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Genetic processes in intergeneric cell hybrids *Atropa* + *Nicotiana*

1. Genetic constitution of cells of different clonal origin grown in vitro *

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Summary. The genetic constitution of the cell hybrids *Atropa belladonna* + *Nicotiana chinensis*, obtained by cloning of individual heteroplasmic protoplast fusion products (Gleba et al. 1982) and cultured in vitro for 12 months, has been studied. The study comprised 11 hybrid cell clones of independent origin and included analysis of a) chromosome number, size, morphology, and relative position in metaphase plates, b) multiple molecular forms of the enzymes esterase and amylase, and c) relative nuclear DNA content. The data obtained permit us to conclude that, after one year of unorganized growth in vitro, the cells of most (8) clones had retained chromosomes of both parents, while species-specific elimination of nearly all *Atropa* chromosomes had occurred in three clones. About half of the non-segregating clones possess 120–150 chromosomes including 50–70 of *Atropa* and 50–90 of *Nicotiana*. Other clones are polyploid and possess 200–250 chromosomes with a predominance of either *Atropa* or *Nicotiana* chromosome types. Only a few chromosomal changes (reconstituted chromosomes, ring chromosomes) have been detected. In some metaphase plates, chromosomes of the two parents tend to group separately, indicating non-random arrangement of chromosomes of the two parents within the hybrid nucleus. Cytophotometric studies of the relative nuclear DNA content showed that distribution histograms for cell clones were similar to those of non-hybrid cultured cells. Cell populations were relatively homogenous and do not indicate any genetic instability as a result of hybridization between remote plant species. Biochemical analysis of isoenzyme patterns confirmed that in most cell clones, species-specific multiple molecular forms of esterase and amylase from both parents were present, i.e. genetic material of both parental species was expressed in the cell hybrids.

Key words: *Atropa* – *Nicotiana* – cell hybrids – Somatic hybrids – Chromosome elimination – DNA content

Introduction

Reconstruction of higher plant cells by fusing cells from very distantly related species is one of the most interesting and promising trends of investigations of protoplast fusion. Although the number is still small, successful experiments using this approach have demonstrated that "somatic" hybridization widens the limits of crossability between remote plant species. Several investigators have produced interfamilial cell hybrids capable of continued reproduction: *Nicotiana glauca* + *Glycine max* (Kao 1977; Wetter 1977; Wetter and Kao 1980), *Nicotiana tabacum* + *Glycine max* (Chien et al. 1982), *Nicotiana chinensis* + *Pisum sativum* (Gleba et al. 1983a). Genetic instability (tendency for rearrangement and loss of chromosomes of one of the parents) and incapability of morphogenesis were characteristic for all of these hybrid cell lines. Even more information has been accumulated for intrafamilial, intertribial hybrids. After the first, extensively studied hybrid of *Arabidopsis thaliana* + *Brassica campestris* (Gleba and Hoffmann 1978, 1980), hybrid cells and abnormal plants were recovered for *Daucus carota* + *Aegopodium podagraria* (Dudits et al. 1979), *Atropa belladonna* + *Datura innoxia* (Krumbiegel and Schieder 1979, 1981), *Daucus carota* + *Petroselinum pratense* (Dudits et al. 1980), *Solanum tuberosum* + *Nicotiana tabacum*, *Solanum sucrense* + *Nicotiana tabacum* (Skarzhynskaya et al. 1982), *Atropa*

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belladonna + *Nicotiana tabacum* (Gleba et al. 1983 b). From the data available, it is evident that intertribal hybrids are genetically stable and capable of morphogenesis with formation of plants, although these are abnormal and sterile. However, inferences concerning remote hybrids are still based on an insufficient number of examples. Future investigations should be directed at studies of the greatest numbers of hybrid species combinations possible, and at intensive studies of the genetic constitution of these hybrids.

The detailed study of gene transmission in intertribal cell hybrids and elucidation of the genetic processes in these hybrids is possible only if the chromosomes of both parents can be unambiguously identified in the hybrids. Based on chromosome morphology and other hybrid characteristics, we consider the cell hybrids of *Atropa* + *Nicotiana* to be a suitable model system. We have been able to produce, by mechanical isolation and individual culturing of hybrid products, clones of cell hybrids from fusion of mesophyll protoplasts of *belladonna* (*Atropa belladonna* L.) with callus protoplasts of Chinese tobacco (*Nicotiana chinensis* Fish ex Lehm) (Gleba et al. 1982). Preliminary studies of this material, four to six months after hybridization, demonstrated hybridity of the cloned cells and also relative stability of the hybrid state in a number of clones. The present work was aimed at a more detailed study of the genetic constitution of all available clones and the elucidation of genetic changes that occur in the process of extended (12 months) unorganized in-vitro growth.

