Theor Appl Genet (1992) 83:459–466

THEORETICAL AND APPLIED GENETICS

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

1. Somatic hybrids

W.M. Mattheij, R. Eijlander, J.R.A. de Koning, and K.M. Louwes

Center for Plant Breeding and Reproduction Research CPRO, PO Box 16, NL-6700 AA Wageningen, The Netherlands

Received April 15, 1991; Accepted April 30, 1991
Communicated by G. Melchers

Summary. Somatic fusions between the cultivated potato *Solanum tuberosum* and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter were produced in order to incorporate desirable traits into the potato gene pool. Selection of the putative hybrids was based on a difference in callus morphology between the hybrids and their parents, with the hybrids showing typical purple-colored cells in otherwise green calli. In all, 17 individual calli regenerated to plants. Of the nine plants that could be transferred to the greenhouse, eight showed a hybrid and one a parental morphology. Restriction fragment length polymorphism (RFLP) analysis confirmed the hybrid character in the former group. Chloroplast counts in stomatal guard cells and flow cytometric determination of nuclear DNA content showed that four hybrid plants were tetraploid (4x), one was mixoploid (5x–8x), and the others were polyploid (6x; 8x). Three out of four tetraploid hybrids were found to be fully resistant to *Phytophthora infestans*, and all four hybrids were resistant to *Globodera pallida* pathotypes Pa2 and Pa3. It was further observed that the type and amount of steroidal glycoalkaloids varied among the tubers of the parents and the hybrids. Using the hybrids as female parents in crosses with *S. tuberosum*, viable seeds could be obtained. This demonstrates the potential of these hybrids in practical plant breeding.

Key words: *Solanum tuberosum* subsp. *tuberosum* L. – *Solanum circaeifolium* subsp. *circaeifolium* Bitter – Somatic hybridization – Hybrid characterization – Disease resistance

Introduction

Incorporation of disease resistances into potato (*Solanum tuberosum* subsp. *tuberosum* L.) will be very important for the success of future breeding. An important source of valuable traits are wild *Solanum* species (Hermsen 1977). *Solanum circaeifolium* subsp. *circaeifolium* Bitter is a Bolivian wild species with resistance against *Globodera pallida* (Stone) Behrens, *Erwinia carotovora* (Jones and Bergey et al.) and *Phytophthora infestans* (Mont.) de Bary (Hawkes and Hjerting 1989). Up until now, no successful crosses between this species and *S. tuberosum* have been reported, but recently Louwes et al. (1992)) succeeded in crossing this species with diploid potato clones, albeit with a great deal of effort, and obtained diploid and triploid progeny. As an alternative to these rather cumbersome crosses we investigated the possibility of somatic hybridization of this wild species with a diploid potato clone, in order to obtain highly heterozygous tetraploid hybrids for use in crosses with tetraploid potato lines.

One of the bottlenecks in somatic hybridization is the selection of heterofusion products from the bulk of parental protoplasts. Several different techniques for an early selection in fusion experiments with cultivated *S. tuberosum* have been described. These include selection by micromanipulation (Puite et al. 1986; Mattheij and Puite 1992) and cell-sorting (Puite et al. 1988), selection on difference in performance of parental clones and their hybrids in tissue culture (Austin et al. 1985), including hybrid vigor (Debnath and Wenzel 1987; Deimling et al. 1988; Waara et al. 1989), and selection using differ-

ent molecular (Masson et al. 1989) or biochemical (de Vries et al. 1987) parental markers. Each of these techniques has advantages as well as limitations. Isolation of heterokaryons using a micromanipulator or cell-sorter is independent of the species or genotype used, but sophisticated equipment is needed. Molecular and biochemical traits, such as resistances to antibiotics or amino acid analogues, must be incorporated into the clone of interest with an added risk of changing the original genotype. Hybrid vigor is a simple selection criterium but is not generally applicable (Deimling et al. 1988). Because homozygous diploid clones that showed reduced vigor were used as parents in these fusion experiments, accumulation of increased heterozygosity is expected in the fusion products compared to their parents (Debnath and Wenzel 1987). When applying somatic hybridization in plant breeding programs, highly heterozygous clones should be used in order to obtain the best performance of the hybrid product, while hybrid vigor is not a recommendable selection criterium when heterozygous clones are used.

