Identification of mitotic chromosomes of tuberous and non-tuberous *Solanum* species (*Solanum tuberosum* and *Solanum brevidens*) by GISH in their interspecific hybrids

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Abstract: GISH (genomic in situ hybridization) was applied for the analysis of mitotic chromosome constitutions of somatic hybrids and their derivatives between dihaploid clones of cultivated potato (*Solanum tuberosum* L.) ($2n = 2x = 24$, AA genome) and the diploid, non-tuberous, wild species *Solanum brevidens* Phil. ($2n = 2x = 24$, EE genome). Of the primary somatic hybrids, both tetraploid ($2n = 4x$) and hexaploid ($2n = 6x$) plants were found with the genomic constitutions of AAEE and AAEEEE, respectively. Androgenic haploids (somatohaploids) derived from the tetraploid somatic hybrids had the genomic constitutions of AE ($2n = 2x = 24$) and haploids originating from the hexaploid hybrids were triploid AEE ($2n = 3x = 33$ and $2n = 3x = 36$). As a result of subsequent somatic hybridization from a fusion between dihaploid *S. tuberosum* ($2n = 2x = 24$, genome AA) and a triploid somatohaploid ($2n = 3x = 33$, genome AEE), second-generation somatic hybrids were obtained. These somatic hybrids were pentaploids ($2n = 5x$, genome AAAEE), but had variable chromosome numbers. GISH analysis revealed that both primary and second-generation somatic hybrids had lost more chromosomes of *S. brevidens* than of *S. tuberosum*.

Key words: anther culture, genome, haploid, potato, somatic hybridization.

Résumé : L'hybridation génomique in situ (GISH) a été employée pour analyser le complément chromosomique en mitose chez des hybrides somatiques et leurs dérivés provenant d'un croisement entre un clone dihaploïde de la pomme de terre cultivée (*Solanum tuberosum* L.) ($2n = 2x = 24$, génome AA) et le *Solanum brevidens* Phil. ($2n = 2x = 24$, génome EE), une espèce sauvage diploïde non-tubéreuse. Parmi les hybrides somatiques primaires, autant des plantes tétraploïdes ($2n = 4x$) qu'hexaploïdes ($2n = 6x$) ont été obtenues et celles-ci avaient, respectivement, les formules génomiques suivantes : AAEE et AAEEEE. Des haploïdes androgéniques (haploïdes somatiques) dérivés des hybrides tétraploïdes avaient un génome AE ($2n = 2x = 24$) tandis que les haploïdes dérivés des hybrides hexaploïdes étaient triploïdes AEE ($2n = 3x = 33$ et $2n = 3x = 36$). Suite à une autre ronde d'hybridation somatique entre un *S. tuberosum* dihaploïde ($2n = 2x = 24$, génome AA) et un haploïde somatique triploïde ($2n = 3x = 33$, génome AEE), des hybrides somatiques de seconde génération ont été obtenus. Ces hybrides somatiques de seconde génération étaient pentaploïdes ($2n = 5x$, génome AAEEE) mais présentaient un nombre variable de chromosomes. L'analyse GISH a révélé que les hybrides somatiques, tant de première que de seconde génération, avaient perdu plus de chromosomes du *S. brevidens* que du *S. tuberosum*.

Mots clés : culture d'anthères, génome, haploïde, pomme de terre, hybridation somatique.

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Introduction

In situ hybridization (ISH) is a molecular-cytological method used to analyse chromosome structure and genomic compositions. Species of the Solanaceae family, such as those of the genera *Solanum* and *Lycopersicon*, having relatively small chromosomes and low levels of karyotypic differentation, were first analysed using ISH with repetitive DNA sequences (Visser et al. 1988; Lapitan et al. 1989). Since the demonstration of genomic in situ hybridization (GISH) using total genomic DNA probes (Schwarzacher et al. 1989), cytological analyses of wide hybrids, such as

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intergeneric *Solanum* + *Lycopersicon* (Wolters et al. 1994; Jacobsen et al. 1995; Garriga-Calderé et al. 1997; Gavrilenko et al. 2001), and analysis of hybrids between species that belong to different subgenera (*Solanum tuberosum* + *Solanum nigrum*) (Horsman et al. 2001) have been carried out. However, in GISH analysis there are only a few reports (Wilkinson et al. 1995; Dong et al. 1999) of hybrids between phylogenetically more-related *Solanum* species that belong to the section *petota* (Hawkes 1990).

