Theor Appl Genet (1992) 85:470–479

A cytogenetic and phenotypic characterization of somatic hybrid plants obtained after fusion of two different dihaploid clones of potato (*Solanum tuberosum* L.)

S. Waara[1], L. Pijnacker[2], M. A. Ferwerda[2], A. Wallin[1], and T. Eriksson[1]

[1] Department of Physiological Botany, University of Uppsala, Box 540, S-751 21 Uppsala, Sweden
[2] Department of Genetics, University of Groningen, P.O. Box 14, NL-9750 AA Haren, The Netherlands

Received February 1, 1992; Accepted April 23, 1992
Communicated by R. Hagemann

Summary. Somatic hybrid plants of various ploidy levels obtained after chemical fusion between two dihaploid clones of potato *Solanum tuberosum* L. have been analysed by cytological, morphological and molecular methods. The hybrid nature of tetraploid and hexaploid plants and the genome dosage in hexaploid hybrids were confirmed by Giemsa C-banding. Tetraploid and hexaploid hybrids showed numerical as well as structural chromosome mutations. The latter occurred mainly in the nuclear organizing chromosome. The tetraploid hybrids were more vigorous than the dihaploid parents as demonstrated by an increase in height, enlargement of leaves, increase in the number of internodes, restored potential for flowering and increased tuber yield. The grouping of tetraploid somatic hybrids into various classes on the basis of leaf morphology revealed that plants with a full chromosome complement were more uniform than aneuploids. Many hexaploid somatic hybrids were also more vigorous than the dihaploid parents and could be grouped into two different classes on the basis of floral colour and tuber characteristics, the differences being due to their different dosage of parental genomes. Most of the tetraploid somatic hybrids showed pollen development halted at the tetrad stage as one of the parental clones contained a *S. stoloniferum* cytoplasm. However, one tetraploid plant produced pollen grains with high viability. The chloroplast genome in the hybrid plants was determined by RFLP analysis. All of the hybrids had a cpDNA pattern identical to one parent, which contained either *S. tuberosum* or *S. stoloniferum* cpDNA. A slight preference for *S. tuberosum* plastids were observed in hybrid plants. No correlation between pollen development and plastid type could be detected.

Key words: *Solanum tuberosum* – Somatic hybrids – Cytology – Morphology – cpDNA

Correspondence to: S. Waara

Introduction

The potential use of inter- and intraspecific somatic hybrids of potato in future potato breeding programmes has been widely recognized (Wenzel et al. 1979; Ross 1986; Karp et al. 1988). Characters such as various disease resistance genes have been incorporated by somatic hybridization with wild species such as *S. berthaultii* (Serraf et al. 1991), *S. brevidens* (Barsby et al. 1984; Austin et al. 1985b; Austin et al. 1986; Fish et al. 1987; Fish et al. 1988a), *S. chacoense* (Butenko and Kuchko 1980) and *S. circaeifolium* (Mattheij et al. 1992). Although such approaches will extend the gene pool of potato several backcrossing generations and selections are necessary to obtain a high yielding cultivar with retained disease resistance genes. A more direct approach would be to fuse dihaploid genotypes of diverse genetic origin; the resulting tetraploids could represent a final cultivar or at least an advanced breeding line (Wenzel et al. 1979). Fusions between dihaploid potato and the sexually compatible diploid species *S. phureja* could serve a similar purpose (Puite et al. 1986, 1988; Mattheij and Puite 1992).

A detailed characterization of a large number of somatic hybrids of potato has so far only been carried out on interspecific hybrids (Austin et al. 1985b; Austin et al. 1986; Fish et al. 1988b; Pehu et al. 1989; Serraf et al. 1991). The hybrid plants examined showed an extensive phenotypic variability, which can to some

extent be explained by cytological changes such as the gain of whole chromosome sets (Fish et al. 1988b; Pehu et al. 1989; Serraf et al. 1991) or the loss or gain of a few chromosomes (Austin et al. 1986; Pehu et al. 1989). Other cytological changes such as structural rearrangements, changes in gene copy number and point mutations that frequently occur after protoplast culture and protoplast fusion (Pijnacker and SreeRamulu 1990) might also lead to alteration of the phenotype. Furthermore, protoplast fusion also allows parental organelles to be combined resulting in a number of nuclear and cytoplasmic combinations (Gleba and Sytnik 1984).

In our laboratory somatic hybrid plants were obtained after the fusion of two different dihaploid clones of potato (Waara et al. 1991), which involved the manual selection of hybrid cells between norflurazon-bleached, fluorescein diacetate-stained protoplasts and mesophyll protoplasts. In this paper we present a characterization of these somatic hybrid plants using morphological and cytological methods including Giemsa C-banding. The segregation of plastid type and the influence of the bleaching with norflurazon on the assortment of chloroplasts was possible due to the presence of a *tuberosum* or a *stoloniferum* cytoplasm in the respective parents. The presence of a *stoloniferum* cytoplasm in a potato nuclear background usually results in male sterility, and pollen development is halted at the tetrad stage (Ross 1986). It is also possible that an incompatibility between the nucleus and chondriome is responsible for the reduced fertilty, which is the case in many other cytoplasmatic male-sterility systems (Perl et al. 1990). Consequently, pollen development in the somatic hybrid was also evaluated.

Materials and methods

Plant material

The somatic fusion products of dihaploid *S. tuberosum* (198:21) and dihaploid *S. tuberosum* (67:9) were produced by polyethylene glycol-mediated fusion (Waara et al. 1991). Fusion products were identified by the dual fluorescence emission from chloroplasts in mesophyll protoplasts (red) and by fluorescein diacetate stain in light- and norflurazon-bleached protoplasts (yellow-green). Hybrid cells were manually isolated 2–3 days after fusion and cultured separately. Their hybrid nature was confirmed by isozyme analysis (Waara et al. 1991). The fusion products with bleached protoplasts of 67:9 were numbered 16 and those with bleached protoplasts of 198:21 were numbered 17. Plants derived from different calli were assigned an additional number (e.g. 16:1), and plants regenerated from the same callus were assigned a subsequent letter (e.g. 16:1a). Regenerated plants were maintained as shoot cultures on shoot propagation medium (Carlberg et al. 1983). The dihaploid clone 198:21 was derived from anther culture (Johansson 1986) of cv 'Maria'; which contains a *S. tuberosum* cytoplasm, and 67:9 from the breeding line Y 67-20-40, which contains a *S. stoloniferum* cytoplasm.

