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Characterization of chromosome instability in interspecific somatic hybrids obtained by X-ray fusion between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil

A. Fehér, J. Preiszner, Z. Litkey, Gy. Csanádi, and D. Dudits

Institute of Plant Physiology and Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, H-6701-Szeged, POB 521, Hungary

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Summary. Asymmetric somatic hybrids between *Solanum tuberosum* L. and *S. brevidens* Phil. have been obtained via the fusion of protoplasts from potato leaves and from cell suspension culture of *S. brevidens*. The wild *Solanum* species served as donor after irradiation of its protoplasts with a lethal X-ray dose (200 Gy). Selection of the putative hybrids was based on the kanamycin-resistance marker gene previously introduced into the genome of *Solanum brevidens* by *Agrobacterium*-mediated gene transfer. Thirteen out of the 45 selected clones exhibited reduced morphogenic potential. The morphological abnormalities of the regenerated plantlets were gradually eliminated during the extended in vitro culture period. Cytological investigations revealed that the number of chromosomes in the cultured *S. brevidens* cells used as protoplast source ranged between 28–40 instead of the basic 2n=24 value. There was a high degree of aneuploidy in all of the investigated hybrid clones, and at least 12 extra chromosomes were observed in addition to the potato chromosomes (2n=48). Inter- and intraclonal variation and segregation during vegetative propagation indicated the genetic instability of the hybrids, which can be ascribed to the pre-existing and X-ray irradiation-induced chromosomal abnormalities in the donor *S. brevidens* cells. The detection of centromeric chromosome fragments and long, poly-constrictional chromosomes in cytological preparations as well as non-parental bands in Southern hybridizations with restriction fragment length polymorphism (RFLP) markers revealed extensive chromosome rearrangements in most of the regenerated clones. On the basis of the limited number of RFLP probes used, preferential loss of *S. brevidens* specific markers with a non-random elimination pattern could be detected in hybrid regenerants.

Correspondence to: A. Fehér

Key words: Asymmetric hybrids – Chromosome loss – Protoplast fusion – RFLP analysis – *Solanum*

Introduction

The transfer of genetic information from wild to cultivated species is of considerable significance in crop improvement. Interspecific sexual hybridization in most cases, however, is hindered by incompatibility barriers. Somatic hybridization via protoplast fusion is an alternative way to combine plant genomes (as reviewed by Glimelius 1988) but the fertility of interspecific somatic hybrids depends on the phylogenetic distance between the parental species, and often sterility problems arise (Harms 1983). Moreover, even in the case of fertility, an extensive backcross programme is necessary to eliminate undesired wild traits from the sexual or somatic hybrids between wild and cultivated species. The production of hybrids carrying the complete genome of the recipient species plus a few chromosomes or chromosome fragments of the donor species can be considered as an approach to improve fertility and to shorten the time for subsequent breeding (as reviewed by Dudits and Praznovszky 1985). One possible way to reach this goal is the induction of chromosome instability in the donor protoplasts by irradiation treatment prior to fusion. This strategy has been successfully used to transfer genes between distantly related species (eg. Dudits et al. 1980; Gupta et al. 1984; Somers et al. 1986; Dudits et al. 1987; Gleba et al. 1988) as well as closely related ones (Bates et al. 1987; Muller-Gensert and Schieder 1987; Yamashita et al. 1989; Sacristán et al. 1989; Famelaer et al. 1989; 1990; Sjödin and Glimelius 1989; Bates 1990; Wijbrandi et al. 1990a,b,c).

In spite of the fact that in most of the intrageneric combinations attempted the induced chromosome elimination has been limited (Famelaer et al. 1989, Yamashita et al. 1989; Bates 1990, Wijbrandi et al. 1990a,b,c), this method can provide a tool to incorporate individual donor chromosome or chromosome fragments via recombination into the recipient genome, as has been recently demonstrated by Piastuch and Bates (1990) in *Nicotiana* species. Moreover, in different species combinations it has been observed that the backcrossing of the asymmetric hybrids with the recipient species can extend the elimination of donor chromosomes (Famelaer et al. 1990; Bates 1990; Itoh et al. 1991).

Amongst wild *Solanum* species, *S. brevidens* is of significant interest as a potential source of agronomically important genes for potato breeding. This species possesses resistance to several potato viruses, namely to PLRV, PVY, PVX and to *Erwinia* soft rot. Because of the sexual incompatibility between *S. brevidens* and *S. tuberosum*, somatic hybridization was successfully applied to transfer these resistance traits into potato germ plasm (Helgeson et al. 1986; Austin et al. 1988; Gibson et al. 1988; Pehu et al. 1990). In these hybrids, however, sterility can be a limitation, although in one case female fertility could be observed (Ehlenfeldt and Helgeson 1987).

In the present article we report on our attempts to establish an experimental system for the production of asymmetric somatic hybrids of the above-mentioned two *Solanum* species. We provide morphological and cytological data about the genetic instability of the hybrids after a considerable time of in vitro culture. Furthermore, selectivity in the loss of chromosomes is suggested as a characteristic feature in this fusion combination. The possible significance of the pre-existing chromosome variation in parental donor *S. brevidens* cells cultured in vitro and the reduced effectiveness of irradiation in elimination of the donor chromosomes are discussed.

Materials and methods

Plant material

Solanum brevidens Phil. (PI. 218228) plants were kindly supplied and tested for PLRV resistance by J. Horváth (Pannon Agricultural University, Keszthely, Hungary). Sterile plants of *Solanum tuberosum* L. cv 'Boro' were obtained from Meriklon Research Laboratories, Budapest, Hungary. Plants were maintained and propagated as in vitro shoot cultures on hormone-free MS medium (Murashige and Skoog 1962) in a fitotron chamber under 12 h fluorescent light (52–120 μmol m^{-2} s^{-1}) at 20°/15 °C day/night temperature regime.

