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Somatic hybridization between potato and *Nicotiana plumbaginifolia*

1. Spontaneous biparental chromosome elimination and production of asymmetric hybrids

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Summary. Electrofusion was carried out between mesophyll protoplasts from the transformed diploid *S. tuberosum* clone 413 (2n = 2x = 24) which contains various genetic markers (hormone autotrophy, opine synthesis, kanamycin resistance, β-glucuronidase activity) and mesophyll protoplasts of a diploid wild-type clone of *N. plumbaginifolia* (2n = 2x = 20). Hybrid calli were obtained after continuous culture on selection medium containing kanamycin. Parental chromosome numbers, determined at 2 months after fusion, revealed hybrid-specific differences between the individual calli. On the basis of these differences three categories of hybrids were distinguished. Category I hybrids contained between 8 and 24 potato chromosomes and more than 20 *N. plumbaginifolia* chromosomes; category II hybrids had between 1 and 20 *N. plumbaginifolia* chromosomes and more than 24 potato chromosomes; category III hybrids contained diploid or subdiploid numbers of chromosomes from both parents. The hybrids were evenly distributed over the three categories. After a 1-year culture of 24 representative hybrid callus lines on selection medium the karyotype of 10 hybrids remained stable, whereas 8 hybrids showed polyploidization of the genome of one parent, together with no or minor changes of the chromosome numbers of the other parent. Six hybrids showed slight changes in the hybrid karyotype. The elimination of chromosomes of a particular parent was not correlated to their metaphase location. The processes of spontaneous biparental chromosome elimination leading to the production of asymmetric hybrids of different categories are discussed.

Key words: Diploid *Agrobacterium*-transformed *Solanum tuberosum* – *Nicotiana plumbaginifolia* – Asymmetric somatic hybrids – Biparental chromosome elimination – Karyotypic analysis

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Introduction

Mammalian cell fusion using interspecific combinations have frequently resulted in the production of asymmetric somatic hybrids due to the preferential elimination of chromosomes of one of the fusion partners (Harris and Watkins 1965). Asymmetric hybrids have been extensively used for gene localization on human chromosomes (Kao 1983). The hybrids have also proven to be highly valuable in studying various interactions between the parental genomes at the level of nuclear architecture (Rechsteiner and Parsons 1976), chromosome behaviour during the cell cycle (Kao 1977; Vig and Athwal 1989), the chromosome elimination process (Graves and Zelesco 1988), and gene expression (Puck 1981).

In plants, somatic cell hybridization systems together with regeneration procedures were developed not only for the transfer of desirable genes from one species to the other, but also to achieve gene localization and to study genome interactions (reviews in Gleba and Sytnik 1984; Negrutiu et al. 1989, Heslop-Harrison and Bennett 1990). Plant somatic hybrids like mammalian somatic hybrids (Jakob and Ruiz 1970; Zelesco and Graves 1987), also show a spontaneous gradual elimination of chromosomes. However, the extensive elimination of chromosomes has occurred only rarely in plants (Gleba and Sytnik 1984; Negrutiu et al. 1989). Consequently, several researchers have attempted to force and direct the chromosome elimination process by various methods: (1) through premature chromosome condensation in heterophasic fusions (Dudits et al. 1982), (2) by applying dominant or recessive selection markers (Brunold et al. 1987;

De Vries et al. 1987), and (3) by treating one of the partners prior to fusion with γ- or X-rays (Wijbrandi 1989; Yamashita et al. 1989, Famelaer et al. 1990; Piastuch and Bates 1990). The first method has not been fully exploited, whereas the second one did not result in extensive chromosome elimination. The third method predominantly caused chromosome breakage, which often resulted in chromosome elimination (Famelaer et al. 1990), and in chromosomal rearrangements and the loss of genes (Wijbrandi et al. 1990). For these reasons, this approach might be less useful in gene localization (Wijbrandi 1989) or for transfer of a single or a few intact donor chromosomes (Verhoeven et al. 1991).