One of the wild nontuberous species in the section *petota* is the diploid (2*n* = 2*x* = 24) *Solanum brevidens* Phil. (syn. *palustre* Schlechtd.). *Solanum brevidens* is extremely resistant to various viral diseases that induce tuber-yield losses in cultivated potato and is considerably tolerant to freezing (Hawkes 1990). Attempts to transmit the valuable traits of sexually incompatible *S. brevidens* into cultivated potato (*Solanum tuberosum* L.) by somatic hybridization have been carried out (Austin et al. 1985; Fish et al. 1988; Rokka et al. 1994), followed by subsequent sexual backcrosses (Ehlenfeldt and Helgeson 1987; Jacobsen et al. 1993). Also, in our previous studies, (somato)haploid lines have been produced from male sterile *S. tuberosum* + *S. brevidens* somatic hybrids through androgenesis for potato improvement (Rokka et al. 1995). A new strategy was subsequently proposed and tested involving the refusion of the somatohaploids with dihaploid lines of cultivated potato, resulting in second-generation somatic hybrids (Rokka et al. 2000).

In somatic hybrids, chromosome elimination and structural chromosome changes have frequently been observed (Pijnacker et al. 1987; Wolters et al. 1994; Williams et al. 1993; McGrath and Helgeson 1998). For example, in *S. tuberosum* + *S. brevidens* somatic hybrids, chromosome instability with various degrees of aneuploidy have been described (Fish et al. 1988; Preiszner et al. 1991). Because the karyotypes of *S. brevidens* and *S. tuberosum* are indistinguishable by conventional cytological methods, precise results on the direction of chromosome elimination have not been described.

We previously identified portions of alien (*S. brevidens*) genome in *S. tuberosum* background from the interspecific somatic hybrids (Rokka et al. 1998*a*) using *S. brevidens* specific sequences (Pehu et al. 1990) and fluorescence in situ hybridization (FISH) (Rokka et al. 1998*b*). However, in the present study, we aimed to use GISH for a more accurate analysis of the genomic constitutions of hybrids containing the E genome of *S. brevidens* (Ramanna and Hermsen 1981) in the A-genome background of *S. tuberosum*. We also performed a more in-depth analysis on the chromosomal stability of interspecific potato hybrids and the transmission of parental chromosomes in different generations after subsequent protoplast fusions and haploidizations.

Materials and methods

Plant material

Four tetraploid (2*n* = 4*x*) somatic hybrids (0204, 0205, 0208, and 3004) and three hexaploid (2*n* = 6*x*) hybrids (0502, 0603, and 7001), derived from protoplast fusion between dihaploid (2*n* = 2*x* = 24) line 'Pito 4' of *S. tuberosum* and diploid (2*n* = 2*x* = 24) *S. brevidens* accession CPC 2451 (Rokka et al. 1994), were involved in the study. Figure 1 illustrates the scheme used for the construction of various interspecific hybrids with an indication of their theoretically expected genomic compositions. Two diploid (2*n* = 2*x*) anther-derived somatohaploids (genotypes 3004.1.1.1. and 3004.2.1.2.) from the somatic hybrid 3004 and two triploid (2*n* = 3*x*) somatohaploids (0502.1.1.1. and 0507.1.2.1.) from the hexaploid somatic hybrids 0502 and 0507 (Rokka et al. 1995) were also analysed using GISH. In addition, five pentaploid (2*n* = 5*x*) second-generation somatic hybrids (2701-0105, 2701-0107, 2701-0109, 2701-0302, and 2701-0502), derived from a fusion between triploid somatohaploid 0507.1.2.1. and a dihaploid line of *S. tuberosum* cv. Van Gogh ('Van Gogh 11') (Rokka et al. 2000), were included in the study. Details of the plant material are also presented in Table 1.