Materials and methods

Cells of 11 clones of "somatic" hybrids *Atropa belladonna* + *Nicotiana chinensis* were isolated in April 1981 by using mechanical isolation and individual cloning in microdroplets of single heteroplasmic fusion products. Leaf mesophyll protoplasts from diploid (2n = 72) plants of *Atropa belladonna* and callus protoplasts from an old nearly triploid cell line of *Nicotiana chinensis* (2n = 48) were used. The details of clone isolation and of the preliminary analysis of some of these clones after four to six months of in-vitro growth have been reported by Gleba et al. (1982). After two months of culture, the cells were grown in darkness at 25 °C and 70% humidity on solid B5 medium (Gamborg et al. 1968) containing 2 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l indole-3-acetic acid, 0.5 mg/l 1-naphthaleneacetic acid and 0.2 mg/l kinetin (6-n-furfurylaminopurine) as hormones; the pH was 5.5.

For cytological studies, tissues were fixed in Carnoy's acetic acid-ethanol (1 : 3) for 12–16 h and stained with orcein (1% solution in 45% acetic acid) for 48–72 h at 5–12 °C. In some cases, the material was pretreated with colchicine (0.5%, 1 h, 10–12 °C). Squash preparations were made in 45% acetic acid.

For cytophotometric analysis of nuclear DNA content, the cells were fixed in Carnoy's mixture for 1 h. Hydrolysis was performed in 5N hydrochloric acid at 25 °C for 35 min and stopped by adding ice-cold water. At this point, the material was transferred into Schiff reagent for 2 h at room temperature, washed with three volumes of SO_2 water, dehydrated, and encased in balsam. Determination of relative content of DNA : Schiff in the nuclei was carried out on permanent preparations by a two-wavelength method using a Zetopan (Reichert, Austria) cytospectrophotometer. A 40× objective and an A = 1.35 condenser were used. The wave lengths (505 and 560 nm) were selected by determining the absorption spectra of stained nuclei (Mendelsohn 1969). Determinations for all variants were performed on three independent preparations; 250 nuclei were measured for each variant. The calculations were carried out according to Ornstein and Patau (Patau and Swift 1953; Mendelsohn 1969). For each type of cell, histograms of the empirical distribution of DNA content were plotted and point estimations of the three first characters of distribution (expectation, variance, asymmetry) were made according to Pugachev (1979).

For biochemical analysis, the cells of callus tissue of both parent and cell lines were ground with quartz sand in a mortar and pestle. Soluble proteins were extracted in buffer as described by Medgyesy et al. (1980). The homogenates were centrifuged at 15,000 g for 30 min and the supernatants were used as enzyme preparations. These preparations were subjected to electrophoretic separation in 7.5% polyacrylamide gels according to Medgyesy et al. (1980) at a constant current of 4 mA/gel. Electrode buffer Tris-Tricine buffer (pH 7.5) was substituted for veronal; to stain for esterase activity Brewbaker's method (Brewbaker et al. 1968) was used. For amylase analysis, proteins were subjected to electrophoresis according to Davies (1967). Starch (0.15%) was added into the separation gel. After electrophoresis, gels were incubated in 0.2 M acetate buffer (pH 5.5) which contained 10 mM $CaCl_2$, and were then stained in a I_2 solution.

Results

Chromosomal analysis

Photographs illustrating the results of chromosome investigation in cells of different clones of *Atropa* + *Nicotiana* hybrid cells 11–13 months after hybridization are presented in Fig. 1. Cells of *Atropa belladonna* have 2n = 72 chromosomes, whereas those of *Nicotiana chinensis* have 2n = 48 chromosomes; for morphology and size of parental chromosome types, see Gleba et al. (1982). Eleven hybrid cell clones were analyzed. The clones can be divided in two groups: clones 5, 6, 9, 13, 14 and 15 contained between approximately 120 and 160 chromosomes, whereas clones 1, 3, 7–2, 11 and 12 had higher ploidy and contained more than 200 (most 220–250) chromosomes. The clones also differed in relative numbers of *belladonna* and chinese tobacco chromosomes. No *Atropa*-type chromosomes were found in the cells of clone 14, and in cells of clones 1 and 9, only a few (one to six) *Atropa* chromosomes (in only some metaphase plates) were observed (Fig. 1 a). In contrast, in clones 5, 7–2, and 15, at least 50–60 *Atropa* chromosomes as well as 70–80 tobacco chromosomes could be detected (Fig. 1 b, c). Among the clones of high ploidy, cell lines with a predominance of

