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Chromosomes in somatic hybrids between *Nicotiana plumbaginifolia* and a monoploid potato

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Summary. Leaf mesophyll protoplasts of the monohaploid potato (*Solanum tuberosum* L.) clone H7322 were fused with callus protoplasts of nitrate reductase deficient (NR^-) mutants Cnx 20 and NA 36 of *Nicotiana plumbaginifolia*. Somatic hybrid lines were selected for nitrate reductase proficiency. All callus lines tested appeared to be stable for the retention of the potato chromosome carrying the compensating NR gene when grown for over 1.5 years in the absence of nitrate. Shoots were regenerated from six different fusion lines of Cnx 20 + H7322 24 months after fusion. Chromosomal analysis in callus cultures revealed that in both fusion combinations 40–120 *N. plumbaginifolia* chromosomes were present, as were 9–20 potato chromosomes. Cells with 17 potato chromosomes in combination with a relatively small number (31) of *N. plumbaginifolia* chromosomes were found in one line. Preferential loss of species-specific chromosomes was not observed. Analysis of regenerating tissue from three lines of Cnx 20 + H7322 revealed that after 24 months of culture intra- and intergeneric translocations, fragments and deletions were present. Elimination of the potato and *N. plumbaginifolia* chromosomes had taken place before and after genome doubling.

Key words: Chromosome instability – *Nicotiana plumbaginifolia* – *Solanum tuberosum* – Somatic hybridization

Introduction

Genetic research in the potato (*Solanum tuberosum* L.) is limited compared to other important crops. Until now only two genes have been located by trisomic analysis (Hermsen et al. 1973; Wagenvoort 1982). The potato is autotetraploid and highly heterozygous. Therefore, it is difficult to assign genes to chromosomes. Somatic cell genetic studies may contribute to a better mapping of the potato genome. Interspecific somatic cell hybrids have been used successfully for gene mapping of the human genome (for review see Kao 1983).

Chromosome loss of one of the fusion partners has been observed in interfamily and some intergeneric somatic hybrids of higher plants (Kao 1977; Dudits et al. 1979; Chien et al. 1982; Gleba and Sytnik 1984; Potrykus et al. 1984; Pental et al. 1986). It is suggested that in somatic hybrids of higher plants unilateral chromosome loss proceeds similarly to animal cell hybrids (Evans and Wilson 1984; Gleba and Sytnik 1984). Studies on the elimination of specific chromosomes from the somatic hybrids has been hampered by lack of well-defined karyotypes for the fusion partners used. Chromosome elimination has previously been studied by examining the number of parental chromosomes distinguishable by size and morphology (Kao 1977; Chien et al. 1982; Gleba and Sytnik 1984). Examination of the presence of isozyme forms specific to the parental lines has often been used to complement chromosome studies (Wetter 1977; Gleba and Sytnik 1984).

The identification of the individual somatic metaphase chromosomes of a monoploid potato clone has recently been accomplished (Pijnacker and Ferwerda 1984). This makes it possible to determine which potato chromosomes are present in a somatic hybrid and thus which ones have been eliminated. This kind of experiment requires the choice of an appropriate fusion partner. In the experiments described in this paper, nitrate reductase deficient (NR^-) mutants of *Nicotiana plumbaginifolia* were used as the fusion partners for *S. tube-*

rosum. N. plumbaginifolia and potato chromosomes can be distinguished (Pijnacker and Ferwerda 1984; Mouras et al. 1986; Pijnacker and Mouras 1986) and several biochemically well-characterized NR^- mutants of this species are available (Mendel et al. 1986; de Vries et al. 1986). The karyotypes of the somatic hybrids were followed and special attention was paid to unilateral loss of potato chromosomes.