Anther culture of tetraploid somatic hybrids between *S. tuberosum* and *S. brevidens*

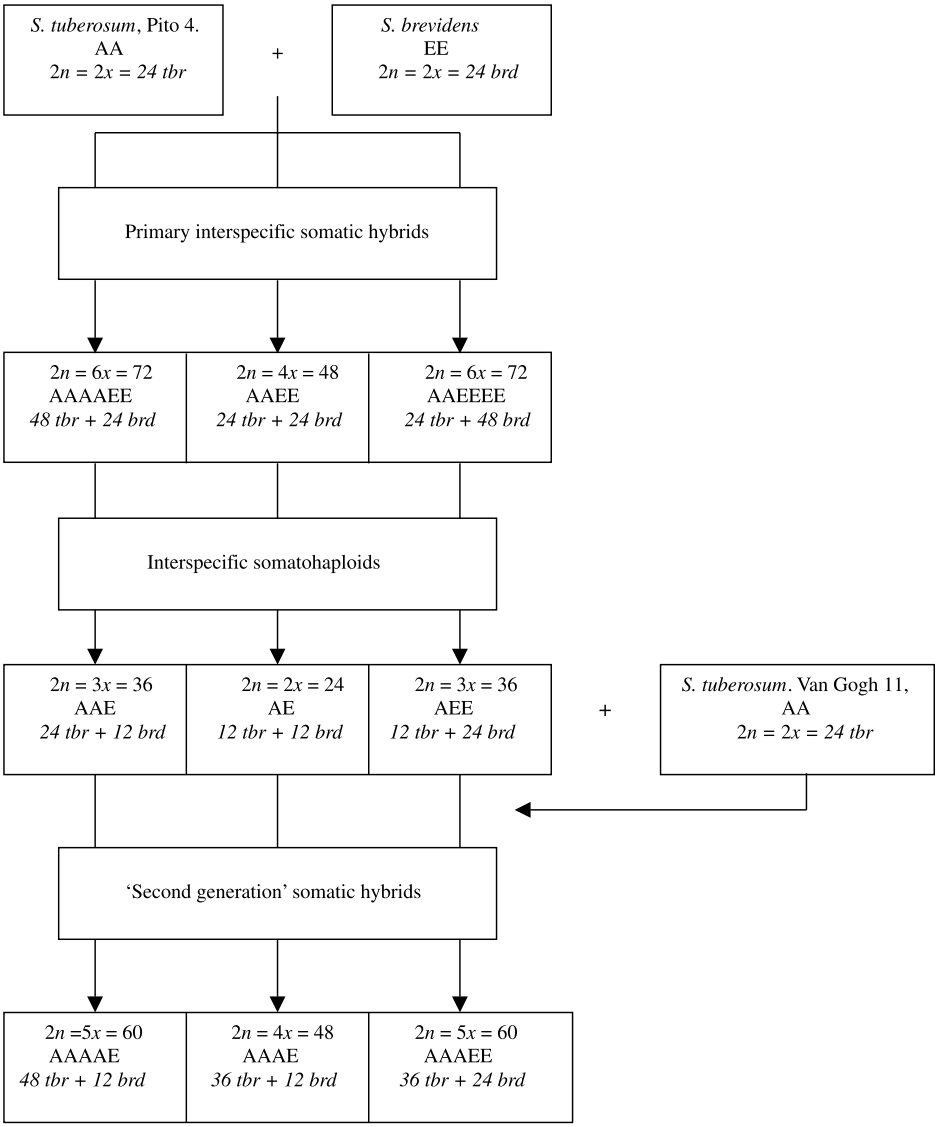
The androgenic potential of 12 tetraploid (2*n* = 4*x*) interspecific somatic hybrids was tested by anther culture using donor plant growth conditions and anther culture protocols previously described by Rokka et al. (1998*c*) (Table 2). The ploidy levels of anther-derived lines were analysed using FACSort Becton–Dickinson flow cytometer (San Jose, Calif.) as described by Rokka et al. (1998*c*).

