacco, *Nicotiana tabacum*. A total of 447 colonies resistant to both inhibitors was selected. Most of them regenerated shoots with morphology similar to one of the earlier obtained and described symmetric somatic hybrids *Nicotiana* + *Atropa*. However, three colonies (0.2%) regenerated vigorously growing tobacco-like shoots; they readily rooted, and after transfer to soil, developed into normal, fertile plants. Unlike their tobacco parental line, BarD, the obtained plants are resistant to kanamycin [they root normally in the presence of kanamycin (200 mg/l)] and possess activity of neomycin phosphotransferase (*NPT II*) with the same electrophoretic mobility as the one of the nightshade line. According to Southern blot hybridization analysis carried out with the use of radioactively labeled cloned fragments of the *Citrus lemon* ribosomal DNA repeat, as well as with *Nicotiana plumbaginifolia* genus-specific, interspersed repeat Inp, the kanamycin-resistant plants under investigation have only species-specific hybridizing bands from tobacco. Cytological analysis of the chromosome sets shows that plants of all three lines possess 48 large chromosomes similar to *Nicotiana tabacum* ones (2n = 48), and one small extra chromosome (chromosome fragment) similar to *Atropa belladonna* ones (2n = 72). Available data allow the conclusion that highly asymmetric, normal fertile somatic hybrids with a whole diploid *Nicotiana tabacum* genome and only part (not more than 2.8%) of an *Atropa belladonna* genome have been obtained without any pretreatment of a donor genome, although both these species are somatically congruent.

Key words: Somatic cell fusion – Asymmetric hybrids – *Atropa* – *Nicotiana*

Introduction

Partial transfer of the genetic material from alien species into given, so-called gene introgression is considered to be a powerful approach to plant breeding. However, sexual incompatibility between species, increasing with phylogenetic remoteness, was and remains a restriction for the more intensive use of the genetic diversity within the plant kingdom. Now this restriction can be overcome, in principle, due to possibilities offered by somatic hybridization. No phylogenetic barriers exist for the obtainment of the heterokaryocytes (for review, see Gleba and Sytnik 1984) and an entire trend, asymmetric somatic hybridization, has evolved, having the goal to develop workable approaches for gene introgression through fusion of somatic cells.

Chromosome elimination of the donor and integration (exchange) of the donor genetic material into (with) the recipient genome are necessary prerequisites of the gene introgression. Chromosome elimination is a common phenomenon in somatic hybrids between very remote species; however, its direction is unpredictable a priori. In somatic hybrids between related species (intra- and interspecific, intergeneric hybrids) and sometime between quite remote species (intertribal, intersubfamilial hybrids), both parental genomes are stable with usually no signs of chromosome elimination. A number of approaches were proposed to induce directed chromosome elimination in order to transfer only a (few) one chromosome(s), however only irradiation of the donor genome with gamma- or X-rays prior to protoplast fusion gives more or less satisfactory results. Using irradiation, the following were reported: carrot plants possessing one chromosome of parsley (Dudits et al. 1980), *Datura innoxia* with a few (one to three) chromosomes from *Physalis minima* (Gupta et al. 1984), *Nicotiana tabacum* with one chromosome from *Nicotiana plumbaginifolia*

(Bates et al. 1987), and *Nicotiana plumbaginifolia* with one or two chromosomes from *Atropa belladonna* (Gleba et al. 1988). However, asymmetric hybrids obtained with the use of irradiation are characterized by lower viability and tendency to polyploidization of the recipient genome; they are, as a rule, male sterile or completely sterile (Bates et al. 1987; Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989; Agoudgil et al. 1990; Wijbrandi et al. 1990 b). The general conclusion is drawn that highly asymmetric hybrids containing only one or a few donor chromosomes are very rare (Agoudgil et al. 1990), therefore, the development of new or complementary approaches for asymmetric hybridization is a necessity.

Here, we present a study wherein highly asymmetric somatic hybrids between the somatically congruent species *Nicotiana tabacum* and *Atropa belladonna* were constructed. These hybrids combine the whole genome of tobacco with one chromosome (2.8% of the haploid genome) of nightshade and were obtained without any pretreatment of the donor genome.

Materials and methods

Plant material

Nicotiana tabacum L., line BarD, is resistant to phosphinothricin and was obtained in this laboratory after "leaf-disc" transformation of the streptomycin-resistant tobacco mutant, SR-1 (Maliga et al. 1975) with *Agrobacterium tumefaciens* MP90 (pGSFR) (strain kindly provided by PGS, Gent, Belgium). *Atropa belladonna*, line Ab5, is resistant to kanamycin and was described previously (Kushnir et al. 1991 a). Plants were grown under aseptic conditions on hormone-free Murashige and Skoog (1962) medium.