Transformation

Agrobacterium-mediated transformation of *Solanum brevidens* stem segments by using the hypervirulent bacterium strain bo 542 carrying the binary vector pGA 471 (An et al. 1985) and establishment of kanamycin-resistant cell suspension cultures were carried out as described in detail elsewhere (Fehér et al. 1990).

Protoplast isolation, culture and regeneration

Potato protoplasts were isolated from leaf tissues of 4- to 6-week-old preconditioned (2 days 20 °C plus 1 day 4 °C in dark) plants grown in vitro. Isolation and culture were performed in V-KM culture medium (Bokelmann and Roest 1983) as described by Fehér et al. (1989). The regeneration of shoots from the protoplast-derived cell colonies was achieved on the media ("C", "D", "E" media) developed by Shephard (1980).

Protoplast fusion and selection of the fusion products.

Right before the fusion, half of the protoplasts isolated from *S. brevidens* cell culture designated SB 42/4 was irradiated with 200 Gy X-ray (10 Gy/min) in the enzyme solution. The isolation of the callus protoplasts and their fusion with potato leaf protoplasts through the use of polyethylene glycol (PEG) were carried out as described by Deák et al. (1988). After fusion, the protoplasts were cultured without selection for 1 month, and then the microcalli were plated onto medium "C" (Shepard 1980) supplemented with 100 mg l^{-1} kanamycin. Two of the 12 petri dishes contained kanamycin-free medium to determine colony formation frequency. Within 2–3 weeks the outgrowing green calli were transferred to the same medium without kanamycin and regenerated under non-selective conditions as described above.

DNA isolation and hybridization

Plant DNA was isolated from leaves of in vitro or greenhouse-grown plants as described by Sharp (1987). Digestions of the purified DNA were carried out according to the instructions of the manufacturer of the restriction enzymes used (BRL). DNA fragments were separated in 0.8% agarose gels, transferred onto nylon filters (Hybond N, Amersham) by vacuum and fixed by UV irradiation. They were labelled by the random priming method. All Southern hybridizations were performed in a buffer containing 3 × SSC, 0.1% SDS, 10 m*M* TRIS, 1 mM EDTA and 0.25% dried skimmed milk at 65 °C for 1 day.

Washing of the filters following hybridization was carried out under stringent conditions (2 × SSC and 0.1% SDS 2 × 10 min, room temperature; 0.1 × SSC and 0.1% SDS 3 × 30 min, 65 °C). Filters were exposed to X-ray films using one amplifying screen (Cronex Lightning Plus, DuPont) at −70 °C for 4–7 days. Radioactive probes were removed from the filters according to the manufacturers (Amersham) instruction.

RFLP analysis and detection the NPT-II gene sequence

Restriction fragment length polymorphism was detected using the probes TG8, TG18, TG46, CD2 and CD32 kindly supplied by S.D. Tanksley (Tanksley et al. 1987). Ten micrograms of plant DNA were digested by the restriction enzymes DraI, EcoRI or HindIII (BRL), respectively. Complete digestion was insured by use of restriction enzymes in excess (80 u/10 μg). Labelling and hybridizations were performed under the same conditions as described above.

To detect the *NPT-II* gene sequence in the EcoRI-restricted genomic DNA of the hybrids and the *S. brevidens* transgenic cell lines, the 0.92-kb PstI fragment of the plasmid pNeo (Pharmacia) was used similarly to the RFLP probes.

Neomycin phosphotransferase activity tests

Expression of the *NPT-II* gene in the *S. brevidens* cell lines and the somatic hybrids was shown by the ability for callus formation in the presence of 100 mg l^{-1} kanamycin on the medium described above for the culture of suspension cells. The enzyme activity assays were carried out according to McDonnell et al. (1987) and Reiss et al. (1984), respectively.

Determination of the chromosome numbers

Shoot tips of plants grown in vitro or in the greenhouse were rooted in hormone-free MS medium or in soil. Root tips of small roots were removed, treated with hydroxyquinoline (0.002 *M*), softened in citrate buffer (pH 4.8) containing 10% Pectinase and 1.5% Cellulase, at 37 °C for 30–60 min, followed by fixation in Carnoy-I solution and staining with carbolfuchsin (Kao 1975).

Results

Establishment and characterization of kanamycin-resistant donor S. brevidens cell lines

Early identification of the putative fusion products of *S. tuberosum* and *S. brevidens* was aided by introduction of the bacterial *NPT-II* gene conferring resistance against kanamycin into the genome of the wild, donor species via *Agrobacterium*-mediated transformation of the stem cells (Fehér et al. 1990). Suspension cultures were initiated from primary kanamycin-resistant tissues, which represent independent transformation events. The activity of the neomycin phosphotransferase enzyme in the established cell cultures was detected by colony formation in the presence of 100 mg l^{-1} kanamycin (Fig. 1 A) as well as by the enzyme activity assay (Fig. 1 B). Integration of the kanamycin-resistance gene into the *S. brevidens* genome was proven by Southern hybridization (see e.g. lane 1 of Fig. 3 B).

Kanamycin resistance has been stably maintained in the cultures for more than 3 years in the absence of selection pressure. The cultured cells, however, did lose their morphogenetic potential and could form only brownish microcolonies on medium "C", which was used to induce the greening of potato microcalli derived from protoplasts. In this way, the identification of fusion products on the basis of their kanamycin resistance and greening ability seemed to be reliable.

To induce preferential loss of the *S. brevidens* chromosomes from the fusion products the protoplasts of this donor partner were irradiated with a lethal dose (200 Gy) of X-ray (Fig. 1 C).

