Plant Breeding **118**, 205—213 (1999)

ISSN 0179-9541

Assessment of genetic and phenotypic variation among intraspecific somatic hybrids of potato, *Solanum tuberosum* L.

T. GAVRILENKO[1], R. THIEME[2] and H. TIEMANN[2]

[1] N. I. Vavilov Institute of Plant Industry, B. Morskaya Street 42, 190000 St. Petersburg, Russia; [2] Federal Centre for Breeding Research on Cultivated Plants, Institute of Agricultural Crops, D–18190 Groß Lüsewitz, Germany

With 3 figures and 5 tables

Received May 5, 1998/Accepted October 10, 1998
Communicated by W. Friedt

Abstract

Intraspecific somatic hybrids have been produced by protoplast fusion in eight combinations involving 10 dihaploids ($2n = 2x = 24$) in an attempt to provide new material for potato breeding. Cytological analysis revealed extensive variation in chromosome number, such as aneuploid, aneusomatic and mixoploid hybrids. Most of the hybrids represented the expected chromosome number of 48; however, the frequency of aneuploids reached 50% in some combinations. Some hybrids carried structurally rearranged chromosomes and exhibited a high frequency of aberrant anaphases. Isozyme and random amplified polymorphic DNA (RAPD) patterns of the hybrids from the same fusion combination were uniform. In the field, somatic hybrids showed wide phenotypic variation in 20 morphological characters. There was a significant correlation between certain leaf characters and the ploidy level, which may be used to distinguish the tetraploid hybrids from hexaploids and octoploids. Tuber yield and flowering intensity were highest in tetraploid hybrids ($2n = 4x = 48$). Eighteen of the 73 hybrids reached higher yields than the standard variety 'Adretta'. Floral development and fertility were restored in hybrids derived from fusions between non-flowering or sterile dihaploids.

Key words: *Solanum tuberosum* — cytology — dihaploids — fertility — molecular characterization — somatic hybrids — yield

The breeding system of potato *Solanum tuberosum* L. is complicated because of the tetraploid and highly heterozygous nature of this species. More controlled selection may be achieved at the diploid level with dihaploid clones, but most of these are male sterile or have reduced fertility. The potential of dihaploids for potato breeding has been shown by Chase (1963) through reducing the ploidy level from tetraploid to diploid via pollination with a dihaploid inducer, *Solanum phureja*, intensive breeding at the diploid level, selection of the superior dihaploids and transfer of selected diploid genotypes to the tetraploid level. Recovery of the tetraploid level can be achieved by one of three methods: (1) somatic hybridization of the dihaploids, (2) sexual polyploidization (unreduced 2*n*-gametes) or (3) somatic chromosome doubling (Wenzel et al. 1979, Hermsen 1982, Ross 1986). In both traditional potato breeding and in protoplast fusion programmes heterozygosity, tetraploidy, genetic stability and phenotypic uniformity are the requirements for promising cultivars. Highly heterozygous tetraploids may be produced by sexual polyploidization and somatic hybridization, while the somatic chromosome doubling may lead to increased homozygosity and decreased vigour. Only with sexual polyploidization does meiotic recombination occur, leading to increased genetic variability. In the case of protoplast fusion, somaclonal variation may be induced by the *in vitro* conditions and produce novel genetic variation. With protoplast fusion, completely sterile, non-flowering superior dihaploids or diploid clones which are unable to produce 2*n* gametes may be involved in breeding. By this method, the desired agronomic characters of the parental dihaploids may be combined in one hybrid genotype and large populations of the heterozygous tetraploid somatic hybrids may be obtained in one step without meiotic segregation. Protoplast fusion has recently been extensively used in potato to resynthesize tetraploids through intraspecific fusion of diploids (Mattheij and Puite 1992, Waara et al. 1992, Thach et al. 1993, Cooper-Bland et al. 1994, Möllers et al. 1994). The technique of somatic hybridization of dihaploid potato materials has meanwhile been introduced in commercial German potato breeding programmes.

Genetic instability and loss of fertility of the somatic hybrids, however, is a serious problem when utilizing protoplast fusions in breeding programmes. A detailed characterization of the potato somatic hybrids has revealed cytogenetic changes, including polyploidization, aneuploidy and chromosome rearrangements, which may cause wide variation in morphological characters and generate undesirable variation and loss of important agronomic properties (Fish et al. 1988, Gibson et al. 1988, Pehu et al. 1989, Preiszner et al. 1991). Conversely, somaclonal variation may also result in production of some somaclones with improved agronomic traits (Rietveld et al. 1991). Consequently, for successful breeding it is desirable to minimize the negative and maximize the positive effects of somaclonal variation, especially those affecting important agronomic traits.

Materials and Methods

Plant materials: Dihaploid clones derived from *S. tuberosum* × *S. phureja* crosses and held in the dihaploid breeding collection of the Institute of Agricultural Crops, Groß Lüsewitz, Germany, were selected for their desirable agronomic characteristics such as high starch content, high yield, resistance to *Globodera pallida* and viruses, wart and cooking quality. The following genotypes were used for the fusions: T14 (= GL-6.063 108-86N), T17 (= GL-6.086 006-86), T18 (= GL-6.053 113-84), T23 (= GL-6.052 100-84N), T67 (= GL-6.090 004-86N), T75 (= GL-6.084 007-87N), T76 (= GL-6.089 044-87), T83 (= GL-6.094 001-88), T89 (= GL-6.271 015-88) and T95 (= GL-6.260 001-88). Somatic hybrids were obtained in electrofusion experiments (Sonntag et al. 1996) performed as described by Möllers and Wenzel (1992).

Isoenzyme analysis: Leaves from plants grown *in vitro* were used for isoenzyme analysis of peroxidases and esterases according to the methods described by Thieme et al. (1997).

