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Intertribal Hybrid Cell Lines of *Atropa belladonna* (×) *Nicotiana chinensis* Obtained by Cloning Individual Protoplast Fusion Products *

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Summary. After fusion of isolated mesophyll protoplasts of belladonna (*Atropa belladonna*) with callus protoplasts of Chinese tobacco (*Nicotiana chinensis*) followed by mechanical isolation and cloning of individual heteroplasmic fusion products, 13 cell clones were obtained. The hybrid nature of most of the clones has been confirmed by biochemical (studies of amylase isozymes), cytogenetic (size and morphology of chromosomes) and physiological (peculiarities of cell-growth in vitro) analyses. Study of chromosomes and isozyme patterns in the hybrid cell lines revealed the presence of both parental genomes, without an indication of chromosome elimination, six months after hybridization. In 4 cell lines shootlike structures and plantlets have been produced by means of transfer to organogenesis-inducing media. The data obtained are interpreted as new evidence for the possibility of using non-sexual hybridization for the production of intergeneric, intertribal plant hybrids which cannot be obtained by sexual crossing. From these results the potential of *Atropa* (×) *Nicotiana* hybrids as a model system for genetic studies of distantly related plant species is discussed.

Key words: *Atropa belladonna* – *Nicotiana chinensis* – Heterokaryocyte cloning – Parasexual hybrids

Introduction

The elaboration of methods for somatic cell hybridization has opened new perspectives for genetic analysis and reconstruction of hereditory mechanisms in plant cells. The study of genetic processes in species is one of the most promising trends in non-sexual hybridization research. Preliminary results in this area have been obtained by Kao (1977), Gleba and Hoffmann (1978; 1979; 1980) and Krumbiegel and Schieder (1979). In the first report mentioned, interfamilial cell hybrids (soybean (×) *N. glauca*[1] were obtained; within a few months, these cell lines lost most of the tobacco chromosomal material and revealed no indications for morphogenesis. In our experiments, the clones of an intertribal hybrid (*Arabidopsis* (×) turnip) were obtained in a similar way, and this study demonstrated stability of a part of the clones, as well as the aptitude of some of them for morphogenesis and plant regeneration. The work of Krumbiegel and Schieder (1979), concerning the intertribal hybridization of stramony and belladonna species, demonstrated that hybrid cells obtained were capable of shoot morphogenesis.

The purpose of the present study was to analyze several hybrid cell clones obtained through the fusion of isolated protoplasts of belladonna (*Atropa belladonna*) and Chinese tobacco (*Nicotiana chinensis*). This work is a constituent part of our attempts to elaborate a model system for studies of genetic processes in hybrid cells obtained by cell fusion of distantly related species. Requirements for this model system include in particular: 1) easy production of hybrid cell lines and their active growth; 2) relative (1 year or more) stability of clones (which is necessary for extensive analyses); 3) existence of morphological differences between the chromosomes of both parents; 4) availability of biochemical (genetic) markers of parental chromosomes; 5) ability of hybrid cells for morphogentic reactions and regeneration of entire plants.

The hybridization of *Atropa* and *Nicotiana* species may be of practical interest, being the only possible way to combine in hybrid cells the biosynthetic potentials of

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1 To distinguish somatic hybrids from the sexual hybrids (A×B), and from the graft chimera (A+B), somatic hybrids should be symbolized A (×) B (Hoffmann 1982)

these two genera known as producers of a number of valued secondary constituents such as alkaloids.

Materials and Methods

Cell lines of Chinese tobacco (*Nicotiana chinensis* Fisch ex Lehm.) were used as one parent. The original line was kindly supplied by Prof. M. H. Zenk, Munich, F.R.G. The cells were grown on agarized B_5 medium (Gamborg et al. 1968) and cultured in darkness at 25 °C with transfers onto fresh medium every month. The preliminary tests have demonstrated that a 15 to 20 day-old culture is the best source for protoplasts able to regenerate with great efficiency when cultured in MO-1 medium (Table 1). Mesophyll protoplasts of diploid ($2n=72$) aseptically grown plants of belladonna (*Atropa belladonna* L.) were used as another parent. The line of aseptically grown plants wer provided by Dr. O. Schieder, Cologne, F.R.G.