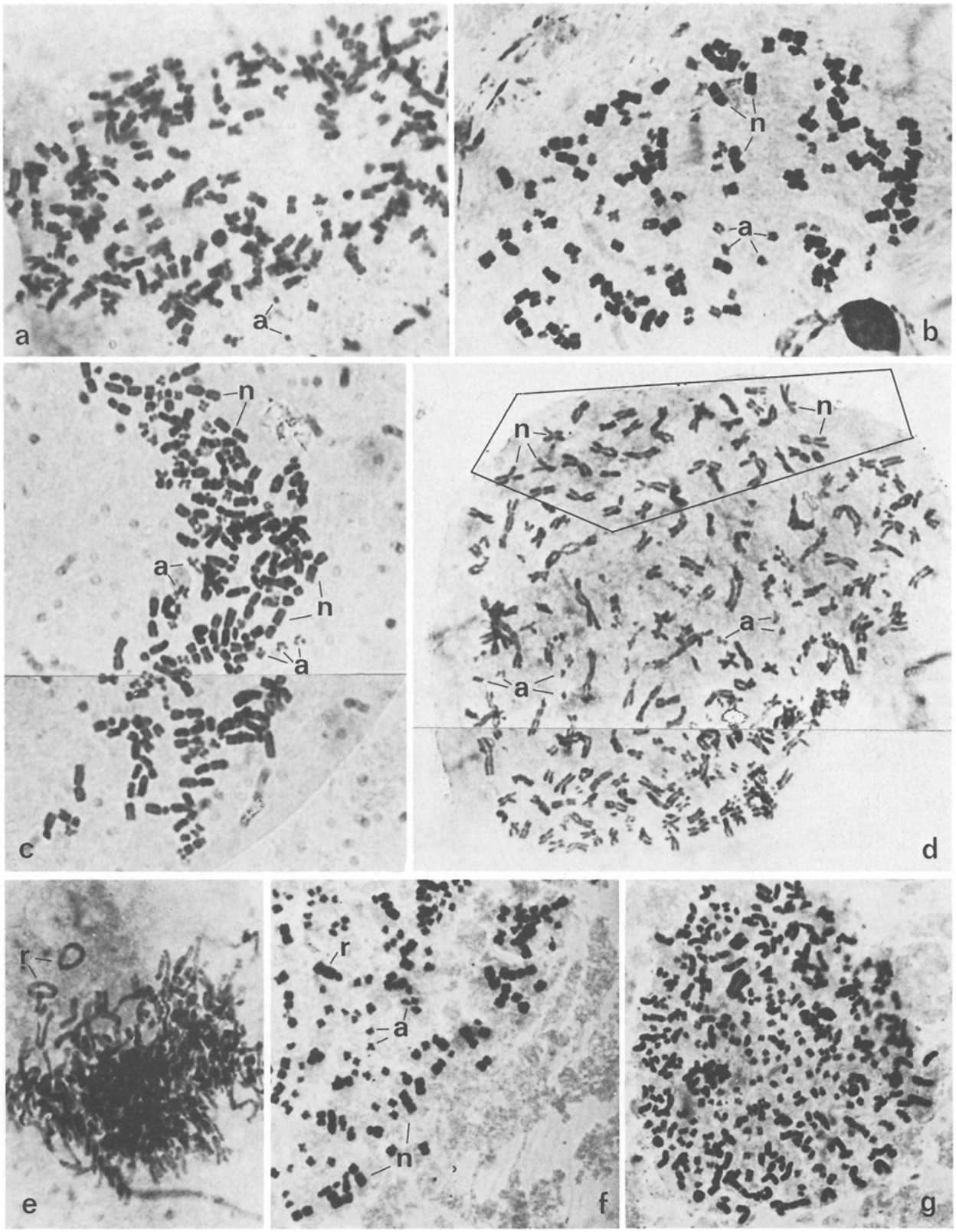

Fig. 1a–g. Chromosome studies in *Atropa belladonna* + *Nicotiana chinensis* hybrid cells. Metaphase plates in cells of the clones AbNc-1 (**a**), AbNc-6 (**b**), NbNc-7-2 (**c**), AbNc-11 (**d**), and AbNc-12 (**f, g**), and anaphase in cells of clone AbNc-11 (**e**). *a* Small *Atropa, n Nicotiana, r* Reconstituted chromosomes. Note the absence of *Atropa* chromosomes in the part (box) of metaphase plate in **d**. ×1300

tobacco chromosomes (line 11; Fig. 1d) or of *Atropa* chromosomes (line 12, Fig. 1f–g) were found. A morphological study of the chromosome types in hybrid cells showed that the great majority of chromosomes corresponded to the parental chromosomes in size and morphology; however, in cells of all the clones, reconstructed chromosomes (ring: Fig. 1a, d, e, g) were also observed.