Up to now, only a few different wild species have been used with success in symmetric fusion experiments with *S. tuberosum* to introduce useful traits (for a recent review, see Ferreira and Zelcer 1989). Fusion of protoplasts of the tetraploid *S. tuberosum* cultivar Priekulsky rannii (2n=48) with those of *S. chacoense* (2n=24) resulted in an aneuploid (2n=60) somatic hybrid with a still high pollen fertility and an absolute field resistance to potato virus Y that was not present in either parent (Butenko et al. 1982). Atrazine resistance was incorporated into potato using an atrazine-resistant biotype of *S. nigrum* L., but no fertile hybrid (Type 3 clones) was obtained (Binding et al. 1982, 1988). The most successful fusion combination of a wild species with the cultivated potato was the fusion between *S. brevidens* and *S. tuberosum* (Barsby et al. 1984; Fish et al. 1988; Helgeson 1989). With this combination, new sources of potato leaf roll virus (PLRV) and *Erwinia* resistance were incorporated into the *S. tuberosum* gene pool.

In this paper we describe the successful somatic hybridization of the diploid wild species *S. circaeifolium* subsp. *circaeifolium* Bitter and a dihaploid *S. tuberosum* L. clone, using a simple morphological selection criterium in the callus phase, and the incorporation of resistance to *Phytophthora infestans* and *Globodera pallida* into these hybrids.

Materials and methods

Plant material

In vitro shoot cultures of *S. tuberosum* subsp. *tuberosum* L. (*tbr*[1]), clone DH81-7-1463 (2n=24), and a clone of *S. circaeifolium* subsp. *circaeifolium* Bitter (*crc1*), BGRC 27058 (2n=24), were propagated on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose, 0.8% agar, pH 5.8 (MS30). Four weeks before protoplast isolation *crc1* shoots were transferred to MS10, i.e., MS medium supplemented with 1% sucrose and 0.8% agar (pH 5.8) *tbr* shoots were then transferred to MS30 supplemented with 0.01 m*M* SAN 9789 (norflurazon; Sandoz AG, Basel, Switzerland) to produce bleached plantlets (Uhrig 1981).

Protoplast isolation, fusion, and culture

Protoplasts were isolated according to Puite et al. (1986). Prior to fusion, green *crc1* and FDA-stained, bleached *tbr* protoplasts were mixed in a 1:1 ratio at a final concentration of 2×10^5 protoplasts per ml in 380 m*M* mannitol. Of this mixture, 1.0 ml was transferred to a multi-well fusion chamber, with 3-mm between the electrodes, and placed in a 60-mm petri dish (Tissue Culture Quality). An alternate current (AC) source of 1 MHz and 100 V/cm output voltage was used to line up the protoplasts, and three direct current (DC) pulses of 1,300–1,600 V/cm were applied to induce membrane fusion. After fusion, 1.0 ml modified ½V-KM medium (Mattheij and Puite 1991) at double strength without mannitol and 30 µl of cefotaxime (final concentration 190 µg/ml), to prevent bacterial infections, was added to the fusion mixture. The petri dishes were sealed with Parafilm and incubated in a growth cabinet at 25 °C under continuous light at 1,000 lx.

The suspension was diluted with 1.0 ml fresh ½ V-KM medium 3 days after protoplast isolation and fusion. Four days later the suspension was transferred to a 94-mm petri dish (Tissue Culture Quality), and 2.0 ml ½ V-KM medium supplemented with 0.2% agarose (SeaPlaque, FMC) was added. When calli had reached a diameter of 1–2 mm (2 weeks after protoplast isolation and fusion) they were transferred to callus growth medium [MS: 1% sucrose, 0.8% agar, 1 mg/l BAP, and 0.2 mg/l NAA (pH 5.8)]. After 2–4 weeks, putative hybrid calli with intermediate morphology were transferred to shoot induction medium [MS: 1% sucrose, 0.8% agar, 1.0 mg/l zeatin, and 0.01 mg/l NAA (pH 5.8)] where they remained for 2 weeks. Shoot proliferation was induced on shoot elongation medium I [MS: 1% sucrose, 0.8% agar, 0.25 mg/l BAP, and 0.1 mg/l GA_3 (pH 5.8)]. As shoot proliferation was poor on this medium, calli were transferred to shoot elongation medium II [MS: 1% sucrose, 0.8% agar, 1 mg/l zeatin, 0.1 mg/l GA_3, and 0.1 mg/l IAA (pH 5.8)] after 3 weeks. Every 2–3 weeks, the calli were transferred to fresh elongation medium II. Shoots were removed upon emergence and maintained in glass culture tubes on MS30 medium. Subculture and propagation of the shoots took place on this medium every 4 weeks.