Callus and mesophyll protoplasts were obtained from tissues treated by a solution of enzymes containing 0.4% Driselase (Kyowa Hakko Kodyo Co, Tokyo, Japan), 0.3% cellulase Onozuka R-10, 0.2% pectinase Macerozyme R-10 (both from Kinki Yakult Mfg. Co, Nishinomiya, Japan) and 0.2% Cellulisin (Calbiochem – Behring Corp. La Jolla, USA) in 0.4 M mannitol. The treatment was carried out for 14–16 h at 25 °C. To obtain the protoplasts from leaves, the epidermis was removed and the solution of enzymes was diluted with MO-1 medium (1 : 1).

Fusion was induced as described by Kao (1977). The mixture of parental protoplasts (belladonna : tobacco 1 : 3) was treated after washing and sedimentation with a solution of polyethylene glycol (PEG, 3,000 d) for 25 min; the high pH – high Ca^{++} buffer solution contained 10% dimethylsulfoxyd (Menczel et al. 1981). Such a treatment resulted in a great number of heteroplasmic fusion products, whereas practically all unfused cells of belladonna were destroyed. The cells were maintained in MO-1 medium (Table 1) at 25 °C under diffuse light for 2–4 days; hybrid products were then isolated and planted as microdroplets in the larger wells of the inner chamber of Cuprak dishes in MO-1 medium as described previously (Gleba and Hoffmann 1978). The hybridization was carried out in April 1981.

For cytological studies, the tissues (roots of parental plants, callus tissue, shoots of regenerants) were fixed in Carnoy's acetic acid/ethyl alcohol (1 : 3) mixture for 12–16 h and stained with orcein (1% solution in 45% acetic acid) for 48–72 h at 10–12 °C. The material was pretreated with colchicine (0.5%, 1 h, 10–12 °C). Squash preparations were made in 45% acetic acid.

Biochemical studies included the electrophoretic separation of proteins in 7.5% polyacrylamide gel (PAG, Davis 1964) containing 1% starch at 3 mA with subsequent determination of amylase isozymes. The plant material was ground in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% 2-mercaptoethanol. The homogenate was centrifuged at 20,000 × g for 20 min, and the supernatant was used for electrophoresis. After electrophoresis the gels were kept in 0.2 M acetate buffer containing 10 mM $CaCl_2$.

Table 1. MO-1 medium developed for the culture of hybrid cells of *Solanaceae*; mineral supplements according to Nagata and Takebe (1971)

Organic compounds	mgl^{-1}	Organic compounds	mgl^{-1}
thiamine	2	glucose	10
pyridoxine	2	3-indolacetic acid	2
biotin	0.5	1-naphthylacetic acid	3
casein	200	kinetin	3
yeast extract	100	zeatin	0.1
egg albumin	100	2,4-dichlorophenoxy-acetic acid	1
nicotinic acid	3		

pH = 5.6

Results

For the present studies of isolation and individual culturing of single fusion products, the procedure of mechanical separation, previously used for obtaining *Arabidopsis* (×) turnip hybrids, has been used. Belladonna (×) tobacco fusion products were shown to be able to divide actively and could be easily recognized, being distinguishable from parental cells even at the stage of 2–3 cell divisions.