We attempted to investigate the spatial arrangement of chromosomes of the two species during mitosis. For this, cells of line AbNc-11 were prepared without using colchicine. A typical metaphase of AbNc-11 is visible in Fig. 1d. Kao (1977) and Constabel et al. (1977) had suggested that the two parental chromosome sets were separated at metaphase during the first few divisions of hybrid cells between distantly related species. Our hybrid cells were monitored after 12 months of growth as callus, and the observations indicated that most of the two parental chromosomes (a and n) were mixed in the hybrid cells. However, in at least 20% of the plates, we detected large zones that contained chromosomes of only one parent, indicating that even after 12 months, a- and n-chromosomes had not completely mixed. For example, the pentagonal region marked in the metaphase in Fig. 1d contains only tobacco chromosomes. In an attempt at testing the hypothesis that n- and a-chromosomes are not randomly distributed in hybrid cells, we computed Mahalanobis' generalized distance D between two samples given by the plane coordinates x^1, x^2 of the n- and a-chromosomes. We designate M_n^i, M_a^i, $i=1,2$, as the mean values of the X^i coordinate for n- and a-chromosomes, respectively, with $d^i = M_n^i - M_a^i$. We denote c_n^{ij} as the covariance of x^i and x^j, $i,j=1,2$, for n-chromosomes and c_n^{ij}, similarly for a-chromosomes. c^{ij} is the average of c_n^{ij} and c_a^{ij}: $c^{ij} = \frac{1}{2}(c_n^{ij} + c_a^{ij})$. $||c_{ij}||$ can be used to designate the matrix inverse to $||c^{ij}||$. Mahalanobis' generalized distance is computed as $D^2 = \sum_{i,j=1}^{2} c_{ij} d^i d^j$. The value of using Mahalanobis' distance is that, with this formula, the choice of the coordinate axes becomes immaterial as D is invariable with respect to any affine transformation of coordinates. The equation: $T = \frac{(N_1+N_2-3)\,N_1\,N_2}{2(N_1+N_2)\,(N_1+N_2-2)} \cdot D^2$, where N_1 and