Protoplast fusion, selection and plant regeneration

Fusion of leaf protoplasts of *S. tuberosum* cv 'Boro' and both irradiated and untreated callus protoplasts of *S. brevidens* cell line SB 42/4 (altogether around 2.5×10^5 protoplasts per fusion treatment) was induced by polyethylene glycol. One month after protoplast fusion, the microcolonies formed were transferred onto agar-solidified medium (medium "C") that supported callus growth and greening. In the case of the fusion of non-irradiated *S. brevidens* protoplasts with the potato protoplast, only a few pale green kanamycin-resistant microcolonies could be obtained; these failed to regenerate, but turned to be brown and died on the regeneration medium (just as parental *S. brevidens* protoclones).

In the fusion experiment where donor protoplasts had been previously irradiated, we estimated the number of protoplast-derived microcolonies subjected to selection pressure to be around 1700 on the basis of the number of green calli developed on the two non-selective plates. Around 2.6% of these colonies (45) had the ability

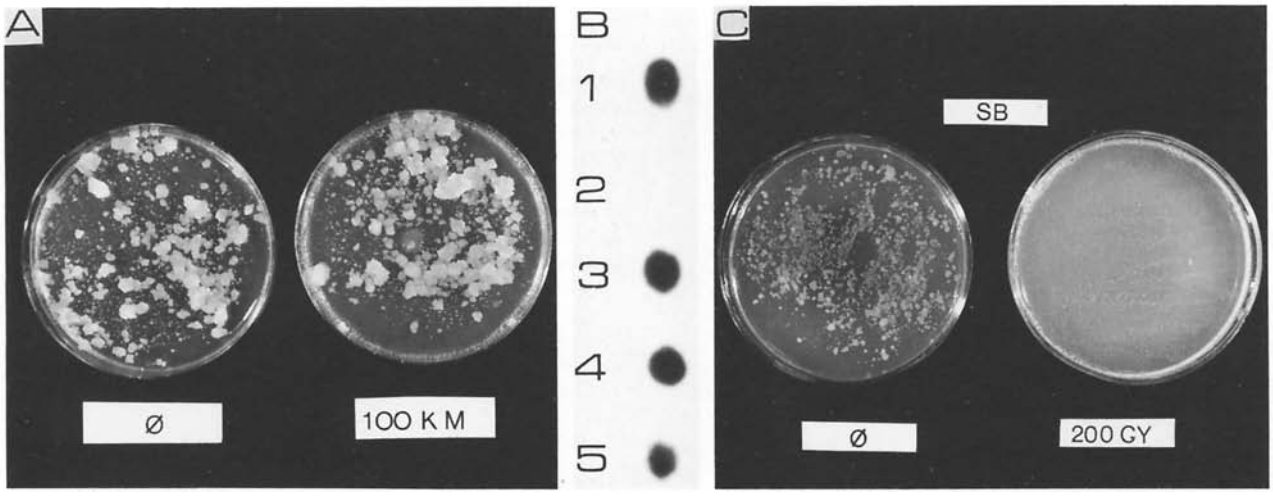

Fig. 1 A–C. Characterization of donor *S. brevidens* cell lines. **A** Colony formation of suspension culture cells of the transgenic cell line SB 42/4 in the absence (0) and presence (100 KM) of 100 mg l^{-1} kanamycin. This concentration completely inhibits the growth of control *S. brevidens* cells (data not shown). **B** Activity of the neomycin phosphotransferase enzyme in a positive control transgenic alfalfa plant (*dot 1*), negative control *S. brevidens* plant (*dot 2*) and three independent transgenic cell lines of *S. brevidens* (*dots 3–5*). **C** 200 Gy X-ray irradiation is lethal for *S. brevidens* (SB 42/4) callus protoplasts and completely inhibits colony formation