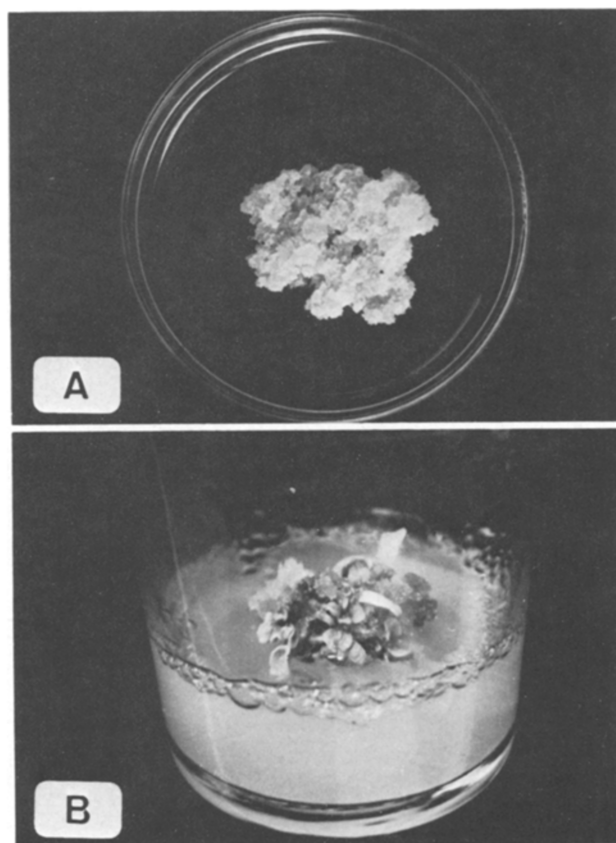

Fig. 1 A and B. Hybrid cells **(A)** and shoot regenerants **(B)** of *Atropa belladonna* (×) *Nicotiana chinensis* obtained from cloned individual protoplast fusion products