Spontaneous chromosome elimination in mammalian and plant somatic hybrids is generally uniparental. However, some mammalian cell hybrids have shown biparental elimination, e.g., Chinese hamster – mouse cell hybrids in which the hybrid-specific elimination of only hamster chromosomes or mouse chromosomes occurred (Zelesco and Graves 1987). In the case of plants, biparental chromosome elimination has been reported in sexual hybrids between *Hordeum marinum* × *H. vulgare* during early embryo and endosperm development (Bennett et al. 1976). The extent of spontaneous (uniparental) chromosome elimination varied between individual hybrids, which were obtained from a particular somatic fusion combination (Pijnacker et al. 1987; Khlebodarova et al. 1988). In general, the preferential elimination of specific chromosomes has not been observed (Kao 1977), although in both mammalian and plant cell hybrids there are some indications of the specific elimination of mostly those chromosomes with suppressed nucleolar activity (Miller et al. 1976; Bennett et al. 1976; Cieplinski et al. 1983; Jørgensen and Andersen 1989; Pijnacker et al. 1989). At present the exact processes underlying chromosome elimination are not known and, therefore, the manipulation of chromosome elimination to be used for the transfer of specific chromosomes and genes is not yet possible.

The present article presents data on karyotypic analysis of somatic hybrid calli between the diploid potato plant clone 413, which contains various genetic markers, including kanamycin resistance, introduced by transformation (De Vries-Uijtewaal et al. 1989), and a diploid wild-type plant clone of *Nicotiana plumbaginifolia*. The character of kanamycin resistance was used for selecting the fusion products. The various hybrid calli revealed complex patterns of spontaneous biparental chromosome elimination and the development of a different karyotypic composition during prolonged culture.

Materials and methods

Plant materials

Plants of the diploid transformed clone 413 of *Solanum tuberosum* (2n = 2x = 24) carrying various genetic markers [hormone autotrophy, opine synthesis, kanamycin resistance and β-glucuronidase (GUS)-activity] (De Vries-Uijtewaal et al. 1989; Gilissen et al. 1991) and a diploid wild-type clone of *Nicotiana plumbaginifolia* (2n = 2x = 20) were used as parents in the somatic hybridization experiments. The plants were grown in vitro as shoot cultures under controlled conditions of 16 h/day light (1 klm/m^2, TL type FTD58W33) and 24 °C in glass jars on solid MS medium (Murashige and Skoog 1962) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (Oxoid), and were subcultured monthly.

Protoplast isolation and fusion

Protoplasts of the parental plant clones were isolated from leaf pieces after an overnight (16 h) incubation in 1% (w/v) Cellulase R10 (Onozuka) and 0.2% (w/v) Macerozyme R10 (Yakult) dissolved in ½V-KM medium (Bokelmann and Roest 1983). This medium contains 0.2 *M* glucose and 0.2 *M* mannitol, but no hormones. During incubation, fluorescein-diacetate (FDA) was added to the potato protoplasts to a final concentration of 2 mg/l for recognition during fusion by means of UV microscopy. Isolated protoplasts were successively washed in solutions of 0.4 *M* mannitol, 0.4 *M* sucrose, and again in 0.4 *M* mannitol. Protoplast yield ranged between 0.5×10^6 and 5.1×10^6 per gram leaf material of potato and between 0.3×10^6 and 0.8×10^6 per gram leaf material of *N. plumbaginifolia*. After isolation the protoplasts were diluted to a concentration of 10^5 per millilitre and then mixed in a 1:1 ratio. The mixture was placed in 0.8-ml samples between the electrodes of a multi-electrode system in a 5-cm petri dish. Electrofusion was carried out using an AC field of 1 MHz and 60 V/cm for alignment of the protoplasts, and fusion was induced by three DC pulses of 1.5 kV/cm (modified according to Puite et al. 1985).