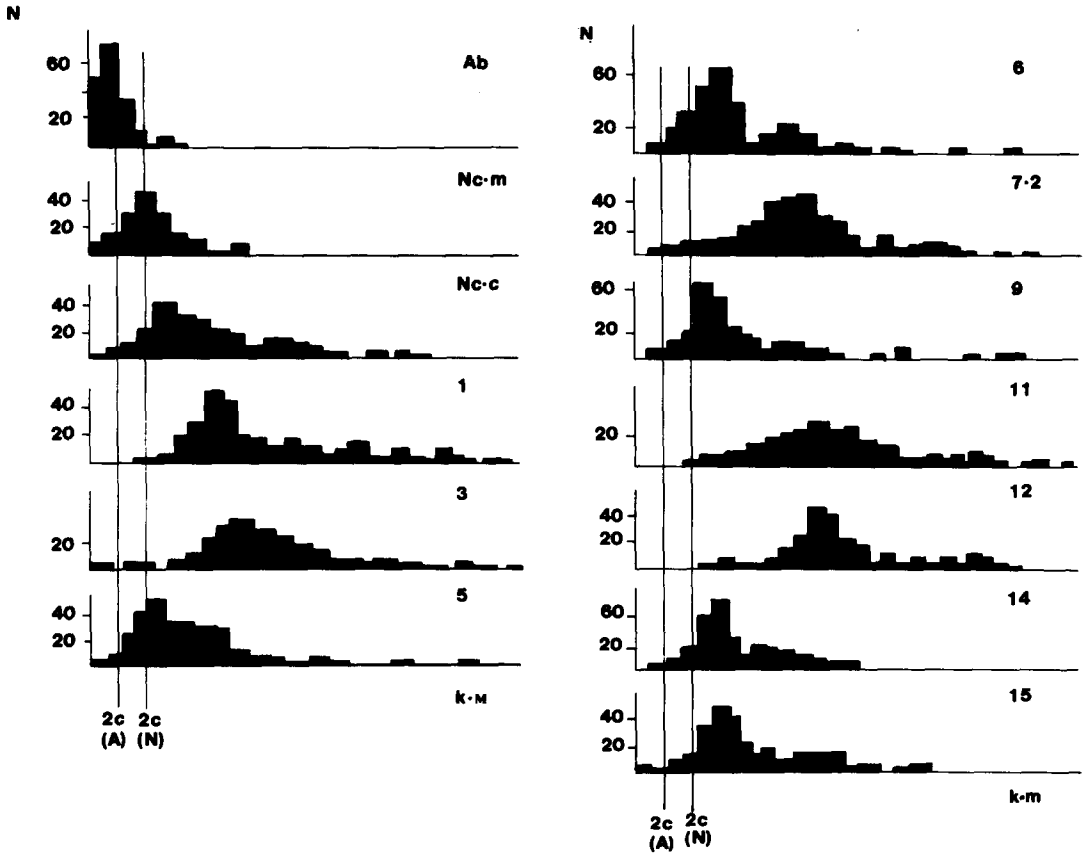

Fig. 2. Histograms of nuclear DNA content distribution in parental cells and somatic cell hybrids *Atropa belladonna* + *Nicotiana chinensis*. *Ab* mesophyll protoplasts of *Atropa belladonna* diploid plants; *Nc-m* mesophyll protoplasts of *Nicotiana chinensis* diploid plants; *Nc-c* callus cells of *Nicotiana chinensis* parental cell line; *1 – 15* different clones of somatic cell hybrids. *N* number of nuclei; *k.m* DNA. Schiff content in arbitrary units. Vertical lines indicate 2c values of *Atropa* (*A*) and *Nicotiana* (*N*)

N_2 are the sample sizes, has a F-distribution with 2 and N_1+N_2-3 degrees of freedom. This permits us to use the F to compare the distribution of parental chromosomes in the hybrid nucleus. Using this method, 25 chromosome spreads were analyzed. The distributions of n- versus a-chromosomes are statistically different ($P=0.95$) in all 25 spreads.

Cytophotometric analysis of nuclear DNA. Histograms of cell hybrid clones (in comparison with leaf mesophyll cells of belladonna and chinese tobacco, and callus cells of the parental strain of chinese tobacco) are presented in Fig. 2A, B. A statistical evaluation of the results of cytophotometry is summarized in Table 1.

The histograms of distribution for mesophyll cells (mesophyll protoplasts) of diploid plants of belladonna and chinese tobacco represent one-peak symmetric curves of normal distribution, indicating that the major portion of the leaf cell population of these species is homoploid (apparently diploid). At the same time, it appears that there is a small (at most a few percent) fraction of polyploid cells with doubled DNA content per nucleus. The cells of the two parental species clearly differ in mass per diploid nucleus ($2C_a=1.42$, $2C_n=4.19$). The histogram of cell distribution for the mass of nuclear DNA for callus cells of *N. chinensis* represents a curve typical for leaf-cell populations in the phase of logarithmic growth with G_1 and G_2. The displacement of the maximum of the distribution from the 2C value shows/indicates that the principal mass of cells of callus line are (nearly) triploid. The cell population of *N. chinensis* is rather uniform for nuclear DNA content. Analysis of histograms of 10 clones of hybrid callus lines of *Atropa* + *Nicotiana* demonstrated that all of them were different with respect to the profiles of distribution. Part of the clones had DNA mass values near to the parental callus line of chinese tobacco (clones 5, 6, 9, 14 and 15), whereas other clones (1, 3, 7–2, 11 and 12) were of higher ploidy, having approximately twice the DNA mass of the lines of low ploidy. These data may reflect the formation of clones from fusion of diploid *Atropa* cell with cells of tobacco with either 3–4n or 6–8n ploidy. The histograms of the hybrid clones also exhibited a bimodal distribution which was more pronounced than that of the callus line of *N. chinensis* (probably a consequence of cloning). It is reassuring that the histograms of the hybrid cells are of normal form despite 12 months in culture. The values of the G maxima of hybrid clones of low ploidy are considerably lower than the sum of masses of DNA from the diploid cells of *belladonna* and the triploid cells of tobacco, indicating that there is partial elimination of chromosomes in hybrid cells, particularly for clones of low ploidy. A statistical evaluation of the results of cytophotometry support these conclusions and is summarized in Table 1.