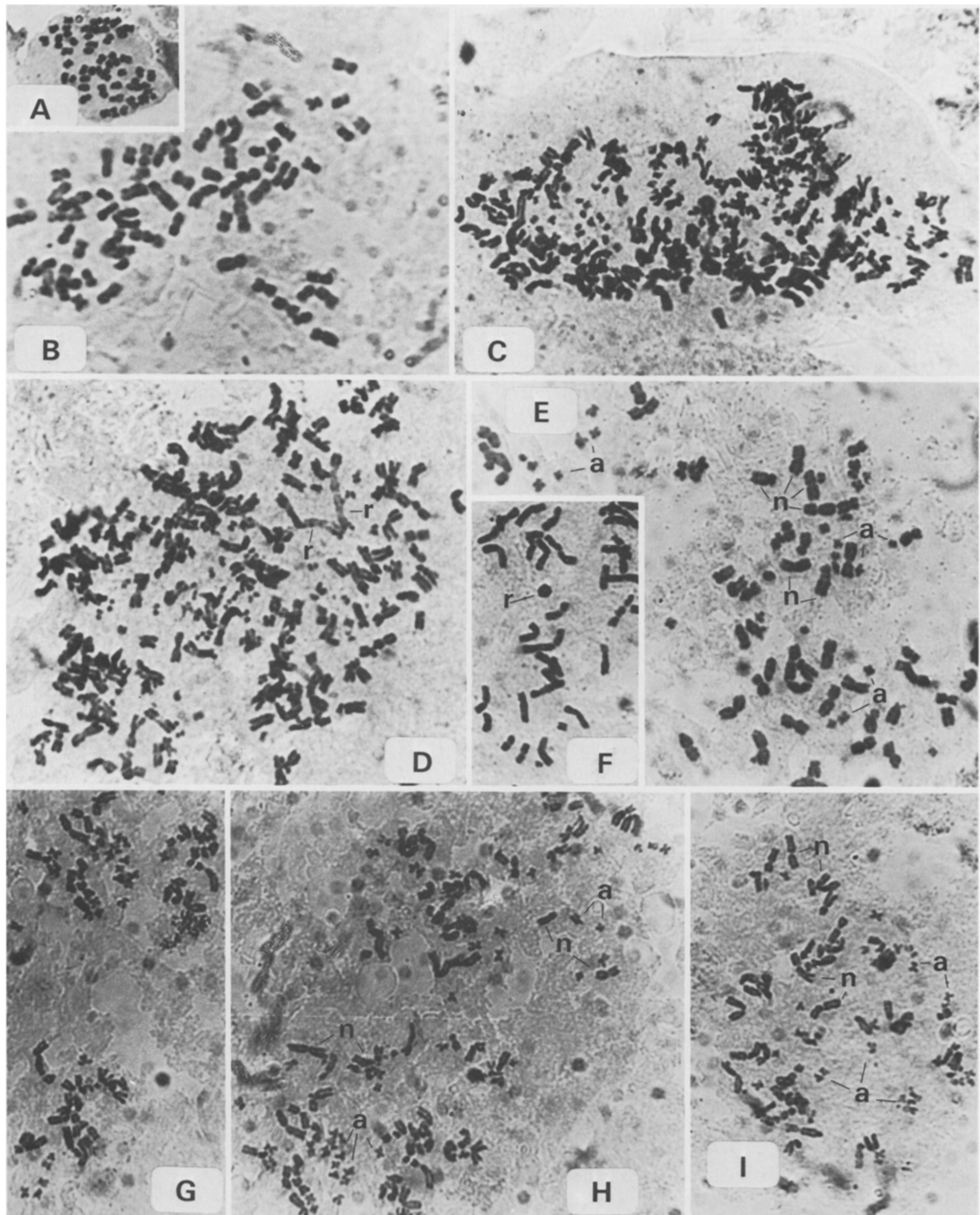

Fig. 2A–I. Cytological analysis of chromosomes in parental cells and hybrids of *Atropa* (×) *Nicotiana:* **A** chromosomes of belladonna; **B** chromosomes of *N. chinensis;* **C–F** chromosomes of the hybrid cell line AbNc-11; **G–I** chromosomes of the line AbNc-5 (a = chromosomes of belladonna type, n = chromosomes of *N. chinensis* type, r = reconstructed chromosomes)

The MO-1 medium developed in our studies for culturing hybrid cells of Solanaceae is given in Table 1. The medium was filter sterilized.

The first division was observed by the end of the second day of culture; the second division by the 4–5th day. Under the same conditions protoplasts of *N. chinensis* divided on the 3rd day while protoplasts of belladonna failed to divide substantially. Thirty-five of the 60 fusion products isolated grew into small colonies, each consisting of several hundred cells. However, only a fraction of the colonies survived subsequent dilutions and transfer to fresh medium. Thirteen colonies were transferred successfully from liquid MO-1 medium onto agar medium. Actively growing cultures of the mentioned cell lines are still maintained.

Cytological analysis of the cell lines isolated as single fusion products were conducted 4–5 months after isolation (Fig. 2). The chromosomes typical for *A. belladonna* (2 n = 72) are shown in Fig. 2A. Chromosomes of belladonna are very small, median or subterminal; the ratio of length between the longest chromosome in metaphase and the shortest one averages no more than 2.5 : 1. Fig. 2B presents a metaphase plate of a *N. chinensis* callus cell (2 n = 48). Chromosomes of this species are median, submedian and subterminal, the ratio between the longest and shortest chromosomes in metaphase is nearly 3 : 1. Study of the callus cell line of *N. chinensis*, used as parental one for hybridization, revealed no visible mitotic anomalies or unexpected chromosomes in this species. Most of the cells under investigation had nearly 70–100 chromosomes, the chromosome number being variable from one metaphase to another. This fact points to the aneuploid nature of the larger part of the plant cell population.

The results of chromosomal analysis of cell line AbNc-11 are shown in Fig. 2C–F. The majority of cells of this line have more than 200 chromosomes (Fig. 2G–D) greatly differing both in length and breadth (Fig. 2E). The ratio in metaphase between the longest chromosome and the shortest is nearly 6 : 1, so that the heterogeneity in length between chromosomes considerably surpasses the one between chromosomes of parental cells. In addition, two types of chromosomes are distinguishable: narrower and smaller ones, coinciding with chromosomes of belladonna in morphology and size, and the wider and larger ones, having no differences compared with the chromosomes of Chinese tobacco (Fig. 2E).

Cytological study of *A. belladonna* (a) and *N. chinensis* (n) chromosome types and their ratio in the clones obtained has shown that the cell line AbNc-11 contains twice as many *Nicotiana* chromosomes as *Atropa* ones. This fact should agree with the assumption that the line in question originated from the fusion of one diploid belladonna cell with one hexaploid or two triploid tobacco cells. Analysis of the chromosomes in line AbNc-11 also revealed some unusual chromosomes: chromosomes forming chains (Fig. 2D, arrows) and ring chromosomes (Fig. 2F, arrow).

Chromosomal study of the cell line AbNc-5 revealed that cells of this line have nearly 150 chromosomes and, as described in the foregoing case, the metaphase plates were shown to contain chromosomes from both, belladonna and tobacco species. The data obtained are presented in Fig. 2G–I. Our study also shows that cells of these lines have nearly equal numbers of *Atropa* and *Nicotiana* chromosomes. Thus, the origin of this line might be explained by the fusion of a belladonna diploid cell with a nearly tetra- or triploid cell of *N. chinensis.*

Chromosomal analysis of the cell lines AbNc-12 and AbNc-15 also revealed the presence of both parental types of chromosomes. These lines were found to be in every respect (chromosome number, relative content of specific parental chromosome types) similar to the line AbNc-5.