GISH analysis

Genomic DNA of *S. tuberosum* and *S. brevidens* was extracted from young leaves of greenhouse-growing plants. Probe DNA (DNA from *S. tuberosum* or, alternatively, DNA from *S. brevidens*) was sonicated until the fragments attained a size of 1–5 kb and then direct labelled with FITC-12-dUTP using a nick translation mix (Boehringer Mannheim, Mannheim, Germany). Blocking DNA (*S. brevidens* or vice versa) was obtained by autoclaving total genomic DNA for 5 min, yielding fragments of 100–500 bp.

Mitotic metaphase chromosome spreads were prepared according to Zhong et al. (1996). In situ hybridization was performed according to Schwarzacher and Heslop-Harrison (1994) with modifications described by Jacobsen et al. (1995) and Kuipers et al. (1997). Slight modifications at the post-treatment step were introduced to reduce a thin layer that covered the cells after GISH treatment of the chromosome spreads. By these post-treatments, a volume of 50 µL of enzyme mixture containing 0.1% w/v Pectolyase Y23 (Seishin Pharmaceutical Co., Ltd. Tokyo, Japan), 0.1% w/v cellulase "Onozuka" RS (Yakult Honsha Co., Ltd. Tokyo, Japan), and 0.1% w/v cytohelicase (Sigma) was added to each slide. The slides were covered by plastic coverslips and incubated in a humid chamber for 15 min at 37°C and washed twice in 2× SSC for 5 min. RNAse and pepsin treatments were prepared as described by Kuipers et al. (1997), but the duration of the pepsin treatment (5 mg/mL in 0.01 M HCl) at 37°C was extended to 30 min. The highest resolution was obtained when the ratio of probe to blocking DNA in the hybridization mixture was increased to 1:100. The hybridization mixture contained 50% v/v deionized formamide, 10% w/v sodium dextran sulphate (SDS), 0.25% w/v SDS in 2× SSC, 2.5 ng of probe DNA/mL, and 0.25 µg of blocking DNA/mL. Chromosome preparations were counterstained with 2 mg DAPI/mL and 1 mg propidium iodide/mL and mounted in Vectashield (Vector Laboratories, Burlingame,

Fig. 1. Proposed breeding scheme to introgress desirable traits of wild species *Solanum brevidens* (E genome, haploid) into the germplasm of cultivated potato *S. tuberosum* (A genome, haploid) using subsequent protoplast fusions and haploidizations. Various alternative pathways and the theoretically expected genomic constitutions of the hybrids are illustrated. Theoretically expected numbers of *S. tuberosum* (*tbr*) and *S. brevidens* (*brd*) chromosomes are shown in italics. Expected chromosome numbers are based on the hypothesis that intergenomic recombination is completely absent.



Calif.). Slides were examined with an Olympus BX 60 microscope using appropriate filters for FITC and DAPI. Digital images were recorded using a colour CCD camera (Sony DXC – 950P, Power HAD, Japan) and analysed with AnalySIS 3.0 (Soft Imaging System GmbH, Münster, Germany). (Gavrilenko et al. 2001). Representative samples of between 5 and 20 well-spread metaphase cells of each plant were examined.

Results

Anther culture of tetraploid somatic hybrids between *S. tuberosum* and *S. brevidens*

Anther culture of the tetraploid interspecific somatic hybrids resulted in eight shoots regenerated through direct embryogenesis from a total number of 2182 anthers isolated from flower buds (Table 2). Of the 12 tetraploid hybrid clones tested, four clones produced embryos, but only one hybrid clone (3004) formed green shoots from embryos derived from three separate anthers (Table 2). Of the eight shoots formed from the hybrid 3004, only five survived during the subsequent maintenance in vitro. Of these, four plants (3004.1.1.1., 3004.1.1.2., 3004.2.1.1., and 3004.2.1.2.) were diploid and one plant (3004.5.2.1.) was tetraploid based on flow cytometric analyses.