Materials and methods

Materials

In this study monohaploid *Solanum tuberosum* plants (2n=x=12) coded Mn 797322 were used. This clone, abbreviated H7322, was produced androgenetically from the interdihaploid family H78.01 (Max Planck Institut, Cologne, FRG), itself an F1 family from cross: interdihaploid *S. tuberosum* (clone HH439)×*S. phureja* (clone H75.1631153). Clone HH439 is an F1 plant from the crossing of the primary dihaploids H31 (originating from the tetraploid 49.540/2) and H149 (originating from the tetraploid 44.1016/10) (for detailed information see Ross and Jacobsen 1975). H7322 was kindly supplied by Prof. Dr. G. Wenzel, Grünbach, FRG. From *Nicotiana plumbaginifolia* we used NR^- lines Cnx 20 (kindly supplied by Dr. I. Negrutiu, Brussels, Belgium) and NA 36 (obtained from Dr. L. Marton, Szeged, Hungary). Cnx 20 belongs to complementation group cnxA; NA 36 represents the *nia* category. Both cell lines are predominantly tetraploid (2n=4×=40). *S. tuberosum* plants were aseptically grown on basal MS medium (Murashige and Skoog 1962), supplemented with 10 g/l sucrose. The plants were grown in a growth cabinet at 24 °C with 14 h light (3,000 lux). The callus cultures from Cnx 20 and NA 36 were propagated on AA medium, which contains asparagine, arginine, glutamine and glycine as nitrogen source (Muller and Grafe 1978), or on MSC medium (Dirks et al. 1985), supplemented with 5 mg/l thiamine. The cultures were grown in the dark at 25 °C. The callus cultures were transferred every 3 weeks to fresh culture medium. The callus and plant culture media were autoclaved.

Protoplast isolation, fusion and culture

Mesophyll protoplasts from *S. tuberosum* were isolated following the procedure as described by Bokelmann and Roest (1983), except for some modifications. We used an enzyme solution containing 0.6% cellulase 'Onozuka' R-10 and 0.12% macerozyme in 0.5 M mannitol. The same enzyme solution was used for the isolation of callus protoplasts from the Cnx 20 and NA 36 mutants of *N. plumbaginifolia.* Incubation was carried out overnight, in the dark. As washing medium we used 0.5 M mannitol. The protoplasts were collected by centrifugation in 0.57 M sucrose. Fusion was carried out following the procedure of Menczel et al. (1981), modified by using 30% PEG 4000. In three experiments, a total of $1 \cdot 10^6$ NA 36 callus protoplasts were fused with an equal number of *S. tuberosum* mesophyll protoplasts. In the other combination, $6 \cdot 10^6$ Cnx 20 callus protoplasts were fused with $9 \cdot 10^6$ H7322 mesophyll protoplasts in four experiments. As controls, homologous fusions of Cnx 20, NA 36 and H7322 as well as heterologous mixtures and cultures of the parental cell lines were used. All were cultured in VKm+AA medium: VKm as described by Bokelmann and Roest (1983), supplemented with the amino acids from AA medium (Muller and Grafe 1978).

After 1–2 weeks of culture the well-growing protoplast suspensions were diluted 1:1 with the same culture medium. After another 2 weeks of culture, the suspensions were diluted again with VKm+AA. MDR15 (Dirks et al. 1985), which contains KNO_3 as nitrogen source, was used for the second dilution in two experiments involving NA 36 and H7322 protoplasts. Further culture was carried out as described in detail for intraspecific *N. plumbaginifolia* fusions (de Vries et al. 1987). The protoplast culture media were filter sterilized. The protoplast cultures were grown in a growth cabinet at 26 °C under continuous illumination of 1,000 lux.

Selection of somatic hybrids and plant regeneration

H7322 protoplasts lack regeneration capacity under the culture conditions used. No division activity was observed after 10 days of culture. The protoplasts turned brown and degenerated. PEG-treated H7322 protoplasts degenerated too. This simplified the selection procedure. Selection of somatic hybrids was carried out as has been described before for experiments in which NR^- mesophyll protoplasts from *N. plumbaginifolia* were fused (de Vries et al. 1987). The growth of putative somatic hybrids was tested on A medium, which contains NH_4NO_3 and KNO_3 as nitrogen source (Muller and Grafe 1978), and on AA medium, supplemented with 50 mM $KClO_3$. NR^- mutants are resistant to the latter compound. NR activity was induced by growing callus for 48 h on A medium. NR activity in vivo was assayed qualitatively according to Jaworski (1971). Calli having NR activity in vivo, growing well on A medium and growing poorly on AA medium+$KClO_3$ were classified as NR^+.