RAPD analysis: Genomic DNA was extracted according to Gebhardt et al. (1989). A set of 80 decamer primers purchased from Operon Technologies Inc. (Alameda, CA, USA) and PrimeZyme DNA polymerase (Biometra, Göttingen, Germany) was used. Polymerase chain reaction (PCR) was performed in 25–100 μl of a reaction mixture containing reaction buffer, 200 μM dNTPs, 0.2 μM primer, 0.5–3 U polymerase and 50–200 ng genomic DNA. The amplification cycle consisted of the following steps: 94°C for 1 min, with 45 repeats of the thermal cycle, 94°C for 1 min (denaturation), 36°C for 1 min (primer annealing) and 72°C for 3 min (primer extension). The cycle was completed with 4 min at 72°C for full synthesis of the products. PCR fragments were separated on 1.5% agarose gels and 7.5% polyacrylamide gels followed by ethidium bromide or silver staining.

Flow cytometry and guard cell characters: DNA histograms were measured using a Partec Cell Analyser CA II equipped for UV excitation and blue light emission (Partec GmbH, Münster, Germany). Ploidy level was determined according to De Laat et al. (1987).

Guard cell characteristics of greenhouse grown hybrids and their parents of three fusion combinations were determined. Each genotype was represented by 3–4 week-old plants grown in a greenhouse under the same conditions. Chloroplast number and guard cell length of 10–20 guard cell pairs were measured on the fifth fully developed leaf of each plant using epidermal peels taken from the centre of the leaf underside.

Chromosome analysis: Chromosome numbers of hybrids were counted using mitotic metaphase preparations from squashed root tips. A minimum of 10 metaphase cells was used from at least two separate roots of each plant. Mitotic and meiotic preparations were stained according to Abramova (1988). For estimation of the cytological instability, only the frequency of mixoploids, aneuploids and aneusomatic plants was taken into account. The proportion of polyploid hybrids (6*x* and 8*x*) was not considered because these could have arisen not only from polyploidization but also from multifusion events.

Morphological analysis: Phenotypes of interdihaploid somatic hybrids were compared with those of the parents and the standard variety 'Adretta' under greenhouse and field conditions. In the first year, the somatic hybrids were grown in a greenhouse; each genotype was represented by three to five clonal copies. Tubers from these plants were harvested and planted in the field in the following year. Eighteen morphological characters were recorded: plant habit and vigour, plant height, number of primary stems, number of internodes, lateral and terminal leaflet indexes (width/length), number of lateral (primary) and secondary leaflets, leaf surface score (1-smooth; 2-rough, 3-ripple and 4-compressed), colour and shape of flowers, flowering intensity score (0–9, where 0-absence of floral buds and 9-profuse flowering, according to Tiemann and Schreiter 1976), tuber weight, shape and yield, number of tubers, depth of eyes, maturity and starch content. Tuber characteristics were evaluated in field experiments using two replicates of 10 plants per genotype. The results were analysed by correlation, ANOVA and pairwise comparisons (Tukey and Bonferroni) using SYSTAT for Windows.

Crossability and fertility: Pollen viability of somatic hybrids was determined by acetocarmine staining. Fertility was examined in reciprocal crosses of the somatic hybrids with three potato breeding lines, GL-96/1, GL-96/2 and GL-Producent.

Results

A total of 650 *in vitro* cultured regenerants were analysed (Table 1). Isozyme and/or RAPD analysis was performed for hybrid identification and for detection of molecular variation in the somatic hybrids.

Molecular analysis

Somatic hybrids of five combinations were identified by isoenzyme analysis. The frequency of somatic hybrids per regenerant was between 27.6 and 95.4% (Table 1). Somatic hybrids contained the isoenzyme bands of both parents. When the parental clones had identical isoenzyme patterns, RAPD analyses were used. Three primers of a set of 80 primers were selected for detecting polymorphism (Fig. 1). Somatic hybrids showed all of the bands of both parents.

In addition, somatic hybrids from certain combinations could be identified at an early stage when grown in a greenhouse by the dominantly-inherited phenotypic traits, such as purple or red anthocyanin pigmentation of the stems or at the underside of leaflets, pubescent stems or broad-crinkled stem wings.

Cytophotometry and guard cell characters

Most of the somatic hybrids were tetraploid (202 tetraploids out of 306 hybrids analysed by flow cytometry). Hexaploids, octoploids and highly chimeric hybrids of 4*x*–6*x*, 4*x*–8*x* and 6*x*–8*x* ploidy levels were also detected by flow cytometry (Table 1).

In addition, the ploidy level of somatic hybrids from three combinations was determined via their guard cell characters, i.e. numbers of chloroplast in stomatal cells and guard cell length. A positive correlation between these two scores was found ($r = +0.82$). The analysis of stomatal cells gave results similar to those obtained by flow cytometry. The correlations between stomatal cell parameters and ploidy level detected by flow cytometry were positive and significant ($P < 0.001$).

Chromosome number

Chromosome numbers of root tip cells of the parental clones indicated that eight dihaploid clones used in fusions were diploid and had 24 chromosomes. Chromosome counts confirmed the ploidy level determined by flow cytometry and stomatal guard cell analysis (Fig. 2). Examination of 143 somatic hybrids chosen at random from eight combinations revealed extensive variation in chromosome number: polyploid, mixoploid, aneuploid and aneusomatic hybrids at the tetraploid, hexaploid and octoploid levels.

Tetraploidy is the first requirement of selected somatic hybrids of potato. It was found that 65% of the somatic hybrids studied were tetraploid, but in some combinations the proportion of aneuploid tetraploids reached 50% (Table 1). Of 88 tetraploid hybrids, 17 were aneuploids and eight were aneusomatic. Most of the 4*x* aneuploids were hypotetraploid, lacking one to three chromosomes and four of them were hypertetraploid with one to four additional chromosomes. Aneusomatic hybrids had variable numbers of both euploid and aneuploid tetraploid cells (the proportion of hypotetraploid cells varied from 23 to 45%). In total, 63 euploid 4*x* hybrids ($2n = 4x = 48$) were selected from eight fusion combinations. The frequency of aneuploids increased significantly at the high ploidy levels. Among the 6*x* hybrids 50% were aneuploid, lacking four to 14 chromosomes and all 8*x* hybrids studied were hypo-octaploid (Table 1). The high frequency of aneuploids among the 6*x* and 8*x* somatic hybrids indicates that at these levels there was less stability than in 4*x* potatoes.

Microchromosomal fragments and telocentrics were observed in several cytological preparations of the hybrids.