The DNA content of the diploid somatohaploids (1.60–1.69 pg) was half of that of the tetraploid hybrid 3004 (3.38 pg) using chicken red blood cells (DNA content 2.33 pg) as an internal DNA standard (Galbraith et al. 1983; Rokka et al. 1995).

GISH analysis

All protoplast fusion-derived primary somatic hybrids, their somatohaploids, and second-generation somatic hybrids ana-

Table 1. Observed chromosomal composition of somatic hybrids between *Solanum brevidens* and *S. tuberosum* and their androgenic and second-generation hybrid progenies identified by GISH.

Genotype	No of cells analysed	2*n*	No. of chromosomes* derived from *S. brevidens*	No. of chromosomes* derived from *S. tuberosum*
Primary somatic hybrids: *S. brevidens* (EE) + *S. tuberosum* 'Pito 4' (AA)				
Tetraploids (4*x*)				
0204	6	47	23 (–1)	24
0205	18	48	24	24
0208	12	48	24	24
3004	8	48	24	24
Hexaploids (6*x*)				
0502	15	64	42 (–6)	22 (–2)
0603	15	70	46 (–2)	24
7001	13	69	44 (–4)	25 (+1)
Somatohaploids				
Diploids (2*x*)				
3004 1.1.1.	7	24	12	12
3004 2.1.2.	22	24	12	12
Triploids (3*x*)				
0502 1.1.1.	17	36	22	14
0507 1.2.1.	5	33	22	11
Second-generation somatic hybrids: 0507.1.2.1 (AEE) + *S. tuberosum* 'Van Gogh 11' (AA)				
2701-0105	7	55	20 (–2)	35
2701-0107	7	55	20 (–2)	35
2701-0109	10	53	20 (–2)	33 (–2)
2701-0302	13	60	24 (+2)	36 (+1)
2701-0502	17	60	26 (+4)	34 (–1)

*The numbers of the parental chromosomes involved in aneuploids (+, gained; –, lost) are indicated in parentheses.

Table 2. Results of embryo formation and plant regeneration in androgenesis of tetraploid (4*x*) *Solanum tuberosum* + *Solanum brevidens* somatic hybrids.

'Pito 4' + *S. brevidens* somatic hybrid genotype	No. of anthers isolated	Total no. of embryos formed	No. of green shoots regenerated	No. of plants/ploidy level
0201	360	5	0	
0204	140	0	0	
0208	118	2	0	
0214	13	0	0	
1203	180	0	0	
1702	225	0	0	
1703	56	0	0	
1903	277	0	0	
3002	29	0	0	
3004	187	5	8	4/2*x*, 1/4*x**
3301	162	0	0	
3306	435	2	0	
Total	2182	14	8	

*Only five plants analysed by flow cytometry.

lysed using GISH had the theoretically expected genome compositions corresponding to our scheme (Fig. 1). However, deviations in the theoretically expected chromosome numbers were detected in both the A and the E genomes (Table 1). Three of the four tetraploid hybrids (AAEE) analysed were eutetraploid ($2n = 4x = 48$) with the complete chromosome constitution of the parental lines (24 chromosomes of dihaploid *S. tuberosum* and 24 chromosomes of diploid *S. brevidens*). One tetraploid hybrid was an aneuploid ($2n = 4x - 1 = 47$) with one eliminated chromosome of *S. brevidens*.

Hexaploid primary somatic hybrids had the same genomic constitution (AAEEEE), as a result of fusion between two protoplasts of *S. brevidens* and one protoplast of dihaploid

Fig. 2. Chromosome constitutions of somatic hybrid, somatohaploid, and their second-generation somatic hybrid between *Solanum brevidens* (*brd*) and *S. tuberosum* (*tbr*). On the left side, the chromosomes derived from *S. brevidens* parental species fluoresce yellow-green owing to the FITC-labelled *S. brevidens* genomic DNA (probe DNA), whereas chromosomes of *S. tuberosum* fluoresce red owing to the propidium iodide counterstain. On the right side, the corresponding chromosome sets counterstained with 4,6-diamidino-2-phenylindole (DAPI) are presented. (A and B) Hypohexaploid primary somatic hybrid 0502 with genomic constitution of AAEEEE ($2n = 6x = 64$, 42 *brd* chromosomes + 22 *tbr* chromosomes). (C and D) Triploid somatohaploid 0502.1.1.1. with genomic constitution of AEE ($2n = 3x = 36$, 22 *brd* + 14 *tbr*). (E and F) Pentaploid second-generation somatic hybrid 2701-0109 with genomic constitution of AAAEE ($2n = 5x = 53$, 20 *brd* + 33 *tbr*).