Somatic hybrid lines from Cnx 20+H7322 and NA 36+H7322 were further propagated on A medium. Some lines were subcultured on AA medium as well. Shoot regeneration was induced on M339, which is MS medium containing NAA (0.01 mg/l), GA_3 (0.01 mg/l) and zeatin (1 mg/l) and 20 g/l sucrose. We also used RP medium, modified by using MS instead of MP macronutrients (Installe et al. 1985). Occasionally R4S (Negrutiu et al. 1983) was used as non-selective regeneration medium. Regeneration was carried out in a growth cabinet at 24 °C with 14 h light (3,000 lux). For root formation, regenerants were transferred to MS medium, supplemented with 10 g/l sucrose.

Chromosome analysis

Small pieces of callus or regenerating tissue were transferred onto fresh medium (A, M339 or RP medium) and grown in the dark for 3 days. The material was collected for chromosome analysis and either directly fixed or pretreated in saturated α-bromonaphtalene for 5 h at 4 °C before fixation in 3:1 (v/v) absolute alcohol-glacial acetic acid mixture. For a quick chromosome analysis aceto-orcein squashes were made. Detailed chromosome analysis was carried out on air-dried slides, prepared following Pijnacker et al. (1986a), with incubation in the enzyme solution for 60 instead of 45 min. Giemsa C-band pattern analysis of chromosomes followed Pijnacker and Ferwerda (1984). More consistent C-banding was obtained when the slides were immersed in 0.2 M HCl for 1 h at room temperature before the barium hydroxide step (Sumner 1972).

Results

Selection of somatic hybrids

The selection of somatic hybrids was successfully carried out in growth experiments and by assaying NR ac-

Table 1. Fusion and control experiments

Cell type	No. of tested calli[a]	No. of NR^+ calli
Cnx 20 + H7322 fused	740	54
NA 36 + H7322 fused	734	204
Cnx 20 + H7322 mixture	132	0
NA 36 + H7322 mixture	36	0
Cnx 20 fused	222	0
NA 36 fused	235	0
H7322 fused	+[b]	
Cnx 20	95	0
NA 36	70	0
H7322	+[b]	

[a] The callus growth was tested on culture medium containing KNO_3 and NH_4NO_3 as nitrogen sources and on medium to which 50 mM $KClO_3$ had been added

[b] The H7322 mesophyll protoplasts (fused or non-fused) degenerated after 10 days of culture under the culture conditions used

tivity in vivo. Out of 734 tested calli obtained after fusion of NA 36 and H7322 protoplasts 204 were NR^+ (Table 1). In the other combination, Cnx 20 + H7322, 54 out of 740 tested calli were NR^+. The selected calli were maintained as cell lines. In the control experiments, i.e. the homologous fusions of Cnx 20 and NA 36, the heterologous mixtures and the cultures of Cnx 20, all the tested calli were resistant to $KClO_3$ and were not able to proliferate well on A medium. All lacked NR activity in vivo (Table 1).

Examination of cell lines and plant regeneration

In all, 62 well-growing cell lines of NA 36 + H7322 and 25 of Cnx 20 + H7322 were cultured for over 1.5 years on A medium and on AA medium. NR activity could still be induced in all the calli. This indicates that the potato gene responsible for the compensation of the mutant gene in *N. plumbaginifolia* was still present.

The chosen cell lines were also transferred to shoot regeneration medium. No regenerated shoots were obtained from NA 36 + H7322 fusions after 1 year of culture on selective (nitrate containing) shoot regeneration medium. No green sectors indicating that regeneration was in progress were visible. Regenerating calli were neither obtained on the non selective regeneration medium R4S. The callus line NA 36 has lost its regeneration capacity (Marton et al. 1982), which probably affects the process of plant regeneration in somatic hybrids involving this mutant.