Table 1: Molecular and cytological analysis (ploidy level, chromosome counting) of potato somatic hybrids

Fusion combination	Regenerants analysed (n)	Hybrids identified [%] isoenzyme (a), RAPD (b)	Hybrids (n) with ploidy level determined by flow cytometry and chromosome counting (euploids/aneuploids)			
			4*x*	6*x*	8*x*	Mixoploids
T18 (+) T23	25	72.0 a	10 (5/5)	10 (4/3)	1 (0/1)	0
T14 (+) T17	101	37.6 ab	15 (10/5)	10 (4/6)	2 (0/2)	5
T17 (+) T23	18	44.4 a	4 (4/0)	2 (1/1)	0	1
T67 (+) T75	100	95.4 a	38 (12/6)	22 (1/4)	2 (0/2)	3
T75 (+) T83	105	27.6 a	25 (12/4)	5 (3/1)	0	0
T75 (+) T95	100	47.0 b	27 (6/0)	12 (2/1)	1 (0/1)	2
T75 (+) T76	74	56.8 b	26 (8/2)	15 (2/1)	0	1
T75 (+) T89	127	52.8 b	57 (6/3)	7	2 (0/2)	1
Total	650	n = 348	202 (63/25)	83 (17/17)	8 (0/8)	13

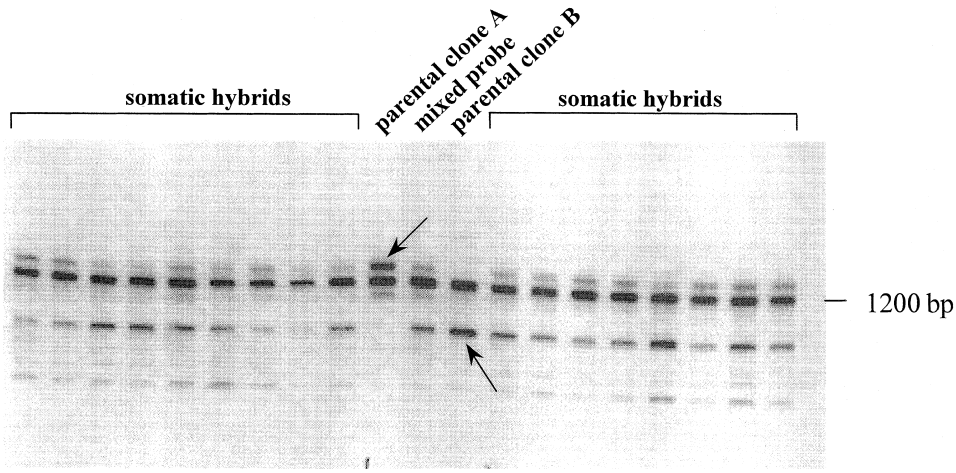

Fig. 1: Identification of somatic hybrids by RAPD-PCR analysis (OPA 05) after protoplast fusion of the two dihaploid potato breeding clones A and B

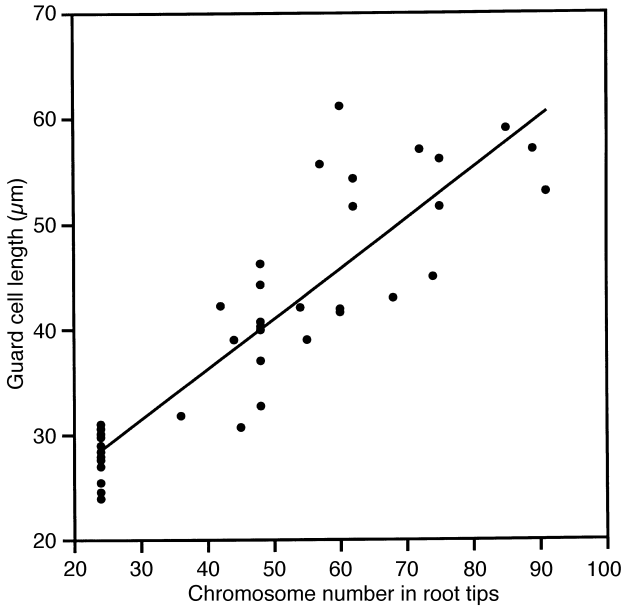

Fig. 2: Relation between chromosome number and length of stomatal guard cells in intraspecific somatic hybrids and parental dihaploids ($y = 17.0985 + 0.4776x$, $r^2 = 0.7887$)

Structural chromosome changes appear to be related to the anomalies during cell divisions (Fig. 3). The 4*x* hybrids showed 67–93% normal mitotic anaphases, 0–17% anaphases with bridges and 3–33% anaphases with one or two laggards. Almost 30% of the tetraploid hybrids revealed the lowest frequency (less than 10%) of mitotic anomalies. This indicates cytological instability, since the observed anomalies may result in daughter cells with various chromosome numbers. Mitotic cells of root tips showed fewer irregularities than microsporocytes. All 4*x* hybrids studied had irregular meiosis but to varying degrees. The frequency of microsporocytes with anomalies varied between hybrids from 19 to 60%. The most common types of irregularity were bridges with and without fragments, lagging chromosomes and micronuclei, resulting in variation of chromosome number in the gametes and a high percentage of sterile pollen.

Morphological variation

The populations of somatic hybrids exhibited high phenotypic variation in leaf, stem, flower and tuber traits. The somatic hybrids and their parents in each combination were grouped into six classes according to their chromosome numbers: (1) diploid parental clones ($2n = 2x = 24$), (2) eutetraploids ($2n = 4x = 48$), (3) aneuploids at the tetraploid level ($2n = 4x \pm$), (4) euhexaploids ($2n = 6x = 72$), (5) aneuploids at the hexaploid level ($2n = 6x \pm$) and (6) hypo-octoploid hybrids ($8x-$). By comparing the morphology of these six classes, the role of aneuploidy and polyploidy in hybrid phenotypic variability became evident.