S. tuberosum. The 6*x* hybrids were hypohexaploids ($2n = 6x = 64–70$), each of them having lost a variable number of chromosomes from *S. brevidens* (Table 1), but only hybrid 0502 had lost two chromosomes from *S. tuberosum* (Figs. 2A and 2B). On the contrary, hybrid 7001 gained one extra chromosome of *S. tuberosum*.

Haploid lines (somatohaploids) derived from the somatic hybrids had the genomic constitution of diploid AE or triploid AEE regenerated from tetraploid (AAEE) or hexaploid (AAEEEE) somatic hybrids, respectively. The diploid somatohaploids analysed (3004.1.1.1. and 3004.2.1.2.) were euploid ($2n = 2x = 24$), containing 12 chromosomes of both *S. tuberosum* and *S. brevidens*, because they were derived from the eutetraploid ($2n = 4x = 48$) somatic hybrid (3004) donor. The triploid ($2n = 3x$) somatohaploid 0502.1.1.1. had the chromosome number of 36, but with 22 chromosomes of *S. brevidens* and 14 chromosomes of *S. tuberosum* (Figs. 2C and 2D). It was regenerated from anther culture of the hypohexaploid ($2n = 6x - 8 = 64$) somatic hybrid 0502 that had 42 chromosomes of *S. brevidens* and 22 chromosomes of *S. tuberosum* (Figs. 2A and 2B). The somatohaploid 0507.1.2.1. was a hypotriploid ($2n = 3x - 3 = 33$) with 22 chromosomes of *S. brevidens* and 11 chromosomes of *S. tuberosum* (Table 1).

The second-generation somatic hybrids, derived from a fusion between triploid somatohaploid 0507.1.2.1. ($2n = 3x = 33$, AEE genome) and dihaploid *S. tuberosum* ($2n = 2x = 24$, AA genome), were pentaploids ($2n = 5x$) with the theoretically expected genomic composition of AAAEE, but none of them had the chromosome number of 57 (the sum of parental chromosomes (22 chromosomes of *S. brevidens* and 35 chromosomes of *S. tuberosum*)), as expected. Two second-generation hybrids (2701-0105 and 2701-0107) had 55 chromosomes, and one secondary hybrid (2701-0109) had 53 chromosomes (Figs. 2E and 2F). The second-generation hybrids 2701-0105, 2701-0107, and 2701-0109 each lost two chromosomes of *S. brevidens*, but the hybrid 2701-0109 also lost two chromosomes of *S. tuberosum*. Each of those hybrids was derived from the same callus. The two remaining second-generation hybrids analysed, 2701-0302 and 2701-0502, were derived from separate calli, and had a higher than expected chromosome number (60 instead of 57). The hybrids 2701-0302 and 2701-0502 gained two and four extra chromosomes of *S. brevidens*, respectively. Hybrid 2701-0302 gained one chromosome, but hybrid 2701-0502 lost one chromosome of *S. tuberosum* (Table 1).