The results of an investigation of multiple molecular forms of esterase in the parental and 10 hybrid cell lines (including two sublines) are presented in Fig. 3; a diagrammatic interpretation of the electrophoretic pattern of the molecular forms of esterase is also included. *Atropa belladonna* callus has six to eight bands of esterase activity, one of which is quite intense (Rf 0.61–0.64), while five are faint but always present (Rf 0.17–0.19; Rf 0.29–0.30; Rf 0.45; Rf 0.52; Rf 0.70–0.72). The remaining faint bands are frequently absent. *N. chinensis* callus has five to seven bands of activity, including two that are intense (Rf 0.20–0.23, and Rf 0.62–0.64) and four that are faint but always visible (Rf 0.14; Rf 0.54; Rf 0.60; Rf 0.74). Four of the esterase bands for *A. belladonna* and four for *N. chinensis* are species specific and may serve as markers (indicated by arrows in Fig. 3). An analysis of the esterase zymograms from hybrid cell lines shows that the hybrid cells have fewer bands than the sum of the bands of the two

Table 1. Statistical analysis of the data for relative DNA content in populations of parental cells and clones of somatic cell hybrids of *Atropa belladonna* + *Nicotiana chinensis*

	M (Mean)	G (Variance)	m (SE)	As (Asymmetry)	Ex (Excess)
Atropa belladonna mesophyll	1.64	1.19	0.08	2.43	8.44
Nicotiana chinensis mesophyll	4.27	2.76	0.23	1.96	5.35
Nicotiana chinensis callus	8.35	4.61	0.29	1.12	0.85
NcAb-1	12.48	6.28	0.39	1.26	0.78
NcAb-3	12.92	5.77	0.36	1.39	2.61
NcAb-5	5.93	3.32	0.21	2.00	6.36
NcAb-6	6.29	3.40	0.22	2.47	9.41
NcAb-7-2	11.79	4.79	0.30	1.24	2.52
NcAb-9	6.71	3.95	0.25	2.53	9.12
NcAb-11	14.31	6.47	0.41	2.05	6.46
NcAb-12	13.29	4.29	0.27	0.98	0.91
NcAb-14	7.18	3.47	0.22	1.54	4.47
NcAb-15	7.37	3.66	0.23	1.37	2.41

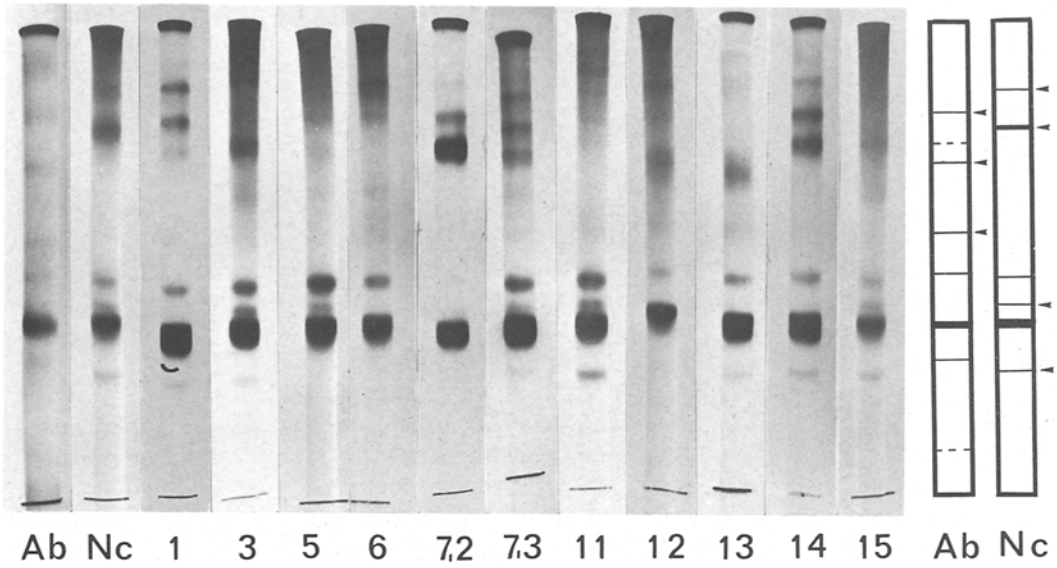

Fig. 3. Electrophoretic analysis of multiple molecular forms of esterase in cells of *Atropa belladonna, Nicotiana chinensis,* and several somatic hybrid cell clones. From *left to right:* Photographs of gels obtained for *Atropa* (*Ab*), *Nicotiana* (*Nc*) callus cells and ten clones (including two subclones) of somatic hybrid cells; a diagrammatic representation of the esterase zymograms of parents is also included. Arrows indicate species-specific bands