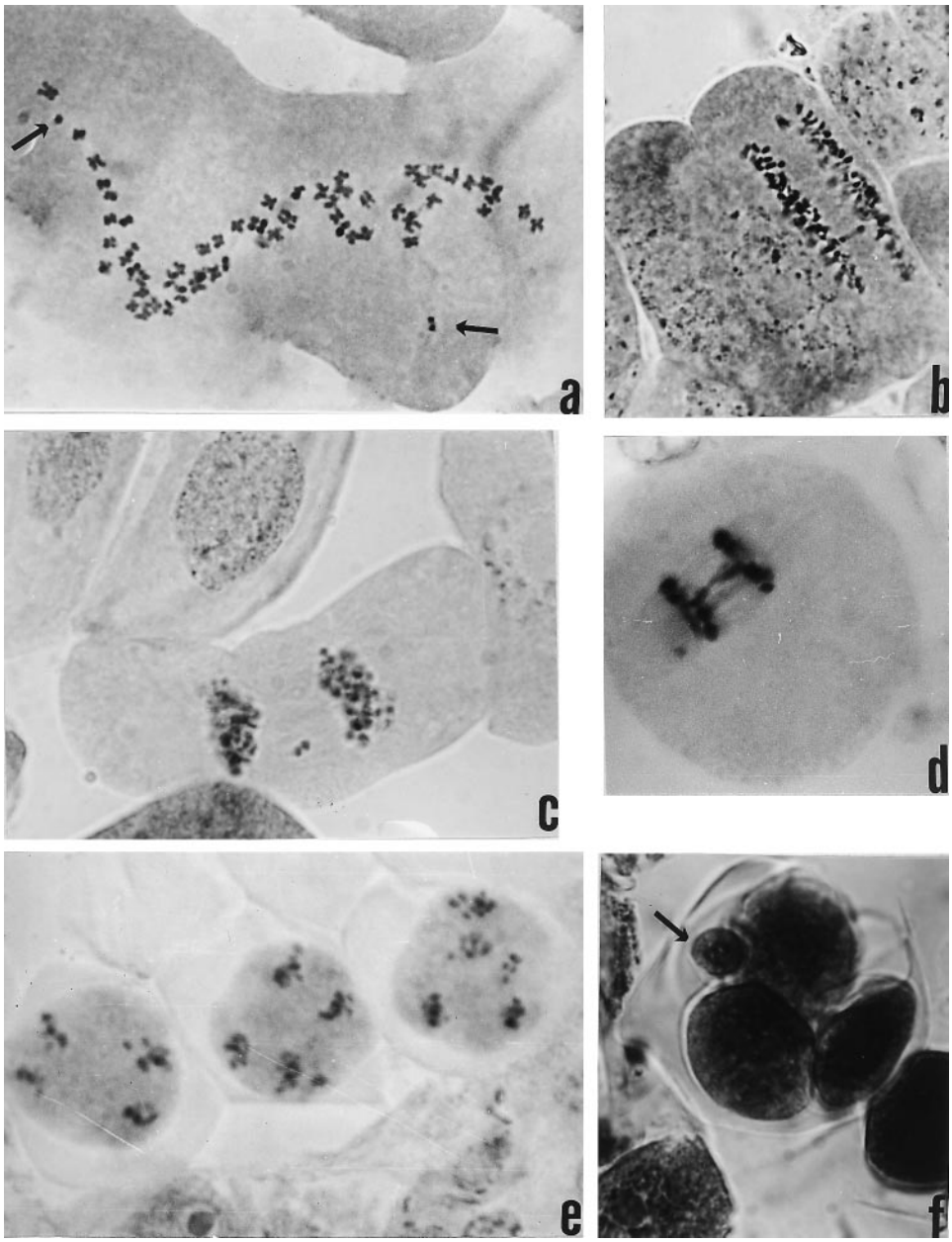

Fig. 3: Mitotic and meiotic anomalies in intraspecific somatic hybrids. a. Mitotic metaphase with chromosome aberrations (arrows); b. mitotic anaphase showing bridge. c–f. Meiotic irregularities in intraspecific somatic hybrids: c,d. anaphase I with bridge and laggard; e. telophase II with multipolar spindles and chromosomes not included in the nuclei; f. tetrad with microcell (arrow)

Tetraploid and hexaploid somatic hybrids were more vigorous than their diploid parents, their leaves were broader and the number of leaflets lower than in the parental clones. Octoploids (class 6) had an abnormal habit, weak morphology, retarded growth, round single leaflets with a compressed surface and their tuber yield was lower than that of the diploid parents; some did not produce tubers. Tetraploid hybrids (classes 2 and 3) were taller, had more numerous lateral and secondary leaflets, and their terminal and lateral leaves were narrower than those of hexaploid or octoploids (classes 4 and 5) from the same combination. The broader leaflets of hexaploid and octoploid hybrids gave lower leaf indices (length/width) compared with tetraploids. This resulted in a strong negative correlation between ploidy level and leaf traits (lateral and terminal leaf indices, average number of secondary and lateral leaflets). Leaflets of 4*x* hybrids were smoother than those of the 6*x* and 8*x* plants, which had rippled or compressed leaf surfaces. A strong positive correlation was observed between ploidy level and leaf surface structure (Table 2). Similar trends were observed in all combinations both in greenhouse and field experiments. There was a weak correlation between chromosome number and the following traits: plant height; number of stems, internodes, flowers and tubers; starch content and depth of tuber eyes (Table 2).

With most characters, euploid and aneuploid hybrids from the same combination and at the same ploidy level could not be distinguished; only flowering intensity and tuber yield were noted to be higher in euploid somatic hybrids than in aneuploids. Maximum tuber yield was observed in hybrids of class 2 ($2n = 4x = 48$). The most promising hybrids were eutetraploids, with tuber yields higher than in the standard cultivar 'Adretta' (Table 3). For effective tuber development high levels of heterozygosity are also necessary. Autotetraploids of genotype T75, generated by homofusion, produced a significantly

Table 2: Coefficients of correlation between chromosome number and morphological characters in interdihaploid potato somatic hybrids