Cytological analysis

Ploidy level was determined in previously unanalysed somatic hybrids by flow cytometry as described by Waara et al. (1989). However, nuclei isolated from the dihaploid clone 67:9 and the tetraploid cv 'Maria' were used as internal standards. Karyotyping was carried out on Giemsa C-banded chromosomes from root tips of at least 3 young greenhouse-grown plants from the first-generation tubers as described by Pijnacker and Ferwerda (1984) and Puite et al. (1986).

Morphological analysis

To examine the phenotype of tetraploid plants derived from the same or different calli, 23 tetraploid somatic hybrids derived from six different calli were compared with the dihaploid parents. In another experiment 6 of the tetraploid somatic hybrids, 19 hexaploid somatic hybrids and 4 octoploid somatic hybrids and the dihaploid parents were examined. From each genotype 3 in vitro-grown plants were established in Vefi pots and transferred after 2 (first experiment) or 3 weeks (second experiment) to 5-l pots. The plants were grown in Rölunda soil (Bålsta, Sweden) under a 16-h daylength supplemented with artificial light of 20 000 lux if the natural light was less than 25 000 lux. The minimum day and night temperatures were maintained at 18 °C and 14 °C, respectively. At the vegetative stage (5–10 weeks after planting) plant height, leaf shape, growth habit, foliage colour, glossiness and anthocyanin pigmentation were recorded; plant height assessment was repeated at flowering. In addition, data on the number of days to flowering as well as on flower morphology were recorded.

Pollen viability was determined by fluorescein diacetate staining as described for *Brassica* by Sundberg et al. (1987) but with 17.5% sucrose (I.-L. Kristiansdottir personal communication). Observations were made on at least three different flowers of each genotype.

Tubers were harvested after 19–20 weeks of culture. The number of tubers obtained per plant, the yield of tubers per plant and average tuber weight were determined. Statistical analysis was carried out by Student's *t*-test.

Tubers derived from the above experiments was also planted as described above to evaluate the stability of the somatic hybrid phenotype. Plant habit, leaf morphology, flowering ability, flower morphology and pollen fertility were recorded.

RFLP analysis of chloroplast DNA

Chloroplast DNA (cpDNA) was extracted from greenhouse-grown leaf material (8 g) from tuber-derived plants according to the method described by Hosaka and Hanneman (1987). The cpDNA was restricted by *Bam*HI and *Kpn*I according to the suppliers' instructions. The DNA fragments were separated by agarose gel (0.8%) electrophoresis in TAE buffer (Maniatis et al. 1982), visualized by staining in ethidium bromide and photographed on a UV transilluminator.

Results

Cytological analysis

The ploidy level was first determined by flow cytometry in 27 shoots derived from the same six calli that had previously given rise to tetraploid shoots (see Waara et al. 1991). The latter shoots have also been included in this study (33 different shoots examined).

Three of the calli (16:44, 16:45, 16:63) gave rise to tetraploid shoots only. Calli 16:43, 16:66 and 17:61 produced 6, 5 and 3 shoots, respectively, of which 5, 3 and 2 shoots, respectively, were polyploid (5x, 6x) or mixoploid (4x/5x, 5x/8x, 6x/12x). Only the tetraploid plants were karyotyped. Twenty-one shoots, originating from different calli, were hexaploid according to flow cytometric measurements (see Waara et al. 1991); 8 of these were determined to contain four (haploid) genomes of 67:9 and two genomes of 198:21 based upon isozyme dosage (Waara et al. 1991), and 11 shoots contained two genomes of 67:9 and four genomes of 198:21, gave roots and were karyotyped. The octoploid plants were not karypotyped.

The chromosome number of one of the fusion partners, i.e. 198:21, appeared to be 25 instead of the expected 24 (Fig. 1a, b). The additional chromosome was one of the smaller ones and could not be identified. Heteromorphism occurred extensively in both parental karyotypes. The differences between the 4 longest chromosomes were used to establish somatic hybridity and the number of parental complements involved. The main distinguishing features were differences in the C-banding pattern(s) on the short arm of chromosome 1 and 2 (nuclear organizer region + distal satellite) and on the long arm of chromosome 3 (Fig. 1a, b). It is worth mentioning that chromosome 2 of 198:21, which had the longest satellited arm, was not found in cv 'Maria'. In the latter only chromosome 2 with less (half) the amount of C-banded chromatin could be detected.

The somatic hybrid nature of all the tetraploid and hexaploid plants could be established by karyotyping the metaphases in the roots of the progeny (Fig. 1c, d). The genome composition of the hexaploids, as established by isozyme analysis (see above), could be confirmed by karyotyping. The tetraploids thus ought to have 49 chromosomes and the hexaploids either 73 chromosomes ($2 \times 24 + 1 \times 25$) or 74 chromosomes ($1 \times 24 + 2 \times 25$). Tables 1 and 2 show that numerical