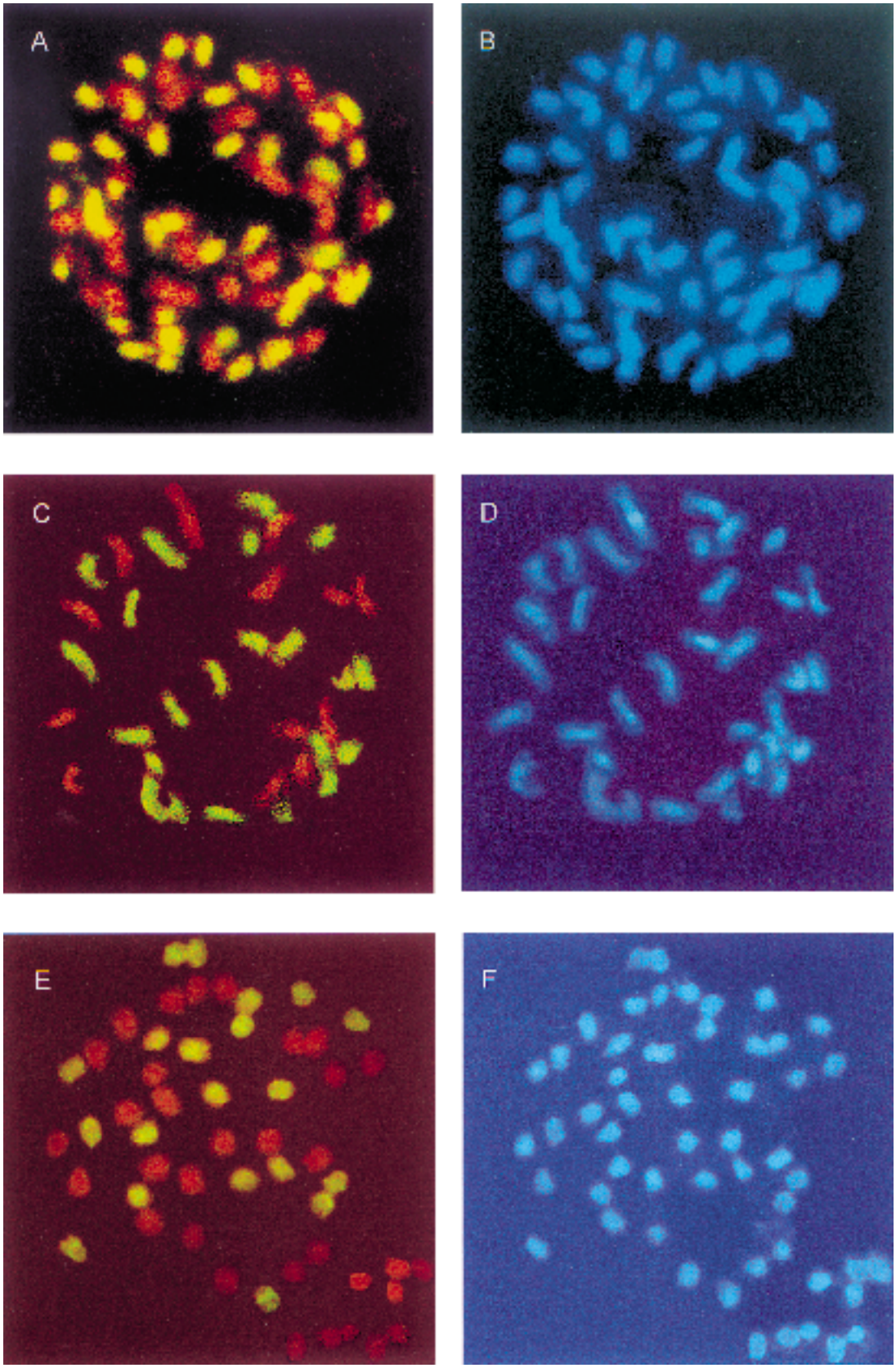
Discussion

In the present study, GISH analysis of interspecific hybrids demonstrated that chromosomes of the A genome of *S. tuberosum* and the E genome of *S. brevidens* can be unequivocally distinguished. For the genetic enhancement of potato using alien genomes, the application of GISH for detailed analysis of interspecific hybrids has great potential. Of the hybrids between *S. brevidens* containing the E genome and *S. tuberosum* containing the A genome, there are no previous reports of chromosome complements detected by GISH except Dong et al. (1999) who were able to discriminate the A and E parental genomes in a related combination (*S. tuberosum* × *S. berthaultii* + *S. etuberosum*).