		Fusion combination				
	Characters compared	T14(+)T17	T75(+)T67	T75(+)T83	T15(+)T18	T18(+)T23
Chromosome number	**Measured traits**					
1	Shoot number	–0.01	—	+0.04	—	—
2	Plant height	–0.29	–0.32	+0.11	0.20	+0.31
3	No. of internodes	+0.03	+0.27	+0.48	–0.50	+0.53
4	Terminal leaf index	–0.77**	–0.78**	–0.82***	–0.69*	–0.91***
5	Lateral leaf index	–0.84***	–0.72**	–0.75**	–0.76*	–0.90***
6	No. of laterals	–0.57	–0.81**	–0.52	–0.82**	–0.76**
7	No. of intermediate leaflets	–0.72**	–0.67	–0.58	–0.83**	–0.66
8	No. of flowers on the first brush	–0.14	–0.41	–0.24	–0.14	–0.13
9	Total tuber number	–0.22	–0.62*	–0.38	—	—
10	Yield	–0.26	–0.61*	–0.01	–0.28	—
11	Starch content	–0.05	—	—	—	—
	Ranked traits					
12	Leaf surface score	–0.70*	+0.88***	+0.70**	+0.89***	+0.75**
13	Plant vigour	–0.19	–0.48	–0.77**	—	—
14	Depth of eyes	—	–0.40	+0.22	—	—
15	Tuber size	—	+0.22	–0.15	—	—

*, **, *** Correlations are significant at P = 0.05, P = 0.01 and P = 0.001, respectively.

Table 3: Mean tuber yield (g) of dihaploid parents and euploid 4*x* (2*n* = 48), aneuploid 4*x* (2*n* = 48 ±), euploid 6*x* (2*n* = 72) and aneuploid 6*x* (2*n* = 72 ±) somatic hybrids

			Yield (min.–max.) of plants with ploidy level			
Fusion combination	Parental yield	Mid-parental value	48	48±	72	72±
T 14 (+)	895	684	1030 a[2]	577 b	407 b	184 c
T 17	472		(525–1303)	(460–770)	(190–624)	(117–250)
NAH/NBH[1]			8/6	3/0	4/0	3/0
T18 (+)	532	464	1073 a	346 b	404 b	294 b
T23	395		(630–1485)	(38–570)	(225–538)	(275–313)
NAH/NBH			5/4	4/0	4/0	3/0
T17 (+)	472	550	645			
T23	395		(525–725)	—	—	—
NAH/NBH			4/0			
T75 (+)	481	558	1033 a		160 b	
T76	636		(874–1200)	—	(130–190)	—
NAH/NBH			5/3		2/0	
T75(+)	481	552	958 a	156 b		
T89	623		(833–1143)	—	—	
NAH/NBH			3/1		1/0	
T75 (+)	481	458	1206 a		136 b	
T95	435		(546–1360)	—	(136–137)	—
NAH/NBH			5/4		3/0	
T75 (+)	481	414	698 a	329 b	427 b	
T83	347		(207–946)	(190–435)	(186–760)	—
NAH/NBH			13/0	3/0	3/0	
T75 (+)	481	406	748	564		563
T67	331		(269–1007)	(361–834)	—	(318–872)
NAH/NBH			12/0	5/0		4/0

[1] NAH, number of hybrids analysed (each hybrid was represented by 10 plants); NBH, number of somatic hybrids with yield higher than standard cultivar 'Adretta' (1995/1996; 1077 g per plant).
[2] Identical letters in a row indicate data that do not differ at P = 0.05 (Student t-test).

lower tuber yield than 4*x* hybrids generated by fusions of the dihaploid T75 with other dihaploids (data not shown).

Tuber yield of euploid 4*x* hybrids was also higher than that of their dihaploid parents; heterosis of yield was observed for class 2 (2*n* = 48) in all combinations tested. Tuber yield in somatic hybrids was not correlated with plant maturity. In general, late maturity was expressed by the intraspecific hybrids, even in fusion combinations of early-maturing dihaploids (Table 4). Increase in tuber yield of the eutetraploid hybrids over the mid-parent value (MPV) varied from 52.8 to 143.7%. High yield in these hybrids was mainly due to increased tuber weight, as the mean tuber number was only slightly higher than the mid-parent value. Fusion combinations with the highest yield of the best parent had higher mean yields for 4*x* hybrids (Table 3), but correlation between parental values and mean hybrid yield were not significant because of the large differences

Table 4: Tuber number, weight, shape and maturity (min.–max.) of parental dihaploids and selected somatic hybrids with mean yield higher than standard cultivar 'Adretta' (MPV = mid-parent value, Hy = somatic hybrids. Tuber shape scores: 3 = round, 4 = round oval, 5 = oval)

Genotype	Tubers (n)	Tuber weight (g)	Tuber shape (score)	Maturity
T 18	11.5	43.1	5	4
T 23	20	20.8	4	4
MPV	10.8	20.7	4.5	4
Hy:T18(+)T23	20	69.1	4.3	3.7
n = 3	(18–21)	(52.3–82.5)	(4–5)	(3–4)
T 14	23	38.9	5	4
T 17	13	36.3	5	6
MPV	18	37.6	5	5
Hy:T14(+)T17	20.7	63.9	5	3.5
n = 6	(10–27)	(40.6–116.3)		(2–4)
T 75	10.1	43.7	5	7
T 95	13.9	29.2	5	6
MPV	12	36.5	5	6.5
Hy:T75(+)T95	12.8	102.9	4.2	4.4
n = 4	(9–16)	(67.8–151.1)	(4–5)	(4–5)
Standard cultivar 'Adretta'	12	89.7	3	6

Table 5: Flowering and fertility of the interdihaploid somatic hybrids and their parents under field conditions