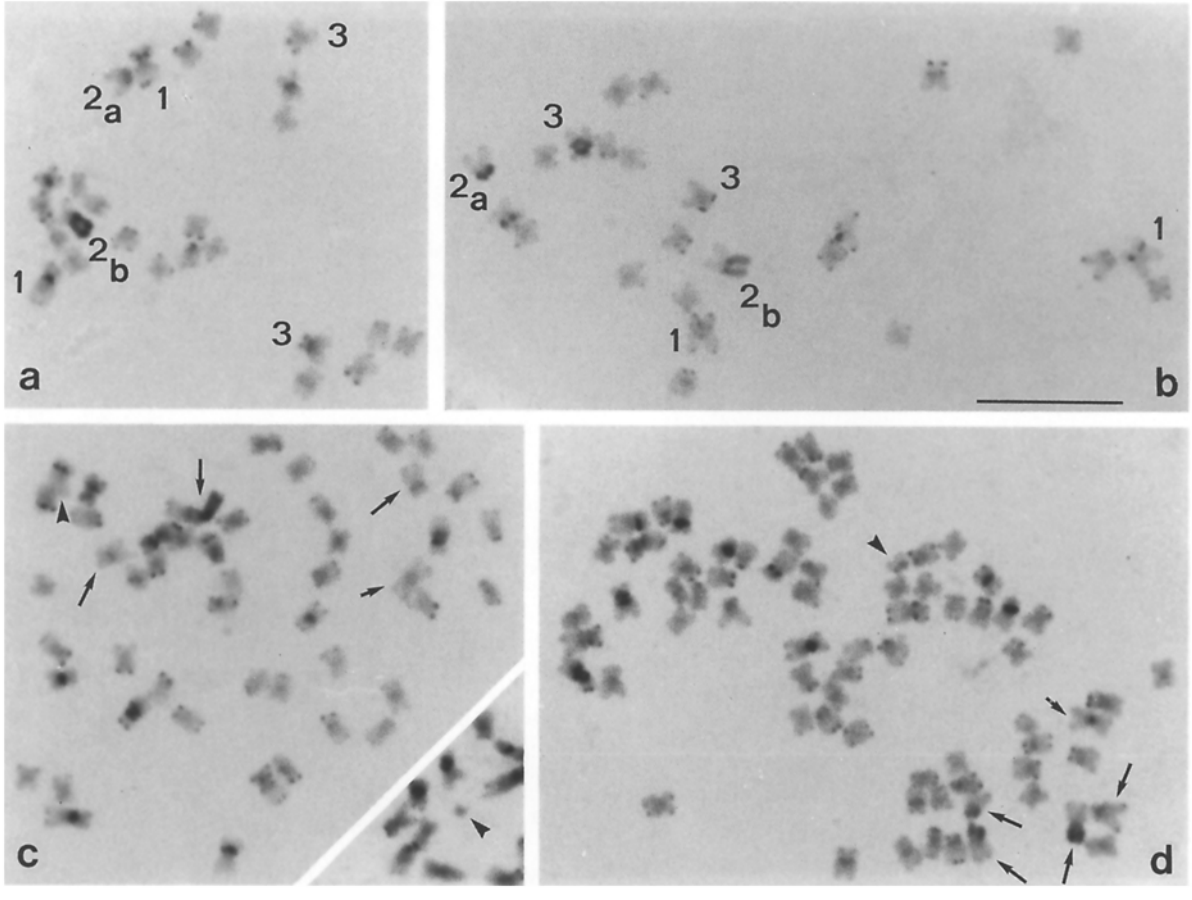

Fig. 1a–d. C-banded metaphases of potato. **a** Dihaploid 198:21 (2x = 25) and **b** dihaploid 67:9 (2x = 24) with chromosomes 1–3 numbered. **c** Somatic hybrid 16:63d (4x = 49): *arrowhead* minute chromosome (distinct in *inset*), *short arrow* isochromosome, *arrows from left to right* chromosome 2b 198:21 with deleted satellite, 2b 67:9 with longer satellite (translocation with 2b 198:21 ?), 2a 198:21, 2a 67:9 missing. **d** Somatic hybrid 16:65b (6x = 73): *arrowhead* deleted chromosome, *short arrow* isochromosome, *arrows from left to right* chromosome 2a 67:9, 2b 67:9, 2b 198:21, 2a 198:21, 2a and 2b 67:9 missing *Bar*: 10 μm

Table 1. Genetical constitution of tetraploid somatic hybrids and the leaf morphology class the plants could be grouped into. The different leaf morphology classes are shown in Fig. 4

Genotype	Chromosome number	Chromosome no. 2		Pollen development[c]	cpDNA[d]	Leaf morphology[e]
		Missing[b]	Mutated			
16:43a	46		b^{I}	p.t.	n.d.	1
16:44a	48		$b^{I}a^{II}$	p.t.	sto	2
16:44b	48		$b^{I}a^{II}$	p.t.	sto	1ab
16:44c	48		$b^{I}a^{II}$	p.t.	tub	2
16:44d	48		$b^{I}a^{II}$	p.t.	tub	1
16:44e	48		$b^{I}a^{II}$	p.t.	tub	1
16:44f	48		$b^{I}a^{II}$	p.t.	tub	3
16:44h	48/49[a]		$b^{I}a^{II}$	p.t.	sto	1
16:44i	48		$b^{I}a^{II}$	p.g.	tub	1
16:45a	49		b^{I}	p.t.	tub	1
16:45b	48			n.f.	n.d.	4
16:45d	50		$b^{I}b^{II}$	p.t.	tub	1
16:45e	48/49		b^{I}	p.t.	tub	1
16:45f	50		b^{I}	p.t.	tub	1
16:63a	48		$b^{I}b^{II}$	p.t.	sto	1
16:63b	48			p.t.	n.d.	5
16:63c	48			p.t.	sto	1
16:63d	49	a^{II}	$b^{I}b^{II}$	p.t.	sto	1
16:63e	48	n.d.		n.f.	n.d.	6
16:63f	49			p.t.	sto	1
16:66a	49		b^{I}	p.t.	tub	1
16:66b	49		b^{I}	p.t.	tub	1
17:61a	49		b^{I}	p.g.	tub	1

[a] The plants are aneusomatic containing two different chromosome numbers within the same roots
[b] Cytological changes of the satellited part of chromosome 2. The letters a and b represent the two morphological different chromosomes. Suffix I is assigned to 198:21, and suffix II to those coming from dihaploid parent 67:9
[c] p.t., Pollen tetrads; p.g., pollen grains; n.f., nonflowering; f.b., floral buds are produced but fall off prematurely
[d] cpDNA; designs from whose cytoplasm the chloroplast genome was derived: sto, *stoloniferum*; tub, *tuberosum*; n.d., not determined
[e] The leaf morphology was grouped into six different classes as shown in Fig. 4

chromosome mutations had taken place in most of the hybrids. Moreover, the progeny of 6 somatic hybrids showed two chromosome numbers (aneusomaty), and examination of the roots of the hexaploid 16:26 indicated plants with either 73 or 74 chromosomes. Both karyotypes of the aneusomatic plants had similar structural rearrangements. The loss of a chromosome could be ascribed to chromosome 2 in several plants, and in the hexaploids it occurred mainly amongst those chromosomes 2 that were identical, i.e. present in twofold. In the tetraploid 16:43a 1 chromosome 1 of the parent 67:9 was lost. The gain of chromosomes (for instances in 16:45f) must also have occurred among chromosome 5–12. Structural rearrangements, i.e. deletions, duplications or translocations, were observed in the short arm of chromosomes 2, in particular in the longest ones ($b^{1}b^{11}$; Tables 1 and 2, Fig. 1e). The tetraploids showed a higher frequency of mutated chromosomes 2 than the hexaploids. Other karyological changes was also observed. For example, the complements of 16:63d, 16:40 and 17:11 had 1 extremely small dot-like chromosome, 16:65 and 17:23 had 1 small chromosome apparently missing one arm and 16:63d and 16:65 had 1 odd metacentric chromosome that might be an isochromosome (Fig. 1c, d).