The first regenerating callus resulting in a distorted shoot was obtained in the Cnx 20 + H7322 somatic hybrid line 2-96 10 months after fusion. From then on 10 other lines also formed green sectors, which developed after some time into teratomata and leaf-like structures. Regeneration was the most successful on RP medium which is normally used for shoot regeneration of *N. plumbaginifolia* (Installe et al. 1985). More normal looking shoots regenerated spontaneously about 18 months after fusion. Also, 24 months after fusion 58 shoots from 6 different somatic hybrid lines had been regenerated, the majority (28) originating from cell line 2-96. The shoots did not exceed 2 cm. Differences in phenotype were observed between plants regenerated from different lines. The leaves varied from lancet- to oval-shaped, all forming a rosette (Fig. 1). Regenerated shoots with oval leaves showed similarities to *N. plumbaginifolia* shoots. Only minor variation in shoots regenerated from the same cell line were observed. All the shoots regenerated from 2-96 had lancet-shaped leaves. The first regenerated plantlets died when transferred to rooting medium. Afterwards most of the shoots could be maintained for a longer period on this medium, but no roots were formed.

Karyotype of H7322 and N. plumbaginifolia

The karyotype of H7322 differs slightly from that of the monohaploid tracing back to cv. Gineke, which has been described (Pijnacker and Ferwerda 1984). The chromosomes of H7322 were numbered in accordance with the numbering in cv. Gineke. Chromosomes 1, 2, 6 and 7 are similar to those of cv. Gineke. The interstitial band in the long arm of chromosome 3 is less distinct. The interstitial band on the short arm of chromosome 4 is wider and in addition this chromosome has an extra band in its long arm. Chromosome 5 contains an extra interstitial band in the short arm. Chromosome 8 has distinct banding of

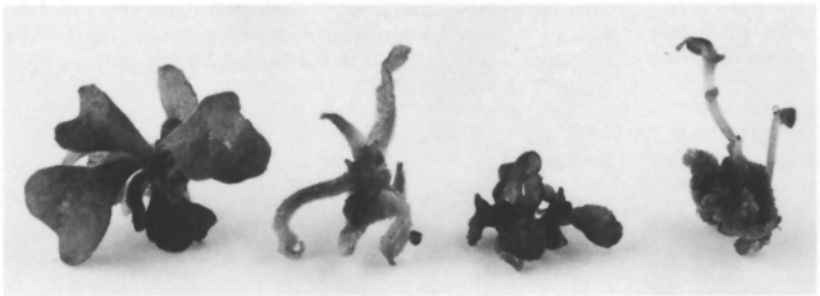

Fig. 1. Regenerated shoots from *Nicotiana plumbaginifolia* (*left*), two somatic hybrids and from *Solanum tuberosum* clone H7322.

the telomeric chromatin of the short arm. The long arm of chromosome 9 is normally shaped with a less distinct band. Chromosome 10 has an interstitial band on its long arm. Chromosome 11 is not metacentric, but submetacentric and has a distinct banding of the telomeric chromatin of the short arm. The centromere of chromosome 12 looks stretched. It has to be stated that one metaphase plate does not always show the bands equally distinct. The karyotype of *N. plumbaginifolia* was as described by Mouras et al. (1986) and Pijnacker and Mouras (1986). Callus cultures consisted predominantly of cells containing 40 chromosomes; a minority had 60 chromosomes.

Chromosome analysis of somatic hybrids

Aceto-orcein squashes were made from some of the cultures of PEG-treated Cnx 20 + H7322 and NA 36 + H7322 protoplasts 1 week after fusion. Somatic hybrids were formed, as in some metaphase plates the small chromosomes of *S. tuberosum* were visible among the ones of *N. plumbaginifolia.* During the first months of culture from time to time orcein squashes were made from NR^+ somatic hybrid calli. These squashes can only be used to investigate whether chromosomes of both partners are present and to estimate the numbers. The potato chromosomes were always present beside *N. plumbaginifolia* chromosomes. As far as could be established, chromosome numbers did not change. Anaphase bridges were never observed. Squashes were also made from calli which were classified as NR^- in the growth experiments for the selection of NR^+ somatic hybrids. Only *N. plumbaginifolia* chromosomes were found in 80 randomly chosen NR^- calli regenerated after fusion of Cnx 20 + H7322 and in 20 NR^- calli of NA 36 + H7322.