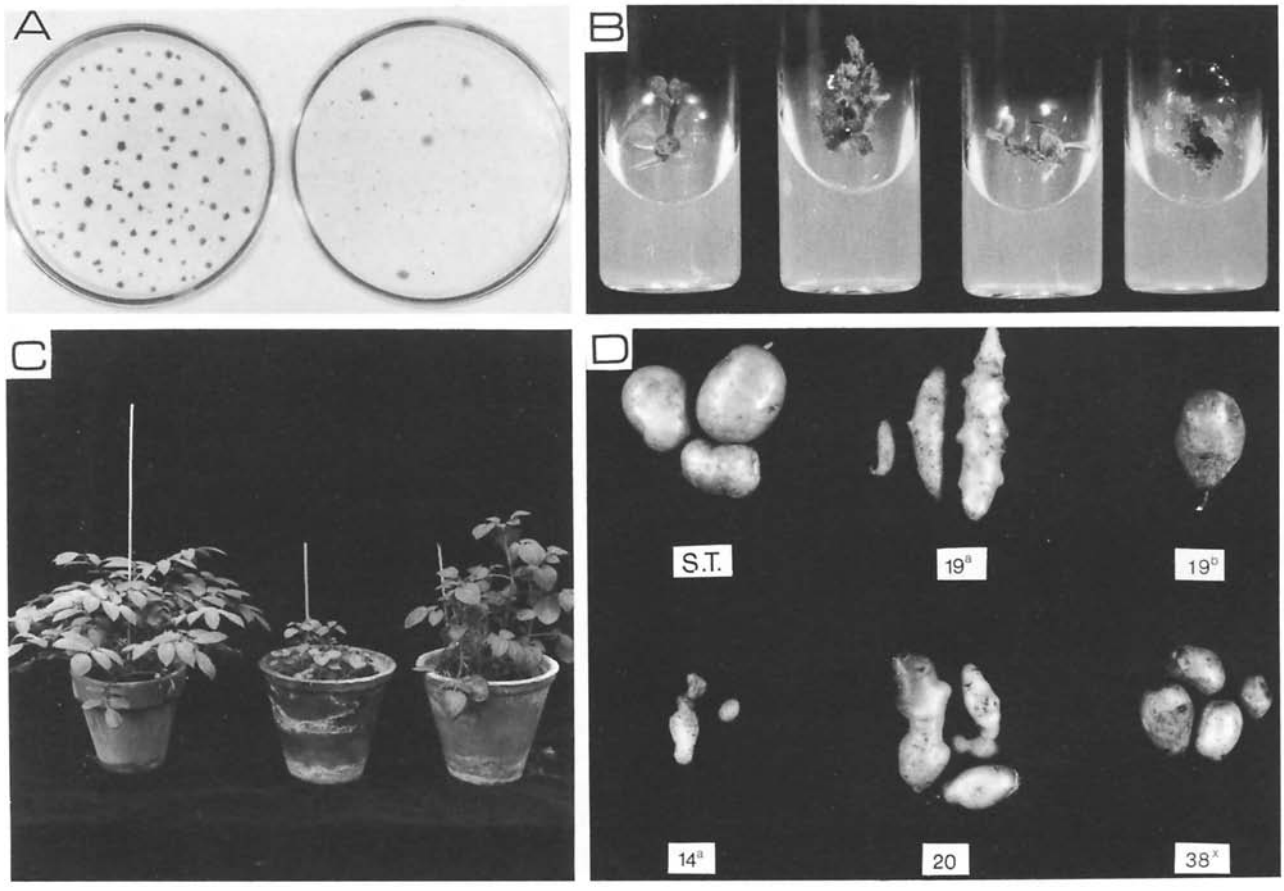

Fig. 2A–D. Selection, regeneration and morphology of somatic hybrids. **A** Formation of cell colonies after the fusion of leaf protoplasts of potato and irradiated cell culture protoplasts of *S. brevidens* in the absence (*left*) and presence (*right*) of 100 mg^{-1} kanamycin. Outgrowing green calli on kanamycin-supplemented medium were considered to be putative fusion products. **B** Shoots with disturbed growth and rooting regenerated from some of the selected clones. **C** One of the asymmetric hybrid plants (TB 38 b, *middle*) and the two parents (*S. brevidens*, *left*; potato, *right*) in the greenhouse. **D** Tuber formation of greenhouse grown potato cv 'Boro' (*S.T.*) and some of the somatic hybrid plants (*19 a* (=TB 19 a_2 in the text) and *19 b* are regenerants of the same callus, the others are independent clones)

to grow and become dark green on kanamycin-containing medium (Fig. 2A). A large portion of *S. brevidens* parental cells was eliminated by the radiation treatment. A few *S. brevidens* protoplasts did survive, but formed brown calli on medium "C". This phenomenon made the unambiguous identification of putative fusion products possible. Kanamycin selection was applied only for a short time (2–3 weeks), and from the identified green colonies shoot were subsequently regenerated under non-selective conditions which provided the possibility for random chromosome segregation.

Shoot regeneration was possible in the case of 13 selected calli. Some of these calli (e.g. TB 10, 14, 19, 38) regenerated several shoots. Shoot induction was difficult and required much more time than the regeneration of the control potato protoclones (6 versus 2 months on medium "D" with monthly subcultures).

Morphology of regenerated plants

The primary regenerants exhibited abnormalities evidenced by disturbed growth and poor rooting (Fig. 2B). They were maintained by monthly subcultures through the propagation of shoot meristem. During a period of almost one year of in vitro culture the phenotype, growth rate and rooting of some of the lines improved significantly. The best growing clones (TB 10a,b, 14a, 19a,b,c, 20a, 38a,b,x) were planted into soil and the others were kept in vitro.

There was a great deal of heterogeneity in the morphology of the plants, even among those regenerated from the same selected colony. However, so far as the overall morphology and the shape and colour of the stems and leaves were concerned, all hybrids showed the potato type phenotype (Fig. 2C). All of them produced

tubers, but the colour (from deep purple to light brown) and the shape varied significantly (Fig. 2D). Most of the hybrids had wavy and distorted leaves probably due to the difference in the growth rate between sectors.

During vegetative propagation in vitro as well as in the greenhouse, primary regenerants segregated into different subclones. The segregation often resulted in plants with improved growth rate and morphology as compared to the primary clone. For example, clone TB 19a had originally several small shoots with small, dark green, distorted leaves, while the subclone TB $19a_2$ could grow into a much higher plant still with several strong shoots bearing large, light green, normal, potato-like leaves. Similarly, definite morphological differences could be observed between the TB 38i and TB $38i_2$ subclones. In both cases cytological investigations revealed intraclonal variation in the number of chromosomes (see later).

Kanamycin resistance in the regenerated putative hybrids

All of the regenerated plantlets were tested for expression of the *NPT-II* gene used for the selection. Both primary callus formation on leaf strips placed onto kanamycin-containing callus induction medium and neomycin phosphotransferase enzyme activity in crude leaf extracts (Fig. 3A) have proved the presence and expression of the antibiotic-resistance gene in all of the putative hybrids, in spite of the fact that regeneration was carried out under non-selective conditions.

As the kanamycin resistance originated from *S. brevidens* and tuber formation is characteristic only of potato, together these properties have provided evidence of the fusion origin of the regenerated clones.

After several (four to six) subcultures, some of the in vitro plantlets were planted into soil in the greenhouse. Four of these clones were further propagated vegetatively through shoot-tip meristem to get enough material for DNA purification and for verification of the presence of the *NPT-II* gene by Southern analysis (Fig. 3B). Interestingly, we found that two clones which had previously given a positive singal in the NPT enzyme activity test (Fig. 3A) had lost the gene during the subcultures, revealing chromosomal instability in the hybrids even at the plant level.