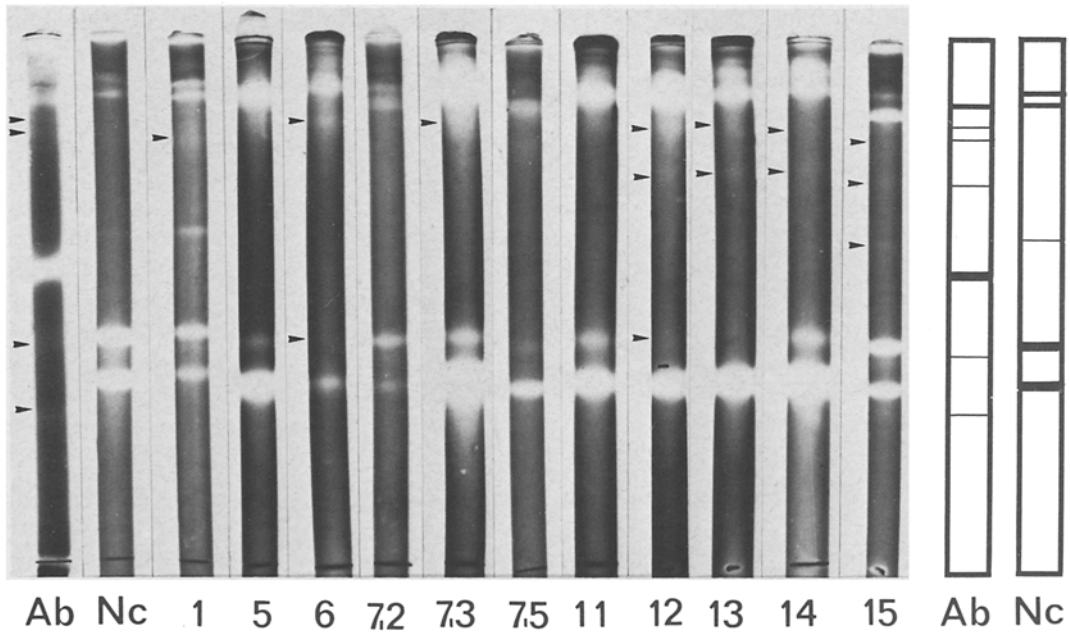

Fig. 4. Electrophoretic analysis of multiple molecular forms of amylase in the cells of *Atropa belladonna, Nicotiana chinensis* and several somatic hybrid cell clones and subclones. From *left to right:* photographs of gels obtained for *Atropa (Ab), Nicotiana (Nc)* callus cells and nine clones (including three subclones) of somatic hybrid cells; also included is a diagrammatic representation of the amylase zymograms of parents. Arrows indicate weak bands

parents, supporting the conclusion on the hybrid nature of cell lines 1, 3, 6, 7–3, 12, 13 and 15. The hybrid lines also differ among themselves in esterase spectra. Thus, the molecular form specific for *N. chinensis* (Rf 0.74) is never present in lines 5 and 6, whereas it is always present in callus cells of other lines. Another molecular form (Rf 0.60), specific for *N. chinensis* is only observed in cell lines 3, 5, 7, 11 and 15. The band (Rf 0.47) specific for *Atropa* is observed in zymograms in the majority of hybrid lines (1, 3, 5, 6, 7–3, 11, 12, 13 and 15). The other band (Rf 0.29–0.30), specific for *Atropa,* is always present in only three lines (7–2, 12 and 13).

Some clones are characterized by new bands of enzymatic activity that were absent in parents (Rf 0.36 for lines 5, 6; Rf 0.41 for line 11, etc.). It is interesting that the cells of two sublines of the clone 7, which were physically separated during the first month of cultivation, differ in their esterase patterns.

The results of the electrophoretic analysis of the multiple molecular forms of amylase (Fig. 4) confirm that in most cell clones, genetic material of both parental species (for example, *Atropa* forms with Rf 0.11–0.13; 0.16–0.17 as well as tobacco forms with Rf 0.38; 0.57–0.59; 0.64–0.67) is present and is expressed. Noteworthy, however, is the fact that despite the presence of all or nearly all *Atropa* chromosomes in the majority of hybrids, the most active specific parental form (Rf 0.42–0.46) of this species is not expressed. The main amylase activity in hybrid cells is the result of the presence of tobacco specific molecular forms.