Protoplast isolation, fusion, and culture. Fully expanded leaves were sliced with a razor blade in 1–2 mm wide strips and macerated in enzyme solution: 0.4 *M* sucrose, 0.2% cellulase onozuka R-10 (Serva), 0.1% cellulysine (Serva), and 0.05% macerozyme (Serva) at room temperature overnight. The next day, the protoplast suspension was filtered through a nylon sieve, viable protoplasts were floated by centrifugation, collected, diluted in salt solution W5 (Menczel et al. 1981), and pelleted. Fusion was induced as described by Menczel et al. (1981). Protoplasts were cultured in 8 p medium (Kao and Michayluk 1975) for 7–10 days and the developing colonies were transferred to medium CO.3 (Kushnir et al. 1991 a). One week later, microcolonies were spread over filter paper discs placed on agar-solidified CO.3 medium supplemented with inhibitors (100 mg/l kanamycin and 20 mg/l phosphinothricin). Filters with colonies were transferred every 7–10 days onto selection medium Sh (basic Murashige and Skoog (1962) medium plus 0.5 mg/l 6-benzylaminopurine, 0.6% agar and inhibitors as above). The same medium was used for shoot regeneration from selected colonies resistant to both inhibitors. Shoots were rooted on Murashige and Skoog (1962) hormone-free medium.

Chromosome analysis. Chromosome spreads were prepared from root tips as described by Pijnacker and Ferwerda (1984).

Biochemical analysis. Neomycin phosphotransferase activity was revealed after nondenaturing polyacrylamide gel electrophoresis of the total soluble proteins according to Reiss et al. (1984). Total cellular DNA was isolated from leaves of the parental lines and hybrids according to Dellaporta et al. (1983), digested with the restriction endonuclease, *Bam*HI, and resulting DNA fragments were separated by electrophoresis in an 0.8% agarose gel. DNA was visualized with ethidium bromide, photographed, and transferred to a nylon membrane as described (Maniatis et al. 1982). Cloned fragments of the *Citrus lemon* ribosomal DNA repeat (Kolosha and Fodor 1986), as well as dispersed *Nicotiana* genus-specific repeated element Inp (Boutry and Chua 1985), were used for preparation of the hybridization probes. Purified fragments were radioactively labeled by nick-translation (Maniatis et al. 1982). Prehybridizations and hybridizations were performed in 0.5 *M* sodium phosphate buffer, pH 7.2, 7% sodium dodecylsulfate (SDS); 1 m*M* ethylenediaminotetraacetic acid (EDTA), and 1% bovine serum albumine (BSA) as described (Church and Gilbert 1984). Membranes were washed in 100 m*M* sodium phosphate buffer, pH 7.2, 1 m*M* EDTA, 1% SDS at 60 °C, three times for 15 min, sealed in a plastic bag, and exposed to X-ray film with intensifying screens.

Results

A total of 447 colonies were selected on media supplemented with kanamycin (100 mg/l) and phosphinothricin (20 mg/l) following protoplast fusion of tobacco, line BarD, and nightshade, line Ab5. Most of the regenerated shoots were similar to shoots of the symmetric *Nicotiana* + *Atropa* hybrids obtained and have been described in previous experiments (Kushnir et al. 1991 b). However, three colonies gave rise upon regeneration to vigorously growing shoots morphologically indistinguishable from tobacco. These lines, 62Ab1, 62Ab2, and 62Ab3, were studied further in detail.

Stem cuttings of these lines rooted in the presence of 200 mg/l kanamycin, whereas the control, BarD, did not. Rooted shoots normally grew in the field, and the resulting plants (Fig. 1) did not differ phenotypically from the starting tobacco cultivar. Flowers were morphologically normal, although anthers shed less pollen in comparison to BarD. After selfing or cross pollination with BarD, normal fruits developed: however, seed yield per capsule was lower in comparison to the wild type (full data about seed viability and inheritance of kanamycin resistance will be presented elsewhere).

Chromosome analysis was greatly facilitated, because metaphase chromosomes of tobacco and nightshade are easily distinguished by size (Gleba et al. 1982, 1983, 1987). About six metaphase plates per plant were counted. All plants in question possessed a diploid ($2n = 48$) chromosome set of tobacco, plus one small chromosome (or chromosome fragment) of nightshade (Fig. 2).