About 8 months after fusion, the investigation of Giemsa C-banded slides from somatic hybrid lines was started on 18 well-proliferating lines of Cnx 20 + H7322 and NA 36 + H7322. However, it was rather difficult to draw conclusions as only a few metaphase plates per callus were found. Roughly tetraploid or hexaploid numbers of *N. plumbaginifolia* chromosomes were accompanied by 9–12 potato chromosomes, indicating that a fusion of one *N. plumbaginifolia* cell with one potato cell had taken place. Numbers of *N. plumbaginifolia* chromosomes varying between hexaploid and 12-ploid were usually accompanied by 15–20 potato chromosomes, indicating that chromosome doubling occurred. The number of potato chromosomes varied considerably and elimination of particular chromosomes could not be established. Calli pre-cultured on shoot regeneration medium had a higher frequency of metaphases and thus could be karyotyped much better. As NA 36 + H7322 cell lines did not show morphogenesis

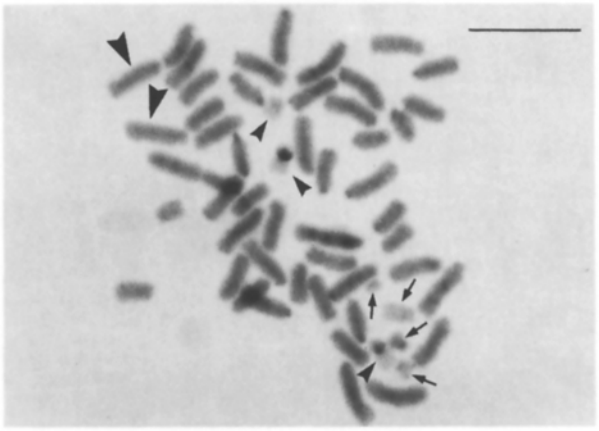

Fig. 2. Metaphase from the somatic hybrid line 2-74. ➸ *Solanum tuberosum* chromosomes; ➤ *Nicotiana plumbaginifolia* chromosomes; → mutated potato chromosomes. Bar = 10 μm

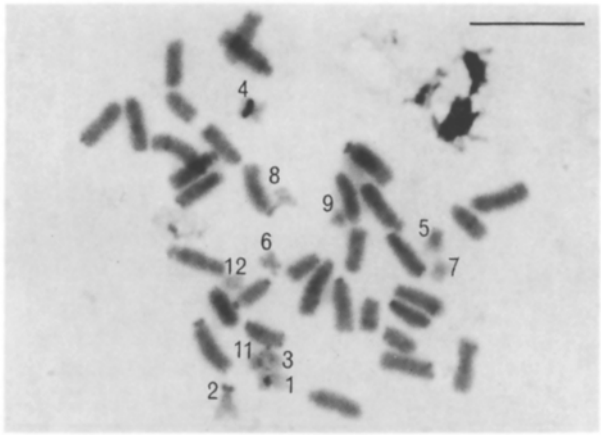

Fig. 3. Metaphase from the somatic hybrid line 2-96. The potato chromosomes are numbered. Bar = 10 μm

further examination was only carried out with Cnx 20 + H7322 hybrid lines, predominantly with lines 2-74, 2-96 and 4-134.

The hybrid line 2-74 is characterized by the presence of 50–60 *N. plumbaginifolia* chromosomes and 5–9 chromosomes which were recognized as potato. Gross structural alterations occurred in the potato and the *N. plumbaginifolia* chromosomes. Chromosome fragments, translocations and deletions of potato chromosomes were observed (Fig. 2). This made it difficult to determine which chromosomes or chromosome parts were eliminated or whether duplications of chromosome segments were present. In some cells, large (86–100) numbers of *N. plumbaginifolia* were present beside 8–10 potato-like chromosomes.