Discussion

After 12 months of unorganized growth, 8 out of 11 hybrid cell clones of *Atropa* + *Nicotiana* preserved evidently most of the genetic material of both parents, supporting our view that the hybrid state in this combination of species is relatively stable. Relative stability for a year and more in culture was also observed for other clones of intertribal cell hybrids previously reported (*Arabidopsis* + *Brassica:* Gleba and Hoffmann 1978, 1979, 1980) (*Atropa* + *Datura:* Krumbiegel and Schieder 1979, 1981).

On the other hand, species-specific elimination of chromosomes is observed in some clones. This phenomenon is also usual for cell hybrids of distantly related plants (interfamilial and some intertribal cell hybrids). In hybrids obtained by fusion of callus and mesophyll cells, the chromosomes of the mesophyll partner have been found to be eliminated (Kao 1977; Gleba and Hoffmann 1978, 1979, 1980; Dudits et al. 1979). We have observed the same in our system, i.e. loss of the chromosomes of *Atropa*, i.e. the mesophyll partner.

Though the hybrid state of cells is associated with some chromosomal rearrangements, no increase of genetic diversity has been observed in the clones. Thus, clones of high ploidy preserved their polyploid nature during 12 months of culture in vitro, whereas clones of low ploidy preserved their ploidy level, nor did distribution of DNA content in the cells of different hybrid cells clones differ noticeably from that of the parental cell lines of *N. chinensis*. In this respect, the *Atropa* + *Nicotiana* cell hybrids are stable and even the selective loss of *Atropa* chromosomes as it can be seen from the histograms (Fig. 2, clones 1, 9 and 14), does not result in a considerable increase of diversity for the character under investigation. As can be seen from Table 1, the contribution of *Atropa* chromosomes to the mass of DNA of the hybrid nucleus is at most 20%, so loss of *Atropa* chromosomes would be difficult to detect cytophotometrically. One can assume, however, that the process of elimination of *Atropa* chromosomes might induce instability and undirected segregation of tobacco chromosomes in lines of high ploidy. In our material this phenomenon is, however, not taking place, i.e., we have the impression that in spite of the process of elimination of chromosomes from one parent, conditions for a highly regular reproduction of the chromosomes of the other parent are preserved in the hybrid cells. This is (quite) in contrast to the other somatic intertribal cells *Solanum* + *Nicotiana* which has been characterized for nuclear DNA content (Skarzhynskaya et al. 1982) and in which an extreme heterogeneity of the cell populations was observed.

Our results also permit us to comment on the degree of integration of chromosomal material from two parental species in common nucleus. In early studies of cell divisions on interspecific somatic cell hybrids, it was found that the chromosomes of the two species were arranged in separate groups, forming two-segment structures in metaphase and anaphase. However, the analyses were limited to either the first mitosis in the newly formed hybrid cell (Kao 1977; Chien et al. 1982) or to the first few mitotic divisions of the hybrid (Constabel et al. 1977). The separation of chromosomes in a mitotic cell after 14 days of culture, demonstrated by Constabel et al. (1977), does not necessarily prove inheritance of spatial separation of chromosomes; the mitosis they observed may have been a delayed first division, where separate grouping is to be expected. We have, therefore, investigated the spatial arrangement of chromosomes in somatic cell hybrids that have been grown in vitro for an extended period of time. In a number of plates, we observed large zones that contained chromosomes of only one parent, suggesting that even after one year of in vitro propagation (minimum of 50 successive cell generations), mixing of parental chromosomes was not complete (Fig. 1). To test the hypothesis that *Nicotiana* and *Atropa* chromosomes are not randomly distributed in hybrid cells, we computed Mahalonobis' generalized distance between two parental chromosome types in common metaphase. We found that the distributions of *Nicotiana* and *Atropa* chromosomes were significantly different ($P = 0.95$) in each case examined. The evident nonrandom distribution of the parental chromosomes was observed in undersquashed preparations, suggesting that chromosome arrangements in squash preparations reflect the arrangement in vivo. As shown earlier by Finch et al. (1981) using three-dimensional electron microscopic estimates of centromere positions

in unsquashed cells, squash spreads quite correctly reflect the spatial arrangement of metaphase chromosomes in vivo.

From the results, therefore, we have concluded that the spatial arrangement of the chromosomes of the two parents, that is formed as a result of somatic cell fusion and formation of common nucleus, is preserved (inherited) at least partially after many successive mitotic divisions upon in vitro culture.

Further studies of this question should consist of analyses of inheritance of the arrangement of distinct chromosomes from two parents in hybrid cells (for example, with nucleolar organizer chromosomes, it should be possible to ascertain, if hybrid nucleoli are formed in hybrid nuclei).

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