Southern blot hybridization analysis was carried out for characterization of the nuclear genomes of the hybrids. Figure 3A shows the hybridization patterns of the lines 62Ab1, 62Ab2, 62Ab3, and their parents, when a cloned fragment of *Citrus lemon* repeat encoding ribosomal RNAs was used for probe preparation. At least six hybridizing bands distinguish tobacco from nightshade

Fig. 1. Flowering plant of the asymmetric somatic hybrid between *Nicotiana tabacum* and *Atropa belladonna*

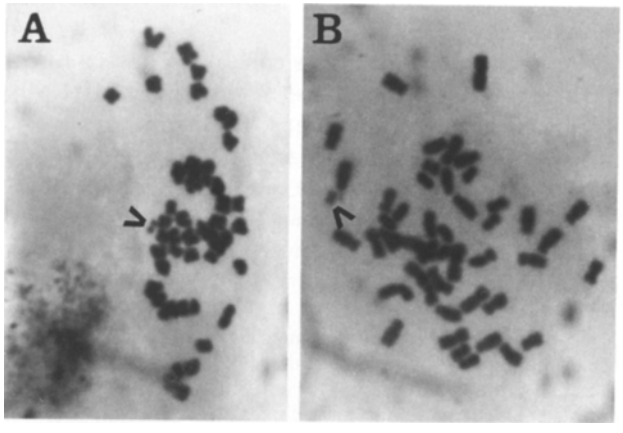

Fig. 2 A and B. The metaphase chromosome spreads of the asymmetric hybrids, line 62Ab1 (**A**) and 62Ab3 (**B**). *Arrows* indicate small nightshade-like chromosomes

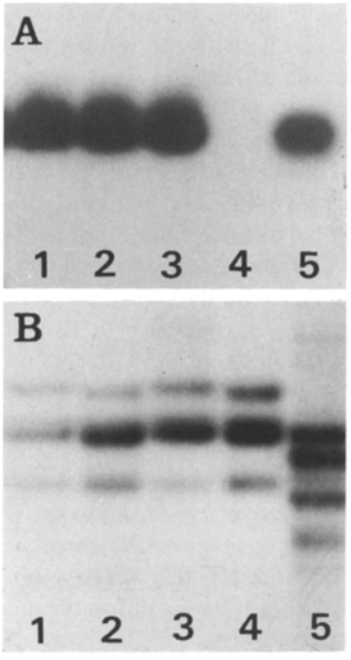

Fig. 3 A and B. Southern blot hybridization and *NPT II* analyses of the asymmetric hybrids *Nicotiana* + *Atropa* and their parental lines. **A** Autoradiogram of the neomycin phosphotransferase activity. The order is as follows: 62Ab1 (*1*); 62Ab2 (*2*); 62Ab3 (*3*); tobacco, line BarD (*4*); nightshade, line Ab5 (*5*). No activity is visible in tobacco, line BarD (*4*), whereas asymmetric hybrids possess activity identical in electrophoretic mobility to nightshade (track *5*). **B** Autoradiogram of the membrane hybridized with a nick-translated fragment of the *Citrus lemon* ribosomal DNA repeat. Total genomic DNA was digested with *Bam*HI. The order is the same as in **A**

after digestion of their total genomic DNA with the endonuclease *Bam*HI. Hybrid plants have only tobacco species-specific hybridizing bands. Subsequent hybridization of the same membrane with dispersed repeat Inp shows no hybridization signal with nightshade DNA, whereas tobacco DNA gives a smear with a few easily recognizable bands. Therefore, the Inp repeat is a useful genus-specific marker. Hybridization patterns of the somatic hybrids with this probe did not differ from tobacco (data not shown).

Plants from the lines in question, as well as their parental lines, were further tested for neomycin phosphotransferase (*NPT II*) activity. The autoradiogram of the *NPT II* test is shown in Fig. 3B. The analyzed plants possessed enzymatic activity of *NPT II*, which migrated under used electrophoresis conditions with the same mobility as one of the nightshade, line Ab5. No activity was observed in tobacco, BarD.

Discussion

The results presented here suggest that tobacco-like, kanamycin-resistant plants of the lines 62Ab1, 62Ab2, and 62Ab3 obtained after fusion of mesophyll protoplasts of phosphinothricin resistant tobacco, line BarD, and kanamycin-resistant nightshade, line Ab5, are highly asymmetric somatic hybrids possessing a whole *Nicotiana tabacum* genome with a very small part of an *Atropa belladonna* genome. These asymmetric somatic hybrids are morphologically indistinguishable from the starting tobacco variety. Hybridization patterns of their *Bam*HI-digested total DNAs obtained after Southern blot hybridization analysis with fragments of *Citrus lemon* ribosomal DNA repeat and Inp interspersed *Nicotiana*-specific repeat are identical to those of parental line

BarD. At the same time, these hybrids, inlike line BarD, are resistant to kanamycin, as follows from a rooting test, and possess *NPT II* enzymatic activity having the same electrophoretic mobility as that of the nightshade line, Ab5.