Despite numerous previous reports of the successful production of interspecific primary somatic hybrids, introgression of the alien genetic material into potato breeding lines has been considerably limited, mainly owing to reduced fertility (Waara and Glimelius 1995). To overcome the high sterility level in the interspecific hybrids, an alternative to the sexual-backcrossing breeding scheme to incorporate desirable traits from wild species *S. brevidens* into cultivated potato genomic background was proposed, using repeated somatic hybridizations and haploid formations (Rokka et al. 1995, 2000). In addition, the purpose was to decrease the dosage of alien *S. brevidens* genome of the primary somatic hybrids. The genome compositions of the primary somatic hybrids, somatohaploids, and second-generation hybrids determined by GISH corresponded to the theoretically expected genomic constitutions as hypothesised in our scheme (Fig. 1), except among the hexaploid primary somatic hybrids with genomic constitution AAAAEE (Fig. 1), which were not detected.

In a range of generations analysed, chromosomes of *S. brevidens* were more frequently lost than the chromosomes of cultivated potato. In addition, extra chromosome gains were rarely detected. Previously, several types of chromosome loss in hybrids have been described as follows: (*i*) random; (*ii*) limited preferential or species-specific chromosome elimination (Garriga-Calderé et al. 1997) or extensive loss, even up to the removal of whole genome of one of the fusion partner (Pental et al. 1986); and (*iii*) preferential loss of the individual chromosomes (Pijnacker et al. 1987). The preferential loss of parental chromosomes can be attributable to a number of factors, such as asynchrony of mitotic cell cycle times, spatial distribution of genomes within the hybrid nuclei (Parokonny et al. 1992), and random mitotic irregularities. Previously, a slower rate of the condensation of the rDNA loci derived from *S. brevidens* was described (McGrath and Helgeson 1998) in *S. tuberosum* + *S. brevidens* somatic hybrids, possibly explaining the loss of the *S. brevidens* chromosomes in our interspecific somatic hybrids.

Besides chromosome loss, within primary somatic hybrids of potato the formation of translocations, isochromosomes, and chromosomal fragmentations have also been detected



(Pijnacker et al. 1987; Wolters et al. 1994; Garriga-Calderé et al. 1997; McGrath and Helgeson 1998). In the secondary interspecific somatic hybrids between potato and *S. nigrum*, intergenomic translocations were found (Gavrilenko et al. 1998). However, in the present study, no chromosome aberrations were observed in the primary or second-generation

somatic hybrids, but some of the somatic-translocation segments might have been too small to be detected by GISH.

In *S. tuberosum* + *S. brevidens* somatic hybrids and their sexual progeny, recombinant chromosomes have also been revealed by RFLP and RAPD analyses (Williams et al. 1990, 1993; McGrath et al. 1994, 1996). Those recombinations might have been caused by somatic translocations and (or) meiotic homoeologous crossing overs. However, recent molecular studies on genomic differentiation have indicated a low probability for homoeological chromosome pairing and meiotic recombination between the A and the E genomes (Perez et al. 1999). This is because of the structural changes (translocations and possibly also inversions and transpositions) accumulated in the E genome that have been revealed in several linkage groups (Perez et al. 1999). In our study, no recombinant chromosomes were found in the somatohaploids analysed, but until now the limited number of somatohaploids might not allow conclusive statements of the intergenomic recombination rate. Some recombinant segments might not have been detected by GISH if they were located in the distal euchromatic chromosomal regions with low amounts of repetitive DNA (Ramanna and Wagenvoort 1976; Peterson et al. 1996). Previous cytological studies of meiotic pairing have revealed multivalent formation in interspecific somatic hybrids between *S. brevidens* and *S. tuberosum*, suggesting the occurrence of intergenomic pairing (Williams et al. 1993). However, the application of conventional cytological methods could not discriminate between intergenomic and intragenomic pairing. Our future studies with meiotic pairing by GISH analysis in the diploid somatohaploids with 12 chromosomes of *S. brevidens* and 12 chromosomes of *S. tuberosum* would describe chromosome pairing affinity between the A and E genomes, and would broaden the knowledge about taxonomic relations between non-tuberous and tuberous *Solanum* species.

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