In conclusion, only 1 tetraploid plant (16:63f) and 2 hexaploid plants (16:7 and 17:77) had not undergone visible chromosomal mutations. The tetraploids originating from callus 16:44 and from callus 16:66 had similar karyotypes. Callus 16:45 and callus 16:63 gave rise to chromosomally different regenerants (Table 1).

Plant morphology

The morphology of dihaploid parents and tetraploids with a full chromosome complement (i.e. 2n = 49) is summarized in Table 3.

Plants of the dihaploid clone 67:9 were erect and fairly tall. Generally only one or a few stems were formed from each planted shoot (Fig. 2). The leaves

Table 2. Genetical constitution of hexaploid somatic hybrids. The plants have been grouped into two classes on the basis of genome dosage from each parent. The first class consists of hexaploids containing four haploid genomes of 67:9 and two genomes of 198:21; the second class consists of hexaploids containing four genomes of 198:21 and two genomes of 67:9

Genotype	Chromosome number	Chromosome no. 2		Pollen development	cpDNA
		Missing	Mutated		
16:7	73			p.t.	sto
16:32	74			p.t.	tub
16:38	72			p.t.	tub
16:40	70	$b^{I}a^{II}$		p.t.	tub
16:46	72			p.t.	sto
16:51	70	$a^{II}b^{II}$	b^{I}	n.f.	n.d.
16:65	72/73	$a^{II}b^{II}$		p.t.	tub
17:11	72/73	$a^{II}a^{II}$	b^{I}	p.g.	sto
17:1	71			f.b.	sto
17:14	75			p.g.	tub
17:23	74			f.b.	tub
17:43	73/74	b^{I}		p.g.	n.d.
17:69	72			p.g.	tub
17:76	72/73			p.g	sto
17:77	74		b^{I}	p.g.	n.d.
17:83	74		$b^{I}b^{I}b^{II}$	p.g.	sto
17:90	73	b^{I}	$b^{I}b^{II}$	p.g.	tub
16:26	73/74			p.g.	tub
16:71	73		b^{I}	p.t.	tub

For explanation of symbols, see Table 1

were fairly dark green and had a rough surface, and anthocyanin pigmentation was observed on the abaxial side of the leaves and on the stems. At tuber harvest the plants were still fairly green with only an indication of yellowing. Potato tubers with a yellow-white flesh were produced from all plants, but the yield was fairly low. The dihaploid clone 198:21 produced several stems and had a bushy appearance (Fig. 2). The leaves were fairly light green, the leaf surface was smooth, and no anthocyanin pigmentation was observed on the plants. At tuber harvest the plants had turned completely yellow, and this dihaploid clone gave both a higher yield and a larger number of potatoes than the dihaploid clone 67:9 (Table 3). However, the average tuber weight of 67:9 was higher than that of 198:21.

The tetraploid somatic hybrids with a full chromosome complement were taller and grew more vigorously than both dihaploid parents (Table 3; Fig. 2). They also often produced more internodes and the leaves were larger. The secondary leaflets were broader in the somatic hybrids than in the dihaploid parent (Table 3). The foliage colour was moderately green and the leaves had a rough surface. The stems were often anthocyanin pigmented. Tuber yield was also higher in these somatic hybrids than in the dihaploid parents. The tubers were round as the potatoes of dihaploid parent 67:9 but bigger in size than those of the respective parents (Table 3; Fig. 3).

The morphology of the tetraploid plants with variant odd numbers of chromosomes (46, 48 or 50) was also examined. In general, they had the same plant appearance as the somatic hybrids with a full chromosome complement although they had a more variable leaf phenotype. In Table 1 the tetraploid somatic hybrids have been grouped into different classes according to their leaf morphology (Fig. 4). For example, plants which have lost 1 chromosome (i.e. contains 48 chromosomes) can be grouped into six different classes while those with a full chromosome complement fall into the same class (Table 1). While statistical analysis of different morphological characters could not separate the $2n=48$ or $2n=49$ somatic hybrids into different groups, the former group showed more variability (i.e. higher standard deviation) than the latter group (data not shown).

Morphological analysis was also carried out to determine the possibility of identifying somatic hybrids of different ploidy level or hexaploids with different genome dosage on the basis of the expressed phenotype. Many of the hexaploid plants resembled the tetraploid somatic hybrids in plant appearance, and grew as tall as the tetraploid hybrids. However, the hexaploids could generally be separated from the tetraploids by their more compressed leaf morphology, poor development of the second and third secondary leaflets (see also Waara et al. 1991) and by the broader first secondary leaflets, all of which resulted in a lower

Table 3. Morphological features of the dihaploid parents and tetraploid somatic hybrids (2n = 4x = 49)

Character	Dihaploid parent 67:9	Somatic hybrids	Dihaploid parent 198:21
Plant habit	Erect, a few stems	Moderately bushy	Bushy
Leaf colour	Dark green	Intermediate	Light green
Leaf surface	Rough	Rough	Smooth
Leaf index (see Table 4)	1.6	1.3	1.5
Anthocyanin pigmentation	Weak	Weak	No
Maximum height (cm)	76 ± 6^a	108 ± 11^b	80 ± 4^a
Number of internodes	19 ± 1^a	23 ± 2^b	19 ± 0^a
Plant maturity	Late	Late	Moderately early
Tuber shape	Round	Round	Oval
Tuber colour	Yellow-white	Yellow-white	Yellow-white
Tuber weight (g)	9.5 ± 2.6^a	13.6 ± 2.1^b	6.0 ± 1.3^c
Tuber yield (g/plant)	67 ± 35^a	284 ± 64^b	201 ± 19^c

Different letters indicate significant differences at $P < 0.05$ calculated with Student's *t*-test

leaf index than that of the tetraploids (Table 4). The top leaf was also more rounded in the hexaploids and the leaf edges often rolled. A few plants were also variegated. All of the hexaploid plants produced tubers with yellow-white flesh.