The results of a biochemical study of multiple molecular forms of amylase in the cells of parental and hybrid lines are presented in Fig. 3. Cells of belladonna contain 2 types of active bands: one very slow broad (Rf 0–0.2) and one with an intermediate type of

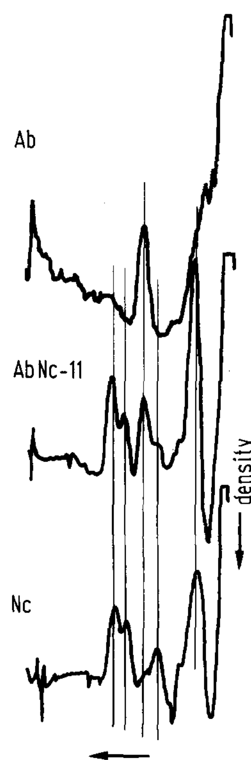

Fig. 3. Densitograms of the amylase multiple molecular forms in cells of *A. belladonna* (Ab), Chinese tobacco (Nc), and the hybrid cell line AbNc-11 of *Atropa* (×) *Nicotiana*, as revealed by PAG electrophoresis

mobility (Rf 0.4–0.44). Cells of Chinese tobacco also revealed activity in a high-molecular fraction (Rf 0.12–0.15) and three active bands of an intermediate type were present (Rf 0.36–0.37, Rf 0.52–0.55 and Rf 0.59–0.61). In some cases in tobacco a very weak reaction in the Rf 0.43–0.45 region was observed. This was similar, but not identical, to one of the molecular forms of belladonna amylase (Fig. 2). In most of the studied lines (AbNc-5, -6, -9, -11, -12, -13, -15) isozymes of both parental types were revealed, though in some of them certain molecular forms were absent. For example, the Rf 0.5 band was absent in the cells of line AbNc-5, but in the lines AbNc-9 and AbNc-10 some additional weak bands appeared which were not visible in the parental lines.

The results of electrophoresis on PAG of the total soluable protein in the cells of all lines studied (except AbNc-14) show the presence of a species-specific protein with Rf 0.08. This protein had previously been revealed in the cells of belladonna, but not in those of the other parent.

The experiments in which morphogenesis was induced were conducted 4–6 months after hybridization. In order to clarify the effect of the media, the cells of lines obtained from heteroplasmic fusion products and tissues of Chinese tobacco, were plated parallel. We could not induce a single morphogenetic event in the callus tissues of Chinese tobacco. After transfer of the cells to Linsmaier and Skoog (1965) medium, containing 8 mgl^{-1} kinetin and 1.5 mgl^{-1} benzylaminopurine, as well as a decreased concentration of sucrose (1.5%), we were able to induce shoots and leaves (Fig. 1) in the cultured lines AbNc-5, -11, -12, -13, -15.

Discussion

The results of these studies unequivocally confirm the hybrid nature of cell lines obtained by protoplast fusion of plants belonging to taxonomically different tribes. The described combination of species reveal vigorous growth of hybrid products (we agree with the opinion of Schieder 1980, on the existence of heterosis effects in some hybrids of somatic cells). The differences in the size of the parental chromosomes allow the analysis of the parental chromosomal material in hybrids. In addition, there is direct evidence that a certain morphogenetic activity can be switched on in the material obtained. As a consequence, it is possible to discuss the potential of the cell hybrids *Atropa* (×) *Nicotiana* as a model system for studies of genetic and epigenetic processes in distantly related hybrids.

Preliminary studies have also demonstrated the possibility of obtaining hybrids of the *A. belladonna* (×) *N. tabacum* combination (Gleba et al. in preparation). Thus, if necessary, the experimental system may include a number of tobacco mutants which have been recently obtained and characterized. The purpose of our future work is the utilization of the *Atropa* (×) *Nicotiana* hybrid lines for 1) the investigation of the mutation process in hybrids of intertribal cells; 2) the analysis of the morphogenetic ability of hybrid cells with different ratios of parental chromosomal material and 3) the possibility of obtaining asymmetrical chromosome combinations in distantly related hybrids by pre-irradiation of one of the parents before hybridization.

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