Analysis of ploidy level and number of chromosomes

The ploidy level was determined by chloroplast counts in the stomatal guard cells, as well as by measurements of the nuclear DNA content using a flow cytometer. The exact number of chromosomes was determined only for the tetraploid hybrid regenerants.

In order to count the number of chloroplasts in stomatal guard cells, leaves were collected early in the morning from well-developed plants. The lower epidermis was removed, transferred to 96% alcohol and, after approximately 1 hour, placed in a drop of a diluted iodine-potassium iodide solution (Lugol's solution). The number of chloroplasts in 100 stomatal guard cells was counted using light microscopy.

To measure the nuclear DNA content by flow cytometry, nuclei were isolated from small pieces of leaf material according

[1] Abbreviations according to Huaman and Ross (1985)

to Galbraith et al. (1983). Leaf material was chopped in a cooled, slightly modified buffer described by de Laat and Blaas (1984) that contained 0.2% instead of 1% Triton X-100, and DAPI (2 mg/l) instead of ethidium bromide as a fluorochrome, while 0.1% β-mercaptoethanol was added. To isolate nuclei, the suspension was filtered through a 30-µm mesh nylon filter. Within 15 min the stained nuclei were analyzed by flow microfluorometry with an ICP22 (Ortho Diagnostic Systems, Beerse, Belgium) flow cytometer, using excitation filters KG-1, BG-38, UG-1, and TK 450 and emission filter BP 440–500.

Exact numbers of chromosomes were determined using root tips from fast-growing greenhouse-grown plants. These root tips were collected in a saturated solution of α-mono-bromo-naphthalene in water. After 1.5 h at room temperature, roots were fixed in a mixture of alcohol and acetic acid 3:1 for 24 h and stored until analysis. They were then washed in demineralized water for 10 min and hydrolyzed in 1 *N* HCl at 60 °C for 8 min, followed by a second washing in demineralized water for 10 min. The surplus water was then removed and the roots were transferred to Feulgen reagent. Only meristematic, purple-colored roots were washed in demineralized water and treated with a 5% pectinase (SERVA, Heidelberg, Germany) solution for 14 min at 40 °C, followed by another washing step with demineralized water for 10 min. Tips (1–1.5 mm) were removed from the roots and squashed in half a drop of 1.5% aceto-carmine. Chromosome counts were performed on at least six different metaphase plates.

Meiotic analysis and fertility

For meiotic analysis, flower buds of greenhouse-grown plants were fixed in a 3:1 mixture of absolute alcohol and a saturated glacial acetic acid solution with ferric acetate, and stored for 24–48 h. The flower buds were then transferred into 96% alcohol for 15 min. The anthers were isolated from the flower buds and transferred to the 'Snow' stain (Snow 1963) and incubated for 6 h at 60 °C. They were then squashed in 45% acetic acid.

Male fertility was tested by staining the pollen with lactophenol fuchsin. Female fertility was tested by crossing hybrids with the tetraploid (2n = 48) cultivars 'Amsel' and 'Désirée' and the diploid (2n = 24) clones KW84-19-2471 and SY7, the latter being a desynaptic clone performing first-division restitution (FDR) and thus a functional tetraploid.

Morphological assessment and molecular analysis of regenerants

Rooted shoots from putative hybrid calli were transferred to the greenhouse. After several weeks, their morphology was assessed qualitatively for leaf, flower, and fruit shape and for stem color.

For the molecular analysis of the regenerants, DNA was extracted from 4 g of leaf material according to Dellaporta et al. (1983). Approximately 10 µg DNA per sample was digested to completion with the restriction endonucleases *Eco*RI or *Hin*dIII (2.5 units/µg) for 2 h at 37 °C. After digestion, the fragments were separated by electrophoresis in a 0.8% agarose (Ultrapure) gel and subsequently transferred onto a Hybond-N (Amersham) filter. As probes, insert DNA from clones KG1, KG3, and KG12 from a *S. spegazzinii* genomic library (C. Kreike, personal communication) was labelled with digoxigenin-dUTP and hybridized with the digested plant DNA. Signal detection was performed by incubating the blot with anti-digoxigenin solution, adding AMPPD (Tropix, Bedford/MA) as a substrate, with the resulting light signal being detected on X-ray film (Kreike et al. 1990).