Selection of hybrids

Immediately after fusion, the protoplasts and heterokaryons were cultured in 1.6 ml of liquid ½V-KM medium supplemented with 0.3 mg/l NAA, 0.1 mg/l zeatine, and 10 mg/l kanamycin. The cultures were incubated at 24 °C in continuous light (1 klm/m^2, TL type F48T12). Only hybrids were able to develop in this medium, which appeared to be selective against unfused potato as well as unfused *N. plumbaginifolia* cells. During the following 6 weeks, microcalli/calli were subcultured twice in fresh medium. Afterwards, the calli were isolated and subcultured monthly as individual callus lines in 9-cm petri dishes on solid MS medium supplemented with 5 mg/l NAA, 0.1 mg/l BAP, and 100 mg/l kanamycin under the same culture conditions as used for shoot cultures.

Karyotypic analysis

The contrasting differences in the size and morphology of the metaphase chromosomes of potato (metacentric or submetacentric) (Pijnacker and Ferwerda 1984) and *N. plumbaginifolia* (acrocentric or subacrocentric) (Mouras et al. 1986) enabled an accurate determination of the karyotypic composition (i.e., the numbers of chromosomes of *S. tuberosum* and *N. plumbaginifolia*) of the hybrids. At 2 months after fusion the karyotypic composition of 49 calli obtained from four separate experiments was determined in four to ten well-spread Feulgen-stained metaphase cells per callus as described previously (Sree Ramulu et al. 1985). Optimum frequencies of metaphase cells were observed in calli at 3–5 days after subculturing. On the basis of karyotypic composition as well as callus growth rate, 24 hybrid lines were selected for further characterization of karyotypic composition at two different culture times, i.e., 8 and 12 months.

Plant regeneration

For plant regeneration, the procedures described earlier by Bokelmann and Roest (1983) and Installé et al. (1985) were used. In the latter, the RP medium constituents were substituted for those of MS.

Results

Karyotypic analysis of the somatic hybrids

Four separate experiments on fusions between *S. tuberosum* and *N. plumbaginifolia* protoplasts resulted in 274 calli that grew on selection medium (½V-KM) containing 10 mg/l kanamycin. In general, these calli continued growth on MS with 100 mg/l kanamycin. Forty-nine calli were selected for further analysis.

Table 1 shows data on the karyotypic analysis of the 49 hybrid callus lines carried out at 2 months after selection. Based on differences in the numbers of parental chromosomes, the individual hybrids were classified into three categories. Each of the hybrids of category I contained a specific number of potato chromosomes, which varied between 8 (H25) and 24 (H17), an had more than 20 *N. plumbaginifolia* chromosomes. Category II hybrids had hybrid-specific chromosome numbers that varied between 1 (H2) and 20 (H67) *N. plumbaginifolia* chromosomes and more than 24 potato chromosomes. Hybrids of category III had diploid (H22, H42) or subdiploid numbers of chromosomes form both parents. Four callus lines (H3, H7, H63 and H64) classified in category II did not contain any *N. plumbaginifolia* chromosomes. These lines might have escaped from selection, might be cybrids, or might have developed form hybrid cells that completely eliminated all of their *N. plumbaginifolia* chromosomes. One hybrid (H45) had a high number of potato chromosomes (55) and more than the diploid number of *N. plumbaginifolia* chromosomes (27). Therefore, this hybrid did not belong to these categories. The deviation from the mean number of chromosomes of both parents in the individual hybrids ranged between 2 and 6. The mean value of deviation over all hybrids (except the four hybrids mentioned below) were 3.1 for potato and 2.9 for *N. plumbaginifolia*. Four hybrids showed higher deviation in the chromosome number of only one parent, i.e., potato (ranging between 10 and 13 chromosomes in hybrids H13, H30 and H33) or *N. plumbaginifolia* (10 chromosomes in H16) (results not shown in Table 1). The total numbers of hybrids belonging to categories I, II, or III were 18, 18, and 12, respectively (Table 1). This even distribution of the hybrids over the three categories was found in each separate fusion experiment (except in experiment 2, from which only one hybrid was selected for karyotypic analysis) (Fig. 1). Metaphase cells of hybrids representative of the different categories are shown in Fig. 2.