Chromosome numbers

S. tuberosum is a tetraploid species with 2n = 48 chromosomes, while *S. brevidens* is a diploid (2n = 24) species. As far as the cultured cells of this donor species are concerned, however, there was a significant increase and variation in the number of their chromosomes (Table 1).

Cytological investigations were limited to counting the total number of chromosomes in the hybrids and to detecting some chromosomal abnormalities. Poor rooting prevented the analysis of some of the clones (e.g. TB 10a,b, 19a,b, 38a,x), but seven clones (and some subclones of them) were included in these studies (Table 1). Chromosome counts showed a great inter- and intraclonal variation. Except for one subclone (TB $19a_2$) in general all of the somatic hybrids possessed a considerably higher number of chromosomes than the potato parent. All but one hybrid (TB 12i) had, however, fewer chromosomes than the expected sum of the parental chromosome sets (calculated on the basis of the increased chromosome number of *S. brevidens* cells).

The somatic hybrid designated TB 38i had more than 100 chromosomes, likely the result of a multiple fusion event. This clone and clone TB 14a proved to be mixoploid, having two ploidy levels (Table 1). A stable subclone (TB $38i_2$) could be selected from the TB 38i hybrid during in vitro propagation; this hybrid possessed a number of chromosomes (66–72) similar to that previously observed in one root tip of the original clone as well (73).

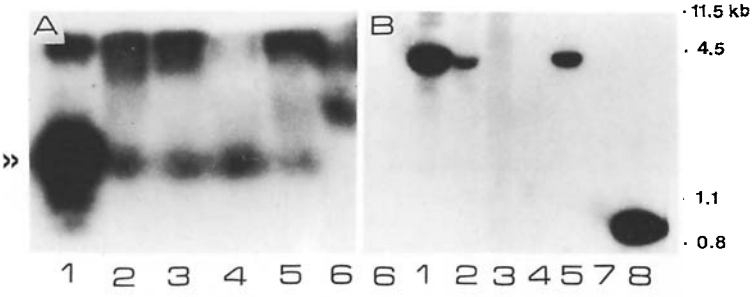

Fig. 3A, B. Fate of the kanamycin-resistance marker in five randomly chosen hybrid clones. Neomycin phosphotransferase activity (indicated by *arrow*) in in vitro-grown plantlets (**A**) and Southern hybridization with the coding sequence of the *NPT-II* gene to the EcoRI-digested total DNA (**B**) of the donor *S. brevidens* cells (*lanes 1*) and greenhouse-grown vegetatively propagated plants of four somatic hybrids (TB 38x, TB 19c, TB 14a, TB 10b in *lanes 2, 3, 4,* and *5*, respectively) and potato (*lanes 6*). *Lane 8* contained the DNA fragment used as the probe

It is likely that the TB 38i clone had two potato and one (most likely partial) *S. brevidens* genomes and that during the vegetative propagation one total potato chromosome set had been lost. Furthermore, a similar loss of one potato genome might have taken place in some cells before or during regeneration of the TB 38 callus, resulting in another regenerant (TB 38b) with the same chromosome number as TB $38i_2$.

The observation of 24 chromosomes in root-tip cells of the TB 14a hybrid, amongst cells with 65–76 chromosomes, further illustrates the chimeric nature of these plants.

Only one of the clones (TB $19a_2$) had around the same (or even less) number of chromosomes as potato. This low chromosome number, however, also seems to result from the loss of chromosomes during the vegetative propagation (see the morphological characterization). The original callus clone likely had 60–66 chromosomes, as did the other regenerants (TB 19c,i). Unfortunately, the chromosome number of the original TB 19a plants have not yet been determined.

Typical metaphase plates of two hybrids are shown in Fig. 4. Small, possibly centromeric chromosome fragments (minichromosomes) were observed in several cytological preparations of the hybrids, and extremely long multiconstrictional chromosomes were also detected (Fig. 4).

Table 1. Chromosome counts in root tips of some of the regenerated somatic hybrids

Clone[a]	Number of chromosomes[c]	% Chromosomes eliminated[d]
St	48	–
Sb (plant)	24	–
Sb.42/4 (callus)	28–40 (6)	–
St × Sb.42/4[b]	76–88	–
TB 2i	63–68 (4)	11–28
TB 12i	84–92 (10)	0– 4
TB 14a	65–76 (5) 24 (3)	0–26
TB 14i	69–76 (5)	0–22
TB $19a_2$	40–46 (3)	39–55
TB 19c	61–64 (2)	16–31
TB 19i	60–66 (5)	13–32
TB 20a	65–70 (6)	8–26
TB 22i	71–78 (2)	0–19
TB 38b	66–72 (4)	5–25
TB 38i	98–119 (5) 73 (1)	4–28
TB $38i_2$	66–72 (5)	5–25

[a] St, *S. tuberosum*; Sb, *S. brevidens*; TB, somatic hybrids. Different numbers mean independent fusion products, while lower case letters, subclones ("i" sign in vitro-grown clones)
[b] Calculated on the basis of the counted chromosomes of the donor cells
[c] The numbers of the investigated plates are in brackets
[d] Minimal and maximal values were calculated concerning the lowest chromosome number observed in the donor cells (28) and the highest of the appropriate hybrid plant (e.g., 68 of TB 2i), and vice-versa

RFLP analysis of hybrids

In attempts to identify restriction fragment length polymorphism (RFLP) markers for characterization of somatic hybrids, several RFLP probes have been hybridized to restricted total DNA of potato and *S. brevidens*. So far five of them have been used to characterize our hybrids: CD2, CD32, TG8, TG18, TG46 (see Tanksley et al. 1987).