Cytological analysis of chromosome sets shows that on metaphase spreads of analyzed plants, in addition to 48 large chromosomes similar to tobacco chromosomes (2n = 48), one small chromosome could be observed. Until linkage analysis of the kanamycin resistance trait and gain/loss of this chromosome (or fragment) or in situ hybridization with *NPT II* encoding DNA sequences on chromosome spreads are carried out, we cannot state with full certainty that it is a nightshade chromosome bearing gene encoding *NPT II*. Nevertheless, because plants of all three asymmetric hybrid lines possess a similar small chromosome and because this chromosome is similar morphologically to nightshade small chromosomes, the more simple explanation is that we have obtained single chromosome addition lines of tobacco and nightshade. If this assumption is true, the asymmetric hybrids reported here possess approximately 2.8% or less of the *Atropa* haploid genome (2n = 72).

Thus, in our experiment, in a low proportion of *Nicotiana* + *Atropa* hybrids (3 out of 447, approx. 0.2%) a very extensive chromosome elimination takes place. It is the more unexpected result of our study, because *Nicotiana tabacum* and *Atropa belladonna*, although phylogenetically quite remote and belonging to different subfamilies of the Solanaceae family (D'Arcy 1979), are somatically congruent (Gleba et al. 1982, 1983, 1987; Kushnir et al. 1991 b). Chromosome of both species have been present in symmetric somatic hybrids for many years. Recently, we have reported asymmetric hybridization experiments between *Nicotiana plumbaginifolia* and *Atropa belladonna*, where different dosages of gamma-irradiation (100–1,000 Gy) were used to induce nightshade chromosome elimination. Only limited chromosome elimination was observed, and retention of the donor chromosomes ranged from 11 to 90% of a diploid donor genome. *Nicotiana* plants with one or two nightshade extra chromosomes were obtained only after two additional backcrosses to the *N. plumbaginifolia* parent.

Limited chromosome elimination after gamma- or X-ray irradiation, as well as weak irradiation dose effects, are common phenomena when asymmetric somatic hybrids are constructed between somatically congruent species. The donor genome was eliminated from 8 to 75% in *Nicotiana plumbaginifolia* + *N. sylvestris* hybrids (Famelaer et al. 1989), from 25 to 100% in *Brassica oleracea* + *Brassica campestris* hybrids (Yamashita et al. 1989), from 33 to 67% in *Nicotiana plumbaginifolia* + *N. tabacum* hybrids (Agoudgil et al. 1990), and from 17 to 69% in *Lycopersicon esculentum* + *L. peruvianum* hybrids (Wijbrandi et al. 1990 a). Somatic hybrids with one or several chromosomes from an irradiated donor were described for carrot + parsley (Dudits et al. 1980), *Datura innoxia* + *Physalis maxima* (Gupta et al. 1984), and *Nicotiana tabacum* + *N. plumbaginifolia* (Bates et al. 1987); however, nothing is known about somatic compatibility of the two former cases. Thus, highly asymmetric hybrids were rarely described, and selection of such hybrids without any pretreatment is very interesting. Noteworthy are recently published experiments where highly asymmetric hybrids between *N. plumbaginifolia* and *N. tabacum* were obtained without any pretreatment (Experiment B in Agoudgil et al. 1990) as well.

In general, the recipient genome in asymmetric "gamma"-hybrids with limited chromosome elimination has the tendency to polyploidization (Gleba et al. 1988; Famelaer et al. 1989; Yamashita et al. 1989; Agoudgil et al. 1990; Wijbrandi et al. 1990 b). In contrast, all the above-described hybrids have a diploid tobacco genome. Asymmetric "gamma"-hybrids are also less viable (assessed as regeneration ability, growth rate, and fertility) than symmetric hybrids, and the higher the irradiation dose, the more pronounced the inhibitory effects are. Hybrids of the 62Ab lines are growing as vigorously as tobacco.

A number of speculations could be proposed to explain our data, as well as those of Agoudgil et al. (1990), but the main conclusion is that some somatic cells can be in a state facilitating rapid asymmetrization of fusion products. Whether or not we can manipulate the direction of asymmetrization and artificially enrich a population of somatic cells in this state is a subject for further experimentation.

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