Table 1. Karyotypic composition in 2-month-old somatic hybrid callus lines obtained after the fusion of leaf protoplasts from the diploid *S. tuberosum* clone 413 (2n = 2x = 24) and a wild-type clone of *N. plumbaginifolia* (2n = 2x = 20) (data pooled from four fusion experiments). The details on the classification of different categories and on ranges of variation in the parental chromosome numbers are indicated in the text

Categories of hybrids								
Category I			Category II			Category III		
Hybrid	S	N	Hybrid	S	N	Hybrid	S	N
H1	17	43	H2	47	1	H9	20	13
H4	23	32	H3[a]	43	0	H22	24	20
H5	20	41	H7[a]	48	0	H26	23	19
H6	20	49	H8	44	14	H32	17	16
H15	12	33	H10	26	13	H42	24	20
H16	18	30	H13	37	11	H46	17	16
H17	24	34	H30	43	6	H47	20	19
H18	14	23	H33	33	10	H50	13	17
H19	16	26	H35	48	2	H55	18	18
H21	11	28	H36	48	11	H70	9	18
H23	10	30	H43	41	11	H74	14	17
H24	13	57	H44	42	2	H75	12	22
H25	8	45	H49	48	3			
H34	11	32	H58	55	10			
H52	13	37	H62	53	6			
H57	12	28	H63[a]	35	0			
H60	12	44	H64[a]	48	0			
H68	12	30	H67	35	20			

S, Number of *S. tuberosum* chromosomes; N, number of *N. plumbaginifolia* chromosomes in the hybrids
[a] Apparent escapes, or cybrids (see text)

The pattern of changes in parental chromosome numbers was analysed in 24 selected hybrid callus lines cultured for 1 year on selection medium containing 100 mg/l kanamycin. Ten hybrid lines, distributed over all three categories, had stably maintained the original composition of the parental chromosomes. Six hybrids showed minor changes, while 8 hybrids exhibited major changes in chromosome composition (Table 2). In these latter hybrids, there had been an increase in the chromosome number of one parent together with no changes or only minor ones (increase or decrease) in the number of the other parent. Hybrid line H74 showed a significant increase in the chromosome numbers of both parents.

Figure 3 presents data on the variation in parental chromosome number between some representative hybrid callus lines. In addition, this figure shows the range of deviation and the direction of alteration of chromosome number changes within these lines that occurred during the 1-year culture period. It is clear from these results that often one or more subpopulations of cells with different chromosome numbers developed within the hybrid calli.

Twenty-two hybrid lines were analysed for spatial arrangement of the parental chromosomes in somatic metaphase cells (Table 3, Fig. 4). Metaphase plates of category I hybrids predominantly showed the presence of small potato chromosomes in the central region and of the large chromosomes of *N. plumbaginifolia* in the peripheral region (type A). Mixed parental chromosome arrangements (type B) were observed in category II and III hybrids. Several hybrids of all three categories contained cells with potato chromosomes located centrally (type A) in addition to cells with mixed arrangements of the parental chromosomes (type B); these hybrids were characterized by having only slightly reduced numbers of either potato (>15) or *N. plumbaginifolia* (>11) chromosomes.

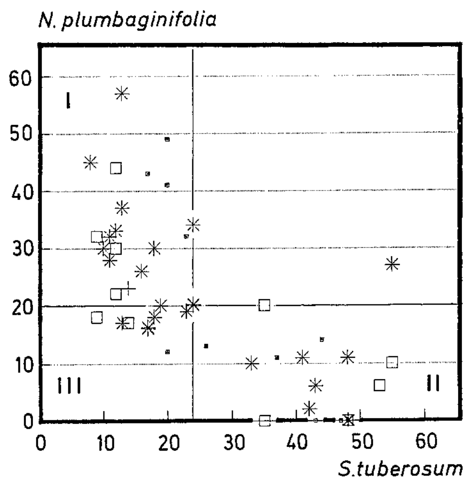

Fig. 1. Parental chromosome numbers in callus lines of hybrids between diploid *S. tuberosum* clone 413 and wild-type *N. plumbaginifolia*. The *lines* x = 24 and y = 20 indicate the diploid number of *S. tuberosum* and *N. plumbaginifolia* chromosomes, respectively, present in the parental plant clones. Details on the classification of hybrids into categories (I, II, III) are given in the text. Each *mark* represents the mean parental chromosome number of one hybrid. Hybrids from four fusion experiments were analysed (■ exp. 1, + exp. 2, * exp. 3, □ exp. 4)

Plant regeneration

Of the 24 hybrid callus lines, only 4 category I hybrids (H21, H24, H25, and H34) and 1 category II hybrid line (H33) showed greening of calli; there was no shoot regeneration.