Amongst these clones, TG8, CD32, and TG18 belong to the same linkage group representing potato chromo-

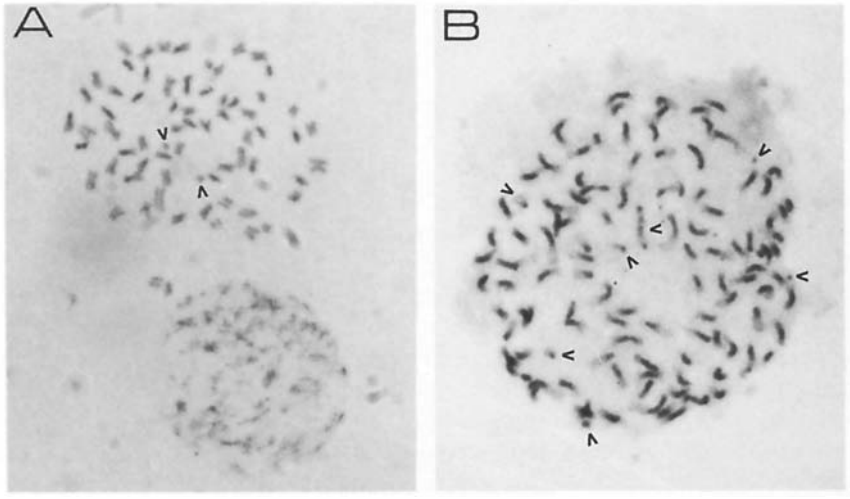

Fig. 4A, B. Typical metaphase plates of two somatic hybrid plants. TB 19c (**A**) possesses 64 chromosomes while TB 38i (**B**) has more than 100 (110) chromosomes. *Arrows* indicate some of the minichromosomes (possible centromeric fragments of the donor) in both preparations and a long, policonstrictional chromosome amongst the chromosomes of TB 38i

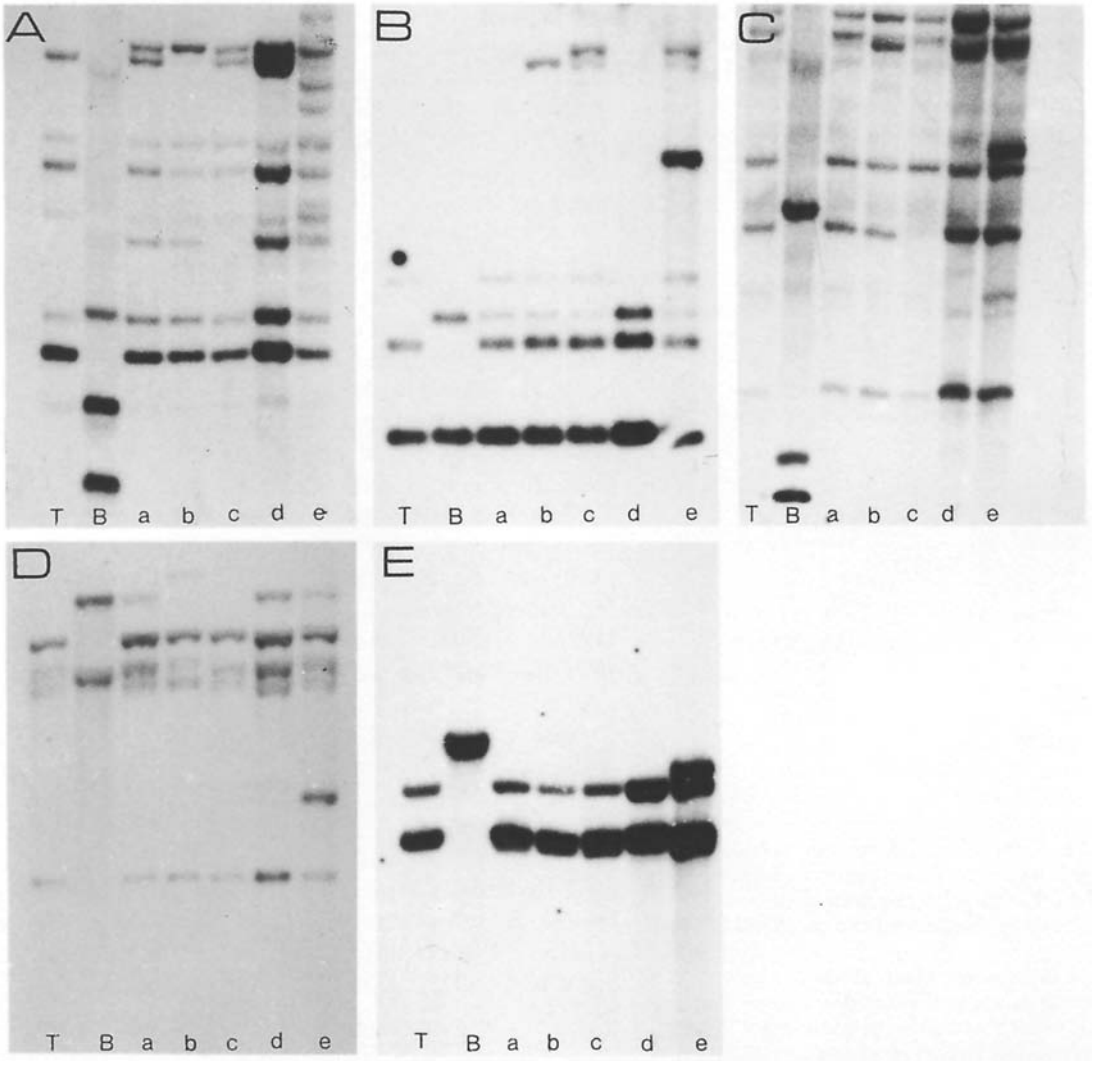

Fig. 5A–E. RFLP analysis of five independent asymmetric hybrid clones. Aliquots of 10 µg of restricted DNA of potato (*lane T*), *S. brevidens* (*lane B*) and asymmetric hybrids TB 12i (*lane a*), TB 14i (*lane b*), TB 19i (*lane c*), TB 20i (*lane d*) and TB 38i (*lane e*) have been analysed by Southern hybridization. The probe/enzyme combinations used were TG8/DraI (**A**), TG18/EcoRI (**B**), CD32/DraI (**C**), CD2/EcoRI (**D**) and TG46/EcoRI (**E**)