In cells of the line 2-96 usually 26–31 *N. plumbaginifolia* chromosomes and 9–11 chromosomes of the potato could be observed. In some cells 18–20 potato chromosomes were present among 67–76 *N. plum-*

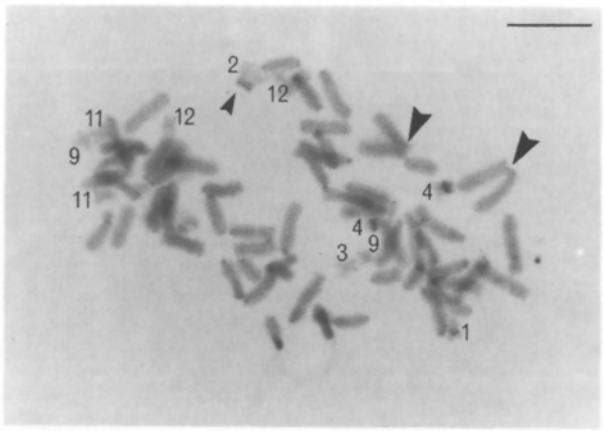

Fig. 4. Metaphase from the somatic hybrid line 2-96. The potato chromosomes are numbered. ← Diplochromosomes of *Solanum tuberosum;* ➤ diplochromosomes of *Nicotiana plumbaginifolia.* Bar = 10 μm

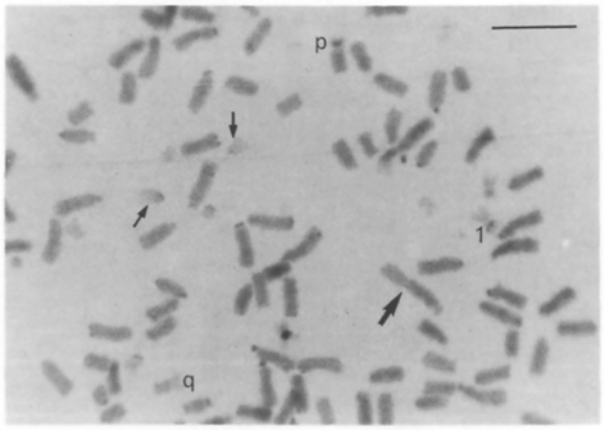

Fig. 5. Part of a metaphase plate from the somatic hybrid line 4-134. → mutated potato chromosomes; ➞ intraspecific translocation. In addition to one complete potato chromosome 1 (1), both chromosome arms (p: short arm; q: long arm) are present for a second time in translocated potato-*Nicotiana plumbaginifolia* chromosomes. Bar = 10 μm

baginifolia chromosomes. In one cell 31 *N. plumbaginifolia* chromosomes were observed beside 17 potato chromosomes, with at least chromosome 1 present in duplo. Thus, within one somatic hybrid line, different chromosome combinations can be found. In this line only minor chromosome aberrations were observed, so the potato chromosomes could be identified. In the metaphase shown in Fig. 3, all potato chromosomes were present except for potato chromosome 10. Chromosome 10 was, however, present in other metaphases of the same callus. Figure 4 shows that in this particular cell genome doubling is due to endoreduplication, which resulted in the formation of diplochromosomes of both species.

The chromosome composition of hybrid line 4-134 is characterized by large numbers of chromosomes from both parental lines. Beside 110–112 *N. plumbaginifolia* chromosomes, 10–13 potato chromosomes were present. Figure 5 shows that in addition to one complete chromosome 1, both the chromosome arms are present for a second time in translocated potato-*N. plumbaginifolia* chromosomes. Apparently this translocation occurred after chromosome doubling took place. In the same figure, intrageneric *N. plumbaginifolia* chromosome translocations can be observed. Hybrid line 4-134 is further characterized by the presence of other deleted chromosomes, such as mini-chromosomes and acrocentric potato chromosomes (Fig. 5).

Discussion

Slight differences were observed between the karyotypes of *Solanum tuberosum* clone H7322 and the monohaploid potato tracing back to cv. Gineke, which has been described (Pijnacker and Ferwerda 1984). This is not surprising as H7322 has been produced androgenetically from an F_1 plant, which resulted from an *S. tuberosum* × *S. phureja* cross (see "Materials and methods"). Comparison of the karyotypes of H7322 and *S. phureja* might reveal which of the characteristics of H7322 can be ascribed to the presence of part of the *S. phureja* genome.