Fusion combination	Chromosone number	Genotypes analysed (n)	Flowering intensity score (min–max.)	Pollen viability (%), (min–max.) flowering	Hybrids/flowers/berries/seeds[1] × Producent	× 96/1	× 96/2
T14 (+)	$2n = 24$		5	m.st., n.m.			
T17	$2n = 24$		2	m.st., f.a.			
	$2n = 48$	7	6.0 (3–8)	49.4 (34–75)	3/29/16/84	3/37/27/81	2/24/21/81
	$2n = 48 \pm$	3	1.0 (0–3)	43.1 (45–41)	—	—	—
	$2n = 72$	3	1.3 (1–2)	31.9 (9–75)	1/10/0	1/13/5/0	1/13/4/0
T18 (+)	$2n = 24$		0	n.f.			
T23	$2n = 24$		6	m.st., n.m.			
	$2n = 48$	4	7.8 (7–8)	39.0 (12–77)	3/30/4/20	2/32/14/30	2/29/16/31
	$2n = 48 \pm$	4	2.8 (1–5)	20.6 (dm–41)	1/9/2/0	1/13/3/0	—
	$2n = 72$	4	3.0 (0–5)	18.0 (6–31)	2/21/2/0	2/26/10/4	2/19/2/0
T75 (+)	$2n = 24$		0	n.f.			
T67	$2n = 24$		6	m.st.			
	$2n = 48$	13	2.8 (0–5)	0 (nf, fa, dm)			
	$2n = 48 \pm$	5	1.8 (0–3)	0			
	$2n = 72$	4	1.3 (0–3)	0 (nf, fa, dm)			
T75 (+)	$2n = 24$		0	n.f.			
T83	$2n = 24$		0	n.f.			
	$2n = 48$	13	1.5 (0–4)	0 (nf, fa, dm)			
	$2n = 48 \pm$	3	0.3 (0–1)	—			
	$2n = 72$	3	0.3 (0–1)	0 (nf, fa, dm)			

[1] Hybrids/flowers/berries/seeds = no. of hybrids used in backcrosses with potato breeding lines/no. of pollinated flowers in backcrosses/no. of berries formed/average no. of seeds per berry; nf = non-flowering hybrids; fa = floral anomalies, rudimentary anthers; dm = degeneration of the microspores; m.st. = male-sterile plants, n.m. = normal floral morphology; — = not determined.

between hybrid clones. Large variation in tuber traits of the 4*x* hybrids was also found between combinations (Table 3), which may be due to differences in the combining ability of parental clones. In comparison with the control cultivar 'Adretta' the best fusion combination was T75 (+) T95, whose hybrids from class 2 had greater tuber weights and average numbers of tubers similar to cv. 'Adretta' (Table 4).

Fertility

Most somatic hybrids produced flowers (Table 5). Eutetraploid hybrids had the highest flowering score, whereas that for the aneuploid tetraploids was significantly lower and similar to that of the 6*x* hybrids; all 8*x* somatic hybrids were non-flowering. Pollen stainability of 67 somatic hybrids ranged from 0 to 77%. Aneuploidy had no significant influence on pollen viability and berry formation (Table 5).

Flower development and pollen fertility were restored in somatic hybrids derived from the fusions between non-flowering and/or male-sterile dihaploids. Berry and seed formation in reciprocal backcrosses with potato lines were observed in somatic hybrids (class 2) generated from the fusions between sterile dihaploids.

The parental genotype exerted a significant effect on flowering intensity, floral morphology and fertility of the somatic hybrids. In combinations, which included the profuse-flowering male sterile dihaploids T14 and T23, i.e. T14 (+) T17 and T23 (+) T18, the 4*x* hybrids were also characterized by profuse flowering, fully developed flowers, a higher proportion of viable pollen and self fertility, and they set berries after cross pollinations in both directions. The highest frequency of floral anomalies, rudimentary anthers and male-sterile hybrids was observed in four combinations in which the non-flowering

dihaploid clone T75 was used as a parent. However, male-sterile 4*x* hybrids produced in these combinations were female fertile.

Discussion

Analysis of plants regenerated from protoplast culture have revealed genetic changes at molecular, chromosomal, genomic and plant level (Gleba and Sytnik 1984). A common phenomenon of protoplast fusion experiments is the generation of different ploidy levels. In addition to polyploidization, extensive chromosomal variations, including high percentages of aneuploidy, chromosome chimerism and structural rearrangements were reported in somatic hybrids of potato in both interspecific (Puite et al. 1986, Pehu et al. 1989, Pijnacker et al. 1989, 1992, Preiszner et al. 1991, Mattheij et al. 1992, Cardi et al. 1993, Ward et al. 1994) and intraspecific combinations (Waara et al. 1992, Thach et al. 1993, Rasmussen et al. 1996). Mutations of nuclear or organelle DNA were also detected in fusion products of potato dihaploids (Lössl et al. 1994) and different *Solanum* species (Xu et al. 1993).

In the present study, a population of intraspecific somatic hybrids obtained from eight fusion combinations was used to investigate genetic and phenotypic variation. Somatic hybrids were analysed by cytological, molecular and morphological methods to select genetically stable 4*x* plants with the desired agronomic traits. A high phenotypic variation, under greenhouse and field conditions, was detected among the hybrids from the same combination. This variation could be the result of genome changes or of alterations in gene expression in the hybrid genome.

No polymorphism among somatic hybrids of the same combinations was found using isoenzyme and RAPD analyses. However, the absence of molecular variation does not guarantee genetic stability. The wide range and high level of morphological variation observed between the somatic hybrids indicated genetic instability. At least part of the phenotypic variation was attributed to changes of karyotype in the somatic hybrids.

Polyploidization, aneuploidy or mixoploidy were noted in all combinations. Most of the somatic hybrids were tetraploid, which was confirmed by three independent methods: flow cytometry, stomatal guard cell scores and chromosome counts. Any of these methods may be used, but each has advantages and disadvantages. Stomatal cell analysis is a cheap method for the identification of polyploids but it is not suitable for detection of mixoploid and aneuploid plants. An advantage of flow cytometry is in the detection of mixoploidy, but we could not select aneuploid hybrids from the euploid plants at the same ploidy level by this method. Compared with the first two methods, chromosome counting is an exact method for detecting aneuploids in potato but it is laborious for large numbers of somatic hybrids because of the small size and relatively numerous chromosomes.

In two out of eight combinations, all tetraploids studied had the expected chromosome number ($2n = 4x = 48$) but in some combinations the frequency of aneuploid 4*x* hybrids reached 50%. In addition, somatic hybrids exhibited chromosome bridges, fragments and laggards at mitotic and meiotic anaphases and structurally rearranged chromosomes at metaphases, which indicate karyotypic modifications. The high frequencies of aneuploid and mixoploid hybrids may result from somaclonal variation or pre-existing chromosome instability of the parental clones. The latter possibility seems improbable because parental chromosome complements were stable ($2n = 2x = 24$) and aneusomatic clones were not detected among them. This suggests that there is a significant level of somaclonal variation induced during callus or regeneration phases. The frequency of cytologically unstable hybrids varied from 16 to 52% between combinations. This may indicate that the degree of somaclonal variation is genotype dependent, since the methods of protoplast fusion, culture, and regeneration were identical for all combinations.