some 9 (one copy of the CD32 sequence is also present on potato chromosome 10). TG18 is located close to the centromeric region while the other two markers are at the ends of the two chromosome arms. As can be seen on Fig. 5A, C, the *S. brevidens* specific bands hybridizing with the markers of the chromosome ends are missing from the restricted DNA of the hybrids, while the marker of the centromeric region is present (Fig. 5B). This probably indicates that this chromosome exists in these plants as a centromeric fragment (minichromosome) or alternatively, the translocation of this region might have taken place. The relative intensity of the *S. brevidens* specific band hybridizing with the probe TG18 is reduced: this can be an indication of the loss of the other whole chromosome 9 (except may be in the case of clone TB 20i). Moreover, the absence of all three *S. brevidens* specific bands hybridizing with the maker CD32 indicates the likely absence of the appropriate region of chromosome 10 from the hybrids. Which of the CD32 hybridization band(s) represents the chromosomes 9 and 10 is not known.

The use of probe TG46 (linkage group 11) also revealed the absence of *S. brevidens* specific bands in each of the investigated hybrids (Fig. 4E), but the presence of a new hybridizing band in clone TB 38i could be a result of chromosomal recombination. Similar extra bands could be observed on almost all of the filters in clone TB 38i (in which several chromosomal abnormalities

could also be detected cytologically, Fig. 4B) and in some cases in clones 14i and 19i (Fig. 5).

The CD2 probe belongs to linkage group 12 and is located close to the end of the chromosome arm in potato. The somatic hybrids TB 12i, TB 20i and TB 38i shared the characteristic bands of both potato and *S. brevidens*, while the band specific for the wild species was missing from TB 19i and became larger (probably due to recombination) in TB 14i (Fig. 5D).

The loss of potato specific bands also could be detected in some cases (e.g. in TB 19i, Fig. 5A,C). The data obtained, however, suggest the preferential loss of *S. brevidens* specific markers. Interestingly, in spite of the high chromosome number of the investigated hybrids, we could hardly detect a hybrid RFLP pattern with the used probes, even in that clone (TB 20i) which has about the number of chromosome that a symmetric hybrid should have. Moreover, the investigated hybrids were very similar with respect to the presence or absence of these markers (except CD2). More detailed RFLP analysis of the regenerated somatic hybrid plants is in progress.

Discussion

Selection and regeneration of the putative somatic hybrids

In general, the identification of fusion products after asymmetric somatic hybridization is significantly more complicated than that of cell hybrids with complete parental genomes. Based on the gradual and prolonged period of chromosome loss, the use of transgenic cell lines (Sacristán et al. 1989) or plants (Müller-Gensert and Schieder 1987; Bates et al. 1987; Bates 1990) can open a selective window for the early detection of fusion products regardless of the linkage between the selected marker and any other trait. In the work presented here, only a short period of kanamycin selection was applied in order to allow a random segregation during plant regeneration. This selection strategy may have significance if the desired trait is coded by an unknown gene or genes without linkage with any selectable marker.

The poor regeneration potential of our fusion products could originate from several components. In contrast to the fact that the potato cultivar used as a parent can be regenerated with sufficient efficiency (Fehér et al. 1989), the *S. brevidens* protoplasts were isolated from a non-morphogenic line with a long-term in vitro culture period. The dominant behaviour of the non-morphogenic trait has been demonstrated in other fusion combinations (e.g. Deák et al. 1988). The high degree of aneuploidy and super-optimal chromosome number can also be pointed out as factors responsible for abnormalities in regeneration processes at the early phase.

Genetic instability in the regenerated somatic hybrids

In agreement with previous observations on carrot (+) parsley somatic hybrids (Dudits et al. 1980), the stepwise improvement in the regeneration, rooting and morphology of potato somatic hybrids during in vitro growth as well as vegetative propagation in greenhouse suggests a continuous and prolonged process of chromosome elimination. Direct evidence of genetic instability was obtained by the investigation of the fate of the kanamycin-resistance marker in some of the hybrids. Under non-selective conditions two out of four vegetatively propagated plant clones lost the gene responsible for this character. In cytological preparations, inter- and intraclonal variations and mosaicism were detected. The vegetative segregation of clones into morphologically and cytologically different subclones can also indicate genetic instability. The only putative "highly asymmetric" plant (TB $19a_2$) having a chromosome number even less than that of potato may also be a result of chromosome loss through extended vegetative propagation. The presence of any genetic material from the donor has not yet been detected in this clone.

Chromosomal changes including chromosome loss, the chimeric composition of the regenerants and segregation after vegetative propagation have been described in protoplast-derived potato plants (e.g. Sree Ramulu 1986) as well as in symmetric (Pijnacker et al. 1987; Sree Ramulu et al. 1989) and asymmetric (Sidorov et al. 1987) somatic hybrids of potato. In symmetric somatic hybrids between potato and *S. brevidens* after fusion of the leaf protoplasts of both parents, except for different degrees of aneuploidy, such genetic instability has not been reported (Austin et al. 1985, 1986; Pehu et al. 1989).