Disease resistance and steroidal glycoalkaloid content

Somatic hybrids, their parents, and the control cultivars 'Bildtstar' (susceptible) and 'Pimpernel' (field resistant) were tested for their resistance to *Phytophthora infestans*, in a laboratory experiment in which three leaflets per plant and three plants per genotype were inoculated with 2 drops (9,62 µl) of a zoospore suspension this suspension contained 48,000 zoospores per ml of the physiological race 1.2.3.4.5.6.7.10.11 and was supplied by the Research Institute for Plant Protection (IPO) at Wageningen. The leaves were incubated on wet filter paper at 15 °C, 100% air humidity, with continuous cool white fluorescent tube illumination. The number of inoculation sites showing infection was scored after 7 days.

The resistance to the *Globodera pallida* pathotypes Pa2 and Pa3 and the steroidal glycoalkaloid content were determined as described elsewhere (Louwes et al. 1992).

Results

In the experiments presented here, calli from the two parents could easily be identified by their morphology, calli from *crc1* being brown-yellow with purple-colored cells and calli from *tbr* being bright green. Putative hybrid calli were identified by their intermediate morphology (calli green in color with purple-colored cells). A total of 101 putative hybrid calli was selected. Plants could be regenerated from 17 different calli. These plants were multiplied in glass culture tubes, and 9 of them formed roots and could be transferred to soil in the greenhouse for further analysis.

Morphological assessment

Some distinct morphological characteristics of *crc1* and *tbr* and their putative somatic hybrids are presented in Table 1 and Fig. 1. Anthocyanin formation in the stems of the hybrids was found to be intermediate between the parents, the *tbr* parent having no purple color in the stems, and the *crc1* parent having an intensely purple stem. The hybrids had purple stems with occasional green areas. Leaf shape was also found to be intermediate (Fig. 1 A). *Crc1* leaves never had more than one pair of lateral leaflets, the terminal leaflet being oversized. In *tbr* the terminal and several leaflets were about the same size. The somatic hybrids showed a large terminal leaflet and several pairs of medium-sized lateral leaflets. The other

Table 1. Some morphological characteristics of *Solanum circaeifolium* (BGRC 27058), *S. tuberosum* (DH81-7-1463), and their somatic hybrid

	S. circaeifolium	Somatic hybrid	*S. tuberosum*
Stem anthocyanin	intense	intermediate	absent
No. of lateral leaflets	1–2	>2	>2
Corolla	stellate	semi-stellate	pentagonal
Fruit shape	long	intermediate	round

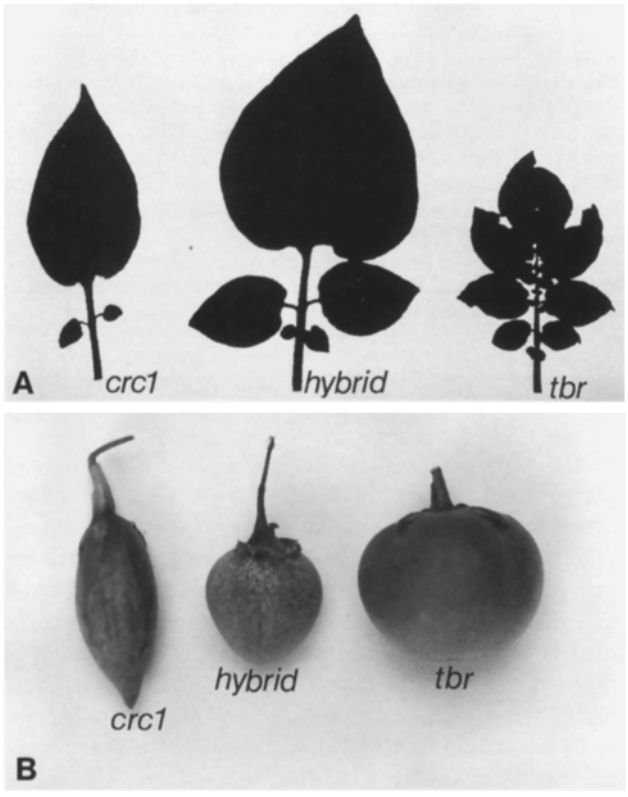

Fig. 1. **A** Leaf shape (shadowgram by a photocopier) and **B** fruit morphology of *Solanum circaeifolium* (*crc1*), *S. tuberosum* (*tbr*), and their putative somatic hybrid (hybrid)