Although cytogenetic instability has been observed in most protoplast fusion experiments, only a few reports exist in which the relation between cytological and phenotypic variation is detailed for a larger population of somatic hybrids of *Solanum* (Fish et al. 1988, Pehu et al. 1989, Preiszner et al. 1991, Waara et al. 1992, Cardi et al. 1993). Only the selection of somatic hybrids with different chromosome numbers (4*x*, 4*x* ±, 6*x*, 6*x* ± and 8*x* classes) and a study of their morphology will provide reliable estimates of the phenotypic effects of polyploidization, mixoploidy and aneuploidy at plant level.

The present results revealed a correlation between frequency of the polyploid somatic hybrids and gross morphological alterations, such as abnormal plant habit, abnormal leaves with round laterals, undeveloped primary and secondary leaflets, and compressed leaf surfaces. Thus, variation in ploidy level may be considered as one of the main sources of phenotypic variability. Similar correlations between ploidy level and morphological traits have been reported for intraspecific (Waara et al. 1992), interspecific (Pehu et al. 1989, Cardi et al. 1993) and intergeneric (Gavrilenko et al. 1994) somatic hybrids involving *Solanum* species. The significant linear regression between ploidy level and some leaf characters (leaf indices, number of primary leaflets, leaf surface score) probably indicates that the development of these morphological traits is due to gene dosage effect. This could be particularly useful where chromosome counts or cytophotometry are needed for numerous regenerants. Grown under the same conditions, 4*x* somatic hybrids may be distinguished from the 6*x* and 8*x* hybrids of the same combination. The frequency of the desired 4*x* hybrids can thus be considerably increased at an early stage by elimination of all plants whose leaves have broad terminal and lateral leaflets, single or no secondary leaflets, ripple compressed leaf surfaces or weak morphology. This will effectively decrease the time and labour costs of breeding potatoes by intraspecific somatic hybridization.

In this study, aneuploidy did not significantly influence most of the morphological traits; this may be due to the buffering capacity of 4*x* genomes, non-specific loss of chromosomes and the polygenic nature of most morphological traits. Aneuploidy only significantly affected flower intensity and tuber yield at the 4*x* and 6*x* ploidy levels; these values were lower in aneuploids in all combinations. It is noteworthy that other investigations of the phenotypic effects of aneuploidy in interspecific somatic hybrids also indicate a decrease in the degree of flowering (Cardi et al. 1993). Despite low floral production, aneuploidy did not significantly influence floral morphology or pollen stainability, which is in agreement with the results of previous studies on potato somatic hybrids (Mattheij et al. 1992, Rasmussen et al. 1996). Other studies have shown that interspecific aneuploid 4*x* somatic hybrids differ from the eutetraploid group in their terminal leaflet index (Pehu et al. 1989), plant height score and number of primary leaflets (Cardi et al. 1993). These trends were also determined in the present study, but only for single combinations. Aneusomatic 4*x* plants were morphologically

indistinguishable from eutetraploid hybrids of the same combination.

The phenotypic characterization demonstrated the general vegetative hybrid vigour of the euploid 4*x* hybrids. Late plant maturity, as revealed in this study, has also been observed in intraspecific potato somatic hybrids (Waara et al. 1992, Mattheij and Puite 1992). The restoration of flower development and male fertility in somatic hybrids derived from fusions of non-flowering or sterile dihaploids could be useful for breeding. However, a high percentage of anaphase irregularities observed in intraspecific somatic hybrids will lead to aneusomatic genotypes, variation of chromosome numbers in the gametes and the loss of fertility. Therefore, somatic hybrids with stable mitotic and meiotic division should be selected for further crossbreeding programmes. Obviously, monitoring of chromosomal stability will be of high value for selecting perspective genotypes within intraspecific somatic hybrids.

The present results are consistent with those of Mattheij and Puite (1992) and Möllers et al. (1994): a number of somatic hybrids ($2n = 4x = 48$) produced significantly higher yields than their dihaploid parents, which, in some combinations, was due to increased tuber weight, i.e. an improvement in the same traits in the standard cultivar 'Adretta'. At the same time, tetraploid ($2n = 4x = 48$) somatic hybrids from the same combination, which theoretically should be uniform, exhibited great variation in tuber yield between the hybrid clones (Table 3). Further investigations on the stability of cytoplasmic genomes (Lössl et al. 1994) might reveal the origin of this variation.

Acknowledgements

The authors thank H. Baumann for assistance with biochemical and molecular analyses, and Dr K. Sonntag and I. Müller for the participation in hybrid production. Thanks are due to Dr L. Novikova for help in statistics and to Dr T. Thieme for advice on ANOVA. Dr T. Gavrilenko was supported by a postdoctoral research grant from the Deutsche Forschungsgemeinschaft, Bonn, Germany.

References

Abramova, L. J., 1988: Methodical Guide 'Chromosome Counting and Chromosome Morphology'. VIR, Leningrad.

Cardi, T., F. D. Ambrosio, D. Consoli, K. J. Puite, and K. S. Ramulu, 1993: Production of somatic hybrids between frost-tolerant *Solanum commersonii* and *S. tuberosum*: characterization of hybrid plants. Theor. Appl. Genet. **87,** 193—200.

Chase, S. S., 1963: Analytical breeding of *Solanum tuberosum*. Can. J. Genet. Cytol. **5,** 359—363.

Cooper-Bland, S., M. J. De Maine, M. L. M. H. Fleming, M. S. Phillips, W. Powell, and A. Kumar, 1994: Synthesis of intraspecific somatic hybrids of *Solanum tuberosum*: assessments of morphological biochemical and nematode (*Globodera pallida*) resistance characteristics. J. Exp. Bot. **45,** 1319—1325.

Fish, N., A. Karp, and M. G. K. Jones, 1988: Production of somatic hybrids by electrofusion in *Solanum*. Theor. Appl. Genet. **76,** 260—266.