Discussion

The present data show that several somatic hybrid calli could be obtained when kanamycin was added immediately after the fusion of mesophyll protoplasts obtained from transformed diploid potato (2n = 2x = 24) and wild-type *N. plumbaginifolia* (2n = 2x = 20). The timing of kanamycin selection appeared to be important, since various other experiments in which selection for kanamycin resistance was carried out between 2 and 6 weeks after fusion did not result in the production of somatic hybrids (L. J. W. Gilissen et al. unpublished results).

Karyotypic analysis of the hybrid calli revealed the biparental elimination of chromosomes. Potato chromosomes were specifically eliminated in some hybrid lines (category I), while *N. plumbaginifolia* chromosomes were predominantly eliminated in other hybrid lines (category II). Also, in some hybrids (category III) both potato and *N. plumbaginifolia* chromosomes were eliminated at differing frequencies. The karyotypes of various hybrids of the three categories appeared to have stabilized by 2

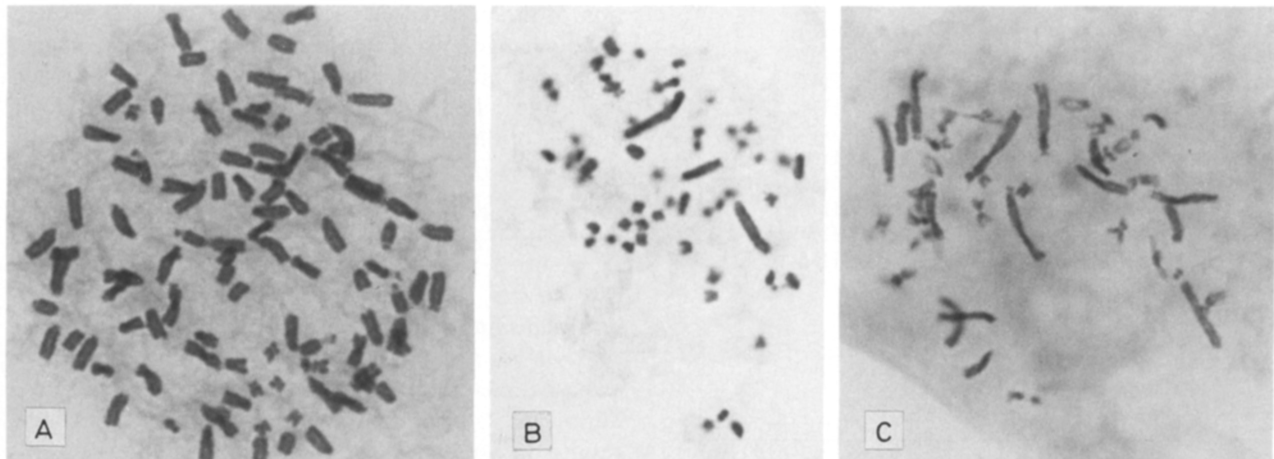

Fig. 2 A–C. Metaphase cells of various callus lines of somatic hybrids between *S. tuberosum* clone 413 and wild-type *N. plumbaginifolia* representative of hybrid category I (**A** H21), category II (**B** H43), and category III (**C** H46)

Table 2. Pattern of changes in parental chromsome numbers in somatic hybrid callus lines after 1 year of culture on selective MS medium containing 100 mg/l kanamycin. For comparison, parental chromosome numbers in the hybrids after 2 months of culture are given in parentheses