Our results demonstrate that it was rather simple to recover large amounts of somatic hybrids of H7322 + *Nicotiana plumbaginifolia* NR^- mutants Cnx 20 and NA 36. The hybrid nature of some of the PEG-treated cells was already confirmed 1 week after fusion. In aceto-orcein squashes *S. tuberosum* chromosomes were present among *N. plumbaginifolia* chromosomes. The chromosomes of both species were always found to lie mixed in the metaphase plate (moderate squashing). This is in contrast to what has been observed in *N. chinensis* + *Atropa belladonna* and *Glycine max* + *Vicia faba* somatic hybrids. In these hybrids, the chromosomes of each parent were localized in separate zones in metaphases during several successive cell cycles in the initial stages after fusion (Gleba and Sytnik 1984).

Chromosome elimination has been observed in some studies involving somatic hybridization of different species belonging to the *Solanaceae.* Somatic hybrid plants of the potato + *N. tabacum* contained less nuclear DNA than the sum of the parental lines (Skarzhynskaya et al. 1982). Different types of asymmetric genome combinations have been observed in somatic hybrid lines of *N. tabacum* + *Hyoscyamus muticus* (Potrykus et al. 1984). Tobacco chromosomes had been eliminated in most of the hybrid lines obtained after fusion between *N. tabacum* mesophyll protoplasts and *Petunia hybrida* cell suspension protoplasts (Pental et al. 1986). In the present experiments, preferential loss of

potato chromosomes did not occur in somatic hybrids obtained after fusion of potato mesophyll and *N. plumbaginifolia* callus protoplasts. Firstly, over 80 NR^+ hybrid cell lines retained the potato chromosome carrying the compensating NR gene when grown in the absence of nitrate for over 1.5 years. Secondly, no potato chromosomes were found in 100 NR^- calli regenerated after fusion of Cnx 20 + H7322 and NA 36 + H7322. Loss of chromosomes might have resulted in somatic hybrids lacking the chromosome bearing the compensating NR potato gene. The present results, however, suggest that all the NR^- calli resulted from non-fused or homologously fused *N. plumbaginifolia* protoplasts. Thirdly, identification of the chromosomes in some NR^+ somatic hybrid lines demonstrated that although the combination of chromosomes can differ within a line, at least in 1 line (2-96) all 12 potato chromosomes were still present after 24 months of culture.

Previous studies reported that the process of regeneration was accompanied by the selection for genotypes with a lower number of chromosomes. Loss of most of the chromosomes from one partner appeared to result in a decreased degree of somatic incompatibility (Dudits et al. 1979; Krumbiegel and Schieder 1981; Potrykus et al. 1984). The regenerated somatic hybrids in our experiments retained high numbers of chromosomes of both parents. This might explain why some kind of somatic incompatibility was observed after the initiation of differentiation. The regenerated shoots from Cnx 20 + H7322 lines looked rather distorted and we did not succeed in inducing root formation. However, line 2-96 is characterized by a relatively low number of *N. plumbaginifolia* chromosomes. This might be related to its regeneration capacity as the majority of the shoots originated from this line.

The observation of diplochromosomes indicates that chromosome doubling through endoreduplication can take place in the hybrid calli. The presence of such chromosomes is common during callus formation from leaf tissue of H7322 (Pijnacker et al. 1986a). Metaphase plates with more than 60 *Nicotiana* chromosomes and over 12 chromosomes of the potato were observed in a part of the lines 2-74 and 2-96 and in all the cells of 4-134. In the former two lines, chromosome doubling apparently took place much after fusion. In line 4-134, doubling of the genome took place soon after fusion. Genome doubling occurred in cells with abnormal chromosome numbers (Fig. 4). Chromosome variability thus may arise before genome doubling. The presence of both a translocated chromosome 1 of the potato and a complete chromosome 1 (Fig. 5) shows that chromosome variability also may arise after genome doubling. This is supported by the observation of monosomics in apparently doubled cells. The exchange between chromosome 1 and the *Nicotiana* chromosomes could be detected because of the morphological differences between the chromosomes of these species. Intergeneric translocations in somatic hybrids of plants have, to our knowledge, never been demonstrated as clearly as in the present study.

The results presented in this paper demonstrate that the fusion of this particular combination of protoplasts under these particular culture conditions does not result in somatic hybrid lines in which potato chromosomes are eliminated preferentially. It is under investigation whether treatment of the potato protoplasts with mutagens before fusion results in somatic hybrid lines with less potato chromosomes, a prerequisite for use in the mapping of potato genes.

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