Gavrilenko, T. A., D. B. Dorokhov, and T. V. Nikulenkova, 1994: Characterization (phenotypic and molecular–RAPD-analysis) of intergeneric somatic hybrids between tomato *Lycopersicon esculentum* and non-tuberous potato species of the *Etuberosa* series. Russian J. Genet. **30,** 1605—1615.

Gebhardt, C., E. Ritter, T. Debener, U. Schachtschnabel, B. Walkemeier, H. Uhrig, and F. Salamini, 1989: RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet. **78,** 65—75.

Gibson, R. W., M. J. K. Jones, and N. Fish, 1988: Resistance to potato leaf roll virus and potato virus Y in somatic hybrids between dihaploid *Solanum tuberosum* and *S. brevidens*. Theor. Appl. Genet. **76,** 113—117.

Gleba, Y. Y., and K. M. Sytnik, 1984: Genetic Engineering in Higher Plants. Springer-Verlag, Berlin, Heidelberg, New York.

Hermsen, J. G. Th., 1982: New approaches to breeding for the year 2000. Proc. Int. Congr. Res. for the Potato in the Year 2000 (CIP), 29—32.

De Laat, A. M. M., W. Göhde, and M. D. C. Vogelzang, 1987: Determination of ploidy of single plants and plant population by flow cytometry. Plant Breeding **99,** 303—307.

Lössl, A., U. Frei, and G. Wenzel, 1994: Interaction between cytoplasmic composition and yield parameters in somatic hybrids of *S. tuberosum* L. Theor. Appl. Genet. **89,** 873—878.

Mattheij, W. M., and K. J. Puite, 1992: Tetraploid potato hybrids through protoplast fusions and analysis on their performance in the field. Theor. Appl. Genet. **83,** 807—812.

——, R. Eijlander, J. R. A. de Koning, and K. M. Louwes, 1992: Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens. Theor. Appl. Genet. **83,** 459—466.

Möllers, C., and G. Wenzel, 1992: Somatic hybridization of diploid potato protoplasts as a tool for potato breeding. Bot. Acta **105,** 133—139.

——, U. Frei, and G. Wenzel, 1994: Field evaluation of tetraploid somatic potato hybrids. Theor. Appl. Genet. **88,** 147—152.

Pehu, E., A. Karp, K. Moore, S. Steele, R. Dunckley, and M. G. K. Jones, 1989: Molecular, cytogenetic and morphological characterization of somatic hybrids of dihaploid *Solanum tuberosum* and diploid *S. brevidens*. Theor. Appl. Genet. **78,** 696—704.

Pijnacker, L. P., M. A. Ferwerda, K. J. Puite, and J. G. Schaart, 1989: Chromosome elimination and mutation in tetraploid somatic hybrids of *Solanum tuberosum* and *S. phureja*. Plant Cell Rep. **8,** 82—85.

——, ——, and W. M. Mattheij, 1992: Microsporogenesis in three tetraploid somatic hybrids of potato and their di(ha)ploid fusion partners. Theor. Appl. Genet. **85,** 269—273.

Preiszner, J., A. Feher, O. Veisz, J. Sutka, and D. Dudits, 1991: Characterization of morphological variation and cold resistance in interspecific somatic hybrids between potato and *S. brevidens*. Euphytica **57,** 37—49.

Puite, K., L. S. Roest, and L. P. Pijnacker, 1986: Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep. **5,** 262—265.

Rasmussen, J. O., J. P. Nepper, and O. S. Rasmussen, 1996: Analysis of somatic hybrids between two sterile dihaploid *Solanum tuberosum* L. breeding lines. Restoration of fertility and complementation *G. pallida* Pa2 and Pa3 resistance. Theor. Appl. Genet. **92,** 403—410.

Rietveld, R. C., R. A. Bressan, and P. M. Hasegawa, 1991: Somaclonal variation in tuber-disc-derived populations of potato. I. Evidence of genetic stability across tuber generations and diverse locations. Theor. Appl. Genet. **82,** 430—440.

Ross, H., 1986: Potato Breeding — Problems and Perspectives. Adv. Plant Breed. 13. Paul Parey, Berlin, Hamburg.

Sonntag, K., R. Thieme, and H. Tiemann, 1996: Protoplast fusion and identification of somatic hybrids of dihaploid genotypes from different origin for potato breeding. Proc. 13th Triennial Conf. Eur. Assoc. Potato Res., Veldhoven, 397—398.

Thach, N. Q., U. Frei, and G. Wenzel, 1993: Somatic fusion for combining virus resistances in *Solanum tuberosum* L. Theor. Appl. Genet. **85,** 863—867.

Thieme, R., U. Darsow, T. Gavrilenko, D. Dorokhov, and H. Tiemann, 1997: Production of somatic hybrids between *S. tuberosum* L. and late blight resistant Mexican wild potato species. Euphytica **97,** 189—200.

Tiemann, H., and J. Schreiter, 1976: Zur Blühintensität und Blütenbiologie bei Dihaploiden von *Solanum tuberosum* L. Biol. Zbl. **95,** 579—588.

Waara, S., L. Pijnacker, M. A. Ferwerda, A. Wallin, and T. Eriksson,

1992: A cytogenetic and phenotypic characterization of somatic hybrid plants obtained after fusion of two different dihaploid clones of potato (*Solanum tuberosum* L.). Theor. Appl. Genet. **85,** 470—479.

Ward, A. C., J. St-J. Phelpstead, A. E. Gleadle, N. W. Blackhall, S. Cooper-Bland, A. Kumar, W. Powell, J. B. Power, and M. R. Davey, 1994: Interspecific somatic hybrids between dihaploid *Solanum tuberosum* L., and the wild species, *S. pinnatisectum* Dun. J. Exp. Bot. **45,** 1433—1440.

Wenzel, G., O. Schieder, T. Przewzny, S. K. Sopory, and G. Melchers, 1979: Comparison of single-cell-culture-derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. **55,** 49—55.

Xu, Y., M. G. K. Jones, A. Karp, and E. Pehu, 1993: Analysis of the mitochondrial DNA of the somatic hybrids of *Solanum brevidens* and *Solanum tuberosum* using non-radioactive digoxigenin-labelled DNA probes. Theor. Appl. Genet. **85,** 1017—1022.