Hybrid categories	Stable hybrid lines	Hybrid lines showing minor changes in chromosome composition			Hybrid lines showing major changes in chromosome composition		
	lines[a]	lines	S	N	clone	S	N
I	H5 H6 H15 H24 H57	H1 H4 H18 H60	15 (17) 30 (23) 8 (14) 11 (12)	35 (43) 48 (32) 35 (23) 55 (44)	H21 H25 H34	10 (11) 10 (8) 10 (11)	87 (27) 84 (45) 60 (32)
II	H8 H10	H13	40 (37)	4 (11)	H30 H33 H36 H43	85 (43) 73 (33) 90 (48) 72 (41)	0 (6) 10 (10) 5 (11) 8 (11)
III	H9 H32 H46	–			H74	90 (17)	42 (14)
Other	–	H45	64 (55)	11 (27)	–		

S, Number of *S. tuberosum* chromosomes, N, number of *N. plumbaginifolia* chromsomes in the hybrids
[a] Chromosome composition of these lines is given in Table 1

Table 3. Spatial arrangements of parental chromosomes in metaphase cells of representative somatic hybrid callus lines during a 1-year culture on selective MS medium containing 100 mg/l kanamycin. Types of spatial arrangements were determined from metaphase plate observation and photographs taken at various times during the 1-year culture period

Hybrid categories	Types of spatial arrangements of parental chromosomes		
	Number of hybrids with central location of potato genome (type A)	Number of hybrids with mixed arrangement of parental genome (type B)	Number of hybrids showing both types of parental chromosome location
I	8	0	3[a]
II	0	4	2[b]
III	0	2	2
Other	0	1	0

[a] Hybrids with more than 15 potato chromosome
[b] Hybrids with more than 11 *N. plumbaginifolia* chromosomes

months after fusion. This indicates that the process of biparental chromosome elimination might have taken place during the initial stage (i.e., the first cell divisions) of hybrid development. This phenomenon of an extensive, spontaneous biparental chromosome elimination has so far not been described for plant somatic hybrids.

Strikingly, the present data show that the three different categories of hybrids occurred evenly in all the experiments (Fig. 1). At present, the exact mechanisms or fac-

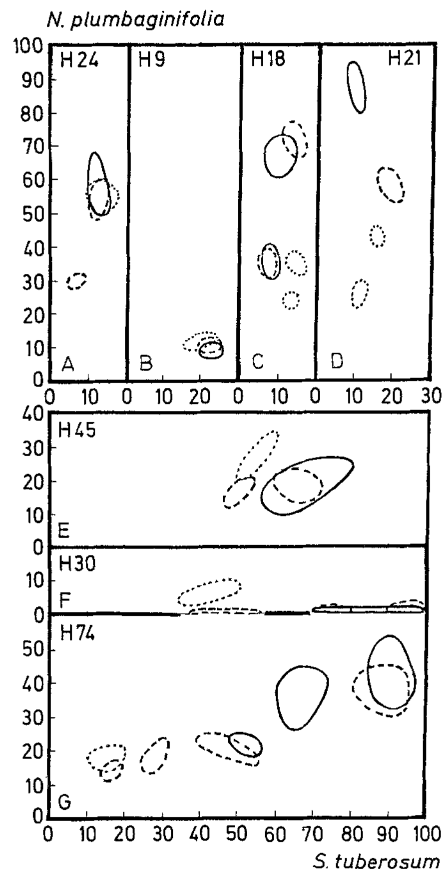

Fig. 3. Pictorialized scatter diagram showing parental chromosome number variation (represented by the areas) in some representative hybrids between *S. tuberosum* clone 413 and wild-type *N. plumbaginifolia* after various periods of culture (····· 2 months, - - - - 8 months, —— 12 months)