Extensive chromosome rearrangements, chimerism and somatic segregation frequently have been observed in interspecific asymmetric somatic hybridizations with other species (Bates et al. 1987; Famelaer et al. 1989; Wijbrandi et al. 1990 a,b,c; Piastuch and Bates 1990), and the intra- and interclonal variability was even more pronounced when the donor partner originated from cell culture (Sacristán et al. 1989). The pre-existing chromosomal instability and variation in cultured cells used as donors in interspecific asymmetric hybridization can be an additional source of cytological abnormalities during the development of fusion products, even when the donor chromosomes are only partially eliminated.

In our experimental system, the pre-existing chromosome variation in the donor *S. brevidens* cells due to their prolonged in vitro culture prior to fusion has to be pointed out as an important factor responsible for the chromosome instability of the selected hybrids during regeneration and vegetative propagation. On the other hand, the fact that we could not regenerate fusion products if the parental donor protoplasts had not been irra-

diated before the fusion indicates the significant role of the irradiation treatment in the induction of chromosome loss in the hybrid cells.

On the basis of the above-mentioned observations, the genetic instability detected in the present hybrids most likely can be ascribed to cummulative factors as the somaclonal variation during the long-term in vitro regeneration and culture and the pre-existing chromosomal instability in cultured *S. brevidens* cells, which might have been further augmented by irradiation treatment. In this respect, the fusion of heterophasic cells derived from suspension culture and leaves, respectively, also has to be considered as a possible component in the generation of wide chromosomal heterogeneity (see: Dudits and Praznovsky 1985).

Efficiency in chromosome elimination

The high chromosome number of the investigated somatic hybrids suggests a limited elimination of chromosomes. An exact determination of the degree of chromosome loss was not possible because of the high chromosomal variability in donor cells and difficulties in the accurate counting of chromosomes due to their high number and the genetic instability in individual plants. The calculated minimum and maximum chromosome loss ranged between 0–32% as far the sum of the parental chromosomes is concerned. This means that in addition to the potato chromosome set there are at least 12 extra chromosomes present in the hybrids (except in subclone TB $19a_2$). Because of the similarities in size and morphology between chromosomes of the fusion partners, cytological investigations could not provide information on the direction of chromosome elimination. Molecular characterization based on restriction fragment length polymorphism between the fusion partners, however, indicated that most likely there is a preferential loss of *S. brevidens* specific sequences from five investigated hybrid clones in spite of their high chromosome number. The similarity between the RFLP patterns of the investigated independent clones (e.g. concerning markers of linkage group 9 revealing the presence of only fragment(s) of this chromosome in each of the hybrids and markers TG 46 and CD 32 detecting the absence of *S. brevidens* specific bands from each of the five independent hybrids) suggests that the elimination was not only species but chromosome specific as well. This can be due to the different sensitivities of the chromosomes to X-ray irradiation and/or preferential elimination during in vitro culture. Non-random elimination of rDNAs was observed in intergeneric asymmetric somatic hybridization between *Atropa belladonna* and *Nicotiana plumbaginifolia* by Gleba et al. (1988). Moreover, non-random chromosome retention has been mentioned as one of the possible reasons for the relative difference in expression frequency of certain markers in interspecific *Nicotiana* asymmetric hybrids (Famelaer et al. 1989). On the other hand, random chromosome loss has been suggested on the basis of isozyme studies in similar experiment with *Brassica* species (Yamashita et al. 1989). Further support of any hypothesis on chromosome loss will require additional RFLP analysis on a high number of somatic hybrids and detailed characterization of their genetic constitution.

In some cases the absence of potato specific fragments was observed, which can be a consequence of recombination events or the somaclonal variability of potato.

Although the number of the hybrids investigated is limited, the data presented here suggest that in this combination of parental species the pre-existing chromosome instability and the irradiation treatment of the donor protoplasts could result in extensive chromosome rearrangement and partial donor chromosome elimination. The irradiation was not sufficient to induce the chromosome instability required for the production of highly asymmetric hybrids. This result is consistent with data obtained in most of the interspecific (and intrageneric) fusion combinations where a large portion of the donor genome was reported to be retained in the asymmetric hybrid plants (Famelaer et al. 1989; Yamashita et al. 1989; Itoh et al. 1991; Sacristán et al. 1989; Bates 1990; Wijbrandi et al. 1990a,b,c). Moreover, even in those cases where the limited transfer of donor genetic material was proven or suggested to occur in interspecific combinations, in addition to extreme donor chromosome elimination partial elimination was also observed (Bates et al. 1987; Sidorov et al. 1987; Sjödin and Glimelius 1989; Agoudgil et al. 1990). The lack of a strong somatic incompatibility between the closely related species can eliminate the influence of one of the key factors that might be responsible for intensive chromosome loss in wide fusion combinations. In donor – recipient fusion experiments with distantly related species (Dudits et al. 1980, 1987; Gupta et al. 1984; Somers et al. 1986), where the transfer of only one or a few chromosomes or traits was observed, the elimination itself was likely greatly enforced by somatic incompatibility reactions, and the irradiation treatment determined first of all the direction of chromosome loss and probably induced the intergenomic recombination events stabilizing donor genes in the recipient genome.

The often incomplete and unpredictable elimination of donor chromosomes in asymmetric hybrids is a serious drawback in the application of this technology for practical breeding purposes. Improvement of this methodology requires a better understanding of the chromosomal and molecular events underlying the process of somatic hybrid formation. Moreover, new ways besides irradiation, should be found, that provide a more reliable control of chromosome elimination and recombination in

hybrid cells. In this respect, the influencing of DNA repair and replication in plant cells is of significant importance.

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