As described in the cytological analysis above the hexaploid plants could be grouped into two classes based upon the genome dosage from each parent. A comparison of most morphological features could not separate these two groups (Table 4, and data not shown). However, they could be separated by their different tuber weight, i.e. total yield was similar but one combination yielded many, small tubers (four genomes of 198:21, two genomes of 67:9) while the other combination of (two genomes 198:21 and two genome 67:9) yided fewer but larger tubers (Table 4). The floral colour of these groups was also different (see below). Both the tetraploid and the hexaploid somatic hybrids remained green even after 19–20 weeks of culture, and when the tubers were harvested many long stolons grew along the inner surface of the pots.

Fig. 2. Plant morphology of dihaploid parent 67:9 (*left*) dihaploid parent 198:21 (*middle*) and somatic hybrid 17:61a (*right*)

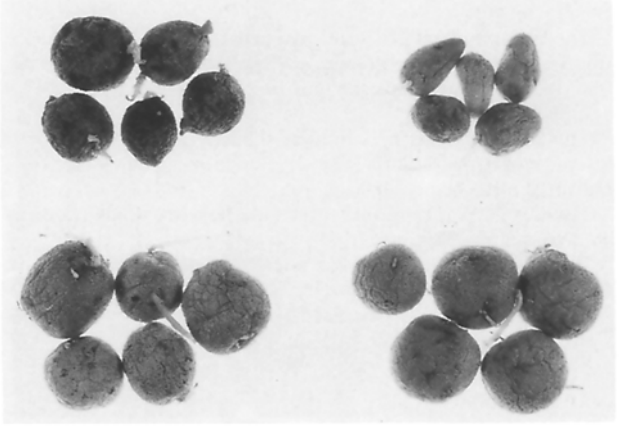

Fig. 3. Tuber morphology of dihaploid clone 67:9 (*top left*), dihaploid clone 198:21 (*top right*) and two tetraploid hybrids, 16:63d (*bottom left*) and 16:66b (*bottom right*)

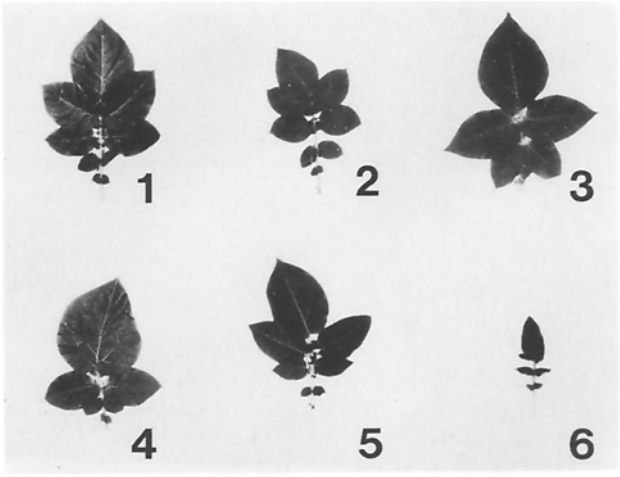

Fig. 4. Variation in leaf morphology in aneuploid tetraploid somatic hybrids (2n = 48). *1* 16:63a, *2* 16:44a, *3* 16:44f, *4* 16:45b, *5* 16:63b, *6* 16:63e

Table 4. A morphological comparision between somatic hybrid plants of different ploidy level and the dihaploid parents

Ploidy class	*Leaf index*[b]	*Plant height*[d]
2x: 67:9	1.647 ± 0.034^{a}	48.3 ± 4.0^{a}
2x: 198:21	1.698 ± 0.011^{a}	33.7 ± 5.0^{b}
4x	1.372 ± 0.087^{b}	48.2 ± 6.9^{a}
6x/a[a]	1.176 ± 0.086^{c}	46.7 ± 5.0^{a}
6x/b[a]	1.127 ± 0.109^{c}	45.0 ± 9.0^{a}
8x	No dissected leaves	33.3 ± 7.5^{b}
	Tuber yield (g/plant)[c]	*Tuber weight (g/tuber)*[e]
2x: 67:9	102 ± 33^{a}	4.7 ± 1.7^{a}
2x: 198:21	240 ± 60^{b}	1.8 ± 3.3^{b}
4x	284 ± 51^{b}	$10.1 \pm 3.2^{c,d}$
6x/a[a]	265 ± 79^{b}	12.1 ± 3.2^{c}
6x/b[a]	308 ± 51^{b}	7.0 ± 2.0^{d}
8x	112.1 ± 68.33^{a}	$6.1 \pm 4.8^{a,b}$

Different letters indicate differences at $P < 0.05$ calculated with Student's *t*-test

[a] The hexaploid plants were grouped into two classes based upon parental genome dosage, a = four (haploid) 67:9 genomes and two 198:21 genomes; b = four 198:21 genomes and two 67:9 genomes

[b] The leaf index was measured as the length/width of the first lateral leaflet on the 5th fully developed leaf after 9 weeks of culture

[c] Tuber yield/plant was measured after harvest, which occurred 19 weeks after planting

[d] Plant height was measured after 9 weeks of culture when floral buds were visible

[e] The tuber weight was assessed after harvest

The octoploid somatic hybrids were all abnormal. The plants were stunted, the leaves were dark green, thick, often variegated and the lateral leaflets were poorly developed. The tuber yield from these plants was also low (Table 4).

Flower morphology and male fertility

The dihaploid parents produced a few floral buds, but most of them fell off prematurely. Fully developed flowers of the dihaploid clone 198:21 were white and contained small, dry, yellowish-white stamens. The anthers contained pollen grains, although only 1% of these were viable. Flowers of the dihaploid clone 67:9 never developed completely, the petals were rudimentary and anthocyanin pigmented, and the small, dry, yellowish-white stamens contained pollen tetrades.

The tetraploid plants with a full chromosome complement (2n = 49) flowered profusely over a period of several weeks. The flowers were white with pink stripes, although the intensity of the stripes could vary. The pistil was always protruding outside the flower bud prior to blossoming, and the anthers were generally thick and yellow-orange. The number of sepals, petals and stamens could differ in individual flowers on the same plants. For example, flowers containing six sepals and six stamens were frequently observed. Pollen development was generally halted in the tetrad stage. One hybrid was fertile and produced pollen grains of which usually 20% was viable. Flower morphology was similar in male-sterile and male-fertile plants. Female fertility appeared to be high as several male-sterile plants set berries without controlled pollination. The floral morphology was similar in the aneuploid tetraploid plants (2n = 46, 48 and 50). They generally flowered profusely and were with one exception male sterile (Table 1).

Of the 19 hexaploid somatic hybrids 18 produced floral buds; of these 16 formed complete flowers (Table 2). Floral morphology was generally similar to that of the tetraploid hybrids but more abnormal flowers could also be formed. There was a clear correlation between floral colour and genome dosage. Hexaploid plants containing four haploid genomes of the anthocyanin-pigmented dihaploid clone 67:9 and two genomes of the unpigmented parent 198:21 had white flowers with pink stripes, while the reverse combination had white flowers. Seven of the flowering hexaploid hybrids were male sterile and produced only pollen tetrads the remaining 9 hexaploid hybrids produced pollen grains of which fewer than 1% were usually viable (Table 2).

Phenotypic stability

The first generation of tuber-derived plants had in general a similar phenotype, flowering ability and pollen fertility as the plants derived from in vitro-grown stocks. However, the tuber-derived plants were less branched.

Chloroplast analysis

The *S. stoloniferum* cpDNA in dihaploid clone 67:9 could be differentiated from the *S. tuberosum* cpDNA in dihaploid clone 198:21 by the RFLP patterns produced by *Bam*HI and *Kpn*I (Hosaka et al. 1984). The *Bam*HI restriction fragment pattern was used to identify the cytoplasm in hybrid plants. Digestion with *Bam*HI showed the presence of a 12-kb band in *S. tuberosum* cpDNA. This band was absent from *S. stoloniferum* cpDNA and had been replaced with two bands, one of 10 kb and one of 2 kb. All of the hybrids had a cpDNA restriction pattern identical to either one of the parental clones. Thirteen of the 19 analysed somatic hybrids and 10 of the 16 analysed hexaploid somatic hybrids contained *S. tuberosum* plastids (Table 1 and 2). Plants arising from the same callus could contain different chloroplast types (Table 1). There was no detectable correlation between pollen development and plastid type (Tables 1 and 2).

Discussion

One of the anther-derived dihaploid parents (198:21) appeared to be aneuploid, with 25 chromosomes, and had a structurally rearranged chromosome 2. Whether this complement arose during meiosis in cv 'Maria' or later during anther culture is not known. Consequently, hypertetraploid and hyperhexaploid somatic hybrids were obtained. A cytological analysis of plants derived from the same six calli revealed that these plants had either four (haploid) genomes or differed in ploidy level (4, 5, 6 genomes), mixoploidy included. The origin of these polyploid shoots, especially when both hexaploid and tetraploid shoots were derived from the same callus, is difficult to explain. They could have originated from genome number changes in the callus phase. Another explanation is that they actually arose from different cell-derived calli. Potato protoplasts do aggregate easily and although extensive care was taken not to pick agglutinated fusion products and to culture them at low densities (Waara et al. 1991), the aggregation of growing cell colonies with different ploidies can not be excluded.

Among the tetraploid plants only 1 plant (i.e. 16:63f) did not have a mutated karyotype. Generally most plants either lost or gained only 1 chromosome and not more. The plants derived from the same callus could have a similar karyotype with, as in 16:44, 1 numerical and 2 structural chromosome mutations. This similarity indicates that the mutational events took place almost simultaneously during early callus formation (or in the part of the callus the shoots were derived from). The mutations in calli from which karyotypically different shoots were grown, apparently occurred at different times. The hexaploids could also undergo similar mutations. The occurrence of genome and chromosome mutations during the callus phase of all types of plants, including somatic hybrids, is a well-known fact (Gleba and Sytnik 1984; Evans and Sharp 1986; Pijnacker and SreeRamulu 1990).

The origin and manner of maintenance of minute chromosomes such as those observed in the tetraploid 16:63d and the hexaploids 16:40 and 17:11 remains an enigma. Minute chromosomes also occurred in somatic hybrids of *S. tuberosum* + *S. phureja* (Pijnacker et al. 1989).

The loss of chromosome(s) 2 and structural rearrangements in the nucleolar organizer bearing the short arm of chromosome(s) 2 occurred in the tetraploid and hexaploid hybrids. Similar mutations have been observed in interspecific somatic hybrids of *S. tuberosum* + *S. phureja* (Pijnacker et al. 1987; Pijnacker et al. 1989) and in a *Petunia* somatic hybrid (White and Ross 1983). The elimination of *phureja* nucleolar chromosomes was preferential and under genetic control, and rearrangements were likely due to nuclear organizer (rDNA) activity. In the present material preferential elimination could not be established. Rearrangements were observed in the tetraploids more than in the hexaploids and particularly in nucleolar chromosomes with the longest C-banded positive arms and possibly the highest amount of ribosomal DNA. These facts also point to a positive influence of nuclear organizer activity on the induction of rearrangements in the short arm.

The hybrid nature of the somatic hybrids studied was established previously by isozyme analysis (Waara et al. 1991). The hybridity was also confirmed by karyotyping. The karyotyping also confirmed the IDH-1 isozyme dosage of the hexaploid hybrids (Waara et al. 1991), supporting the initial observation that the hexaploid hybrids generally consisted of four haploid genomes of the bleached parent and two of the non-bleached.

Tetraploid plants with a full chromosome complement were clearly more vigorous than the dihaploid parents, confirming conclusions drawn from previous studies that hybrid vigour is expressed in various combinations of dihaploid-dihaploid fusions (Austin et al. 1985; Debnath and Wenzel 1987; Deimling et al. 1988; Chaput et al. 1990; Möllers and Wenzel 1992). Furthermore, Mattheij and Puite (1992) also recently obtained extremely high yielding hybrids after the fusion of dihaploid potato with different *S. phureja* genotypes.

Some characters expressed by the tetraploid somatic hybrids such as the anthocyanin pigmentation, late plant maturity and round tubers (67:9), were correlated with only one of the parents. Anthocyanin-pigmentated somatic hybrids have also been obtained between anthocyanin-pigmented *S. brevidens* and

unpigmented *S. tuberosum* (Fish et al. 1988a). The genetic regulation of plant maturity is only poorly understood. However, crosses between early and early genotypes gave 64% early seedlings, while crosses between early and late genotypes only yielded 18% early seedlings (Howard 1970). Thus, more attention should be paid to the maturity time in future fusion programmes. Somatic hybrids with round tubers were also obtained after the fusion of dihaploid genotypes bearing round tubers with genotypes bearing oval tubers, indicating that round is dominant over oval (Möllers and Wenzel 1992).

The tetraploid somatic hybrids that had lost 1–3 chromosomes were clearly more morphological variable than hybrids with a full complement. Among these plants karyotype variation existed but could not be correlated with a specific phenotype. They also appeared with one exception to contain all 4 chromosomes of the 4 largest chromosomes. It is therefore possible that the morphological variability can be due to the loss of different small chromosomes although epigenetic and/or minor genome rearrangements and gene mutations may also be involved. In one hybrid containing 46 chromosomes, 1 chromosome 1 of the parent 67:9 was lost. According to the linkage map established for potato (Bonierbale et al. 1988) the IDH-1 gene is located on chromosome 1. The IDH-1 isozyme dosage of this tetraploid hybrid confirms the cytological data.

The phenotypes of the plants in the two different hexaploid groups were in general similar, although dosage effects on floral colour and tuber characters were recorded. It is possible that the cytological changes that had occurred in most hexaploid plants influenced the phenotype and therefore obscured other genome dosage effects. Genome dosage effects on tuberization characters have also been observed in hexaploid interspecific somatic hybrids between dihaploid potato and the diploid non-tuberizing species *S. brevidens* (Pehu et al. 1989) and between dihaploid potato and the tuberizing species *S. brethaultii* (Serraf et al. 1991).

We have previously observed that mixoploid shoots can be identified during in vitro shoot culture by their very abnormal and weak morphology (Waara et al. 1991). In the study presented here it also became clear that all of the octoploid and many of the hexaploid plants were abnormal, variegated and less vigorous than the tetraploid hybrids. It therefore appears to be rather easy to identify hybrid plants of different ploidy levels in the greenhouse.

The restriction fragment pattern of cpDNA showed that either plastid type could be found in hybrid plants. No effect on the assortment of chloroplasts by norfluorazon bleaching of parental plants was detected, but a slight preference for *S. tuberosum* cpDNA was indicated. Plants from the same callus contain different plastids as described in other fusion combinations (Chen et al. 1977; Glimelius et al. 1981; Menczel et al. 1981).

Both male-sterile and male-fertile plants were produced. Pollen development was not correlated with plastid type, and mtDNA analysis will have to be performed in order to evaluate the mitochondrial constitution in male-fertile and male-sterile plants. Future studies may also reveal if the presence of different chloroplast genomes influences photosynthetic efficiency, maturity and tuber yield.

In conclusion, in the study presented here a low yielding, late-maturing dihaploid clone (67:9) was fused with a moderately early, high yielding dihaploid clone (198:21) resulting in late-maturing somatic hybrids that give a fairly high yield. The production of long stolons in combination with the late maturity makes this particular fusion combination unsuitable for Swedish cultivation. Clearly, information is lacking on the outcome of any one specific fusion experiment since many characters are encoded by polygenes and the interactive forces of these genes are not known. However, general hybrid vigour and the uniformity of tetraploid plants with the full chromosome complement in spite of cytological changes is encouraging for their future use in dihaploid–dihaploid fusion programmes. By the fusion of high yielding, early maturing and cold-adapted dihaploid genotypes somatic hybrids of value also in the Scandinavian countries might be obtained.

Acknowledgements. We thank Mr. B. Blohm, Department of Plant Breeding, Swedish University of Agricultural Sciences, Uppsala for his valuable suggestions on the cultivation of potato plants. The skillful technical assistance of Ms. A. Ottosson, the processing of the photographs of the karyotypes by Mr. H. Mulder and the advice on cpDNA analysis by Dr. E. Pehu, University of Helsinki, Finland is also acknowledged. This work was supported by grants from the Swedish Council for Forestry and Agricultural Research (SJFR).

References

Austin S, Baer BA, Ehlenfeldt MK, Kazmierczak PJ, Helgeson JP (1985a) Intra-specific fusion in *Solanum tuberosum*. Theor Appl Genet 71:49–56

Austin S, Baer MA, Helgeson JP (1985b) Transfer of resistance to potato leaf roll virus from *Solanum brevidens* into *Solanum tuberosum* by somatic fusion. Plant Sci 39:75–82

Austin S, Ehlenfeldt MK, Baer MA, Helgeson JP (1986) Somatic hybrids produced by protoplast fusion between *S. tuberosum* and *S. brevidens*; phenotypic variation under field conditions. Theor Appl Genet 71:682–690

Barsby TL, Shepard JF, Kemble RJ, Wong R (1984) Somatic hybridization in the genus *Solanum: S. tuberosum* and *S. brevidens*. Plant Cell Rep 3:165–167

Bonierbale MW, Plaisted RL, Tanksley SD (1988) RFLP maps based upon a common set of clones reveals modes of

chromosomal evolution in potato and tomato. Genetics 120:1095–1103

Butenko RG, Kuchko AA (1980) Somatic hybridization of *Solanum tuberosum* L. and *Solanum chacoense* Bitt. by protoplast fusion. In: Ferenczy L, Farkas GL (eds) Advances in protoplast research. Akad Kiado Budapest, pp 293–300

Carlberg I, Glimelius K, Eriksson T (1983) Improved culture ability of potato protoplasts by use of activated charcoal. Plant Cell Rep 2:223–225

Chaput MH, Sihachakr D, Ducreux G, Marie D, Barghi N (1990) Somatic hybrid plants produced by electrofusion between dihaploid potatoes: BF15 (H1), Aminca (H6) and Cardinal (H3). Plant Cell Rep 9:411–414

Chen K, Wildman SG, Smith HH (1977) Chloroplast DNA distribution in parasexual hybrids as shown by polypeptide composition of Fraction I protein. Proc Natl Acad Sci USA 74:5109–5112

Debnath SC, Wenzel G (1987) Selection of somatic fusion products in potato by hybrid vigour. Potato Res 30:371–380

Deimling S, Zitzlsperger J, Wenzel G (1988) Somatic fusion for breeding of tetraploid potatoes. Plant Breed 101:181–189

Evans DA, Sharp WR (1986) Applications of somaclonal variation. Biotechnology 4:528–533

Fish N, Karp A, Jones MGK (1987) Improved isolation of dihaploid *S. tuberosum* protoplasts and the production of somatic hybrids dihaploid *S. tuberosum* and *S. brevidens*. In Vitro 23:575–580

Fish N, Karp A, Jones MGK (1988a) Production of somatic hybrids by electrofusion in *Solanum*. Theor Appl Genet 76:260–266

Fish N, Steele SH, Jones MGK (1988b) Field assessment of dihaploid *Solanum tuberosum* and *S. brevidens* somatic hybrids. Theor Appl Genet 76:880–886

Gleba YY, Sytnik KM (1984) Protoplast fusion, genetic engineering in higher plants. Springer, Berlin Heidelberg New York

Glimelius K, Chen K, Bonnett HT (1981) Somatic hybridization in *Nicotiana*: Segregation of organellar traits among hybrid and cybrid plants. Planta 153:504–510

Hosaka K, Hanneman RE Jr (1987) A rapid and simple method for determination of potato chloroplast DNA type. Am Potato J 64:345–343

Hosaka K, Ogihara Y, Matsubayashi M, Tsunewaki K (1984) Phylogenetic relationship between the tuberous *Solanum* species as revealed by restriction endonuclease analysis of chloroplast DNA. Jpn J Genet 59:349–369

Howard HW (1970) Genetics of the potato. Logos Press, London

Johansson L (1986) Increased induction of embryogenesis and regeneration in anther cultures of *Solanum tuberosum* Potato Res 29:179–191

Karp A, Jones MGK, Jones GK, Ooms G, Bright SWJ (1988) Potato protoplasts and tissue culture in crop improvement. In Russell GE (Ed) Biotechnology of higher plants. Anthenaeum Press, Newcastle upon Tyne, pp 1–32

Maniatis T, Fritsch EF, Sambrock J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Mattheij WM, Puite KJ (1992) Tetraploid potato hybrids through protoplast fusions and analysis on their performance in the field. Theor Appl Genet 83:807–812

Mattheij WM, Eijlander R, de Koning JRA, Louwes KM (1992) Interspecific hybridization between the cultivated potato *Solanum tuberosum* suspecies *tuberosum* L. and the wild species *S. circaeifolium* supsp. *circaeifolium* Bitter exhibiting resistance to *Phytophtora infestans* N (Mont.) de Bary and *Globodera pallida* (Stone) Bahrens I. Somatic hybrids. Theor Appl Genet 83:459–466

Menczel L, Nagy F, Kiss ZsR, Maliga P (1981) Streptomycin resistant and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana*: Correlation of resistance to *N. tabacum* plastids. Theor Appl Genet 59:191–195

Möllers C, Wenzel G (1992) Somatic hybridization of dihaploid potato protoplasts as a tool for potato breeding. Acta Bot Neerl (in press)

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

Perl A, Aviv D, Galun E (1990) Protoplast-fusion-derived CMS potato cybrids: Potential seed-parent for hybrid true potato seeds. J Hered 81:434–442

Pijnacker L, Ferwerda MA (1984) Giemsa C-banding of potato chromosomes. Can J Genet Cytol 26:415–419

Pijnacker LP, SreeRamulu K (1990) Somaclonal variation in potato: a karyotypic evaluation. Acta Bot Neerl 39:163–169

Pijnacker LP, Ferwerda MA, Puite KJ, Roest S (1987) Elimination of *Solanum phureja* nucleolar chromosomes in *S. tuberosum* + *S. phureja* somatic hybrids. Theor Appl Genet 73:878–882

Pijnacker LP, Ferwerda MA, Puite KJ, Schaart JG (1989) Chromosome elimination and mutation in tetraploid somatic hybrids of *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 8:82–85

Puite KJ, Roest S, Pijnacker LP (1986) Somatic hybrid potato plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 5:262–265

Puite KJ, Broeke WT, Schaart J (1988) Inhibition of cell-wall synthesis improves flow cytometric sorting of potato heterofusions resulting in hybrid plants. Plant Sci 56:61–68

Ross H (1986) Potato breeding-problems and perspectives. Paul Parey, Berlin

Serraf I, Sihachakr D, Ducreaux G, Brown SC, Allot M, Barghi N, Rossignol L (1991) Interspecific somatic hybridization in potato by protoplast electrofusion. Plant Sci 76:115–126

Sundberg E, Landgren M, Glimelius K (1987) Fertility and chromosome stability in *Brassica napus* resynthesized by protoplast fusion. Theor Appl Genet 75:96–104

Waara S, Tegelström H, Wallin A, Eriksson T (1989) Somatic hybridization between anther-derived dihaploid clones of potato (*Solanum tuberosum* L.) and the identification of hybrid plants by isozyme analysis. Theor Appl Genet 77:49–56

Waara S, Wallin A, Eriksson T (1991) Production and analysis of intraspecific somatic hybrids of potato (*Solanum tuberosum* L.) Plant Sci 75:107–115

Wenzel G, Schieder O, Przwozny T, Sopory SK, Melchers G (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

White JA, Ross H (1983) Cytology of a *Petunia* somatic hybrid. In: Brandham PE, Bennett MD (eds) Key chromosome conference II. Allen and Unwin, London, p 372