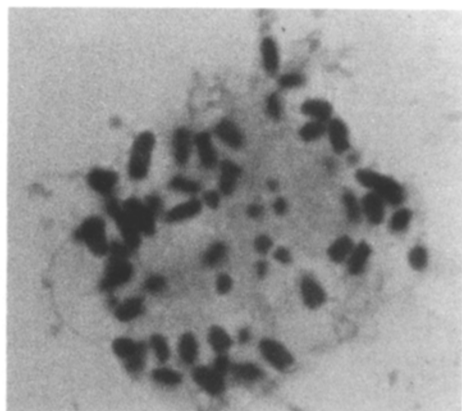

Fig. 4. A metaphase cell of the hybrid callus line H60 showing the chromosomes of *S. tuberosum* clone 413 in central position and the chromosomes of wild-type *N. plumbaginifolia* in the peripheral region (hybrid category I; spatial arrangement type A). For the spatial arrangement type B (chromsomes in mixed arrangement) see Figs. 2B and 2C

tors involved in the induction of spontaneous elimination of chromosomes are not known. Previous studies suggest that one or more nuclear and cytoplasmic factors are involved in chromosome elimination from somatic hybrid cells: interactions at the level of nuclear and cytoplasmic genomes related to phylogenetic distance, differences in cell type, tissue origin, metabolic state, cell volume, cell-cycle phase, cell-cycle duration, and ploidy level of parental protoplasts; and, in addition, the spatial distribution of the parental genomes within the hybrid nucleus and different rates of chromatid separation between parental chromosomes during the anaphase of hybrid cells (Bennett et al. 1976; Kao 1977; Dudits 1982; Graves 1984, Gleba and Sytnik 1984; Bennett 1987, Dudits et al., 1987; Gleba et al. 1987; Zelesco and Graves 1987, Graves and Zelesco 1988; Vig and Athwal 1989; Pijnacker and Sree Ramulu 1990).

Until now, early occurring, bidirectional chromosome elimination has only been reported in sexual hybrids between *Hordeum marinum* × *H. vulgare*. Elimination of the *H. marinum* chromosomes occurred in the embryo, whereas elimination of the *H. vulgare* chromosomes was found to occur in the coenocytic endosperm. Remarkably, in both cases the genome which got eliminated was peripherally located in the metaphase plates prior to elimination in both tissues. The peripheral metaphase location of chromosomes was, therefore, suggested to be related to a predisposition for chromosome elimination (Bennett 1987). However, in a somatic hybrid callus between *Nicotiana chinensis* (+) *Atropa belladonna*, the small chromosomes of the latter parent, located in the central region of the metaphase plate, were subject to extensive elimination in a callus line during prolonged subculture (Gleba et al. 1987). A similar phenomenon was observed in the present study, where progressive elimination of the centrally located potato chromosomes occurred during prolonged subculture of hybrid callus line H18. In addition, in various category II hybrids characterized by mixed parental chromosome locations, extensive elimination of *N. plumbaginifolia* chromosomes had occurred. These results suggest the absence of a correlation between metaphase location and chromosome elimination. Also, in mammalian cell hybrids, where mixed parental chromosome locations have often been found, no clear correlation was obtained between spatial arrangement of metaphase chromosomes and chromosome elimination (Kao 1983; Graves and Zelesco 1988). It should be mentioned, however, that the topology of genome separation can depend on the age of the hybrid (Gleba et al. 1987), subculture conditions, and the method of metaphase slide preparation. Therefore, further investigations on young hybrids using confocal laser scanning microscopy of intact cell preparations could be illuminating in this respect.

None of the hybrid calli regenerated shoots or plants, although regeneration procedures similar to those used in this study have resulted in some plantlets from somatic hybrid calli, developed after fusion of a monohaploid potato plant clone and a tetraploid nitrate reductase-deficient mutant cell line of *N. plumbaginifolia* (De Vries et al. 1987). Recently, however, shoot regeneration was obtained from hybrid calli between potato clone 413 and a diploid *Agrobacterium rhizogenes*-transformed plant clone of *N. plumbaginifolia* using the same regeneration procedures. Karyotypic analysis of these hybrid plants revealed a moderate to strong reduction of the number of potato chromosomes (L. J. W. Gilissen et al. unpublished results).

In practical plant breeding aiming at the transfer of a partial genome (one or few chromosomes carrying useful genes) or at genomic integration, more knowledge on factors inducing chromosome elimination as well as chromosomal integration is highly desirable. In addition, research on the linkage and expression of the introduced genes is important. Some of these topics have been dealt with elsewhere (Gilissen et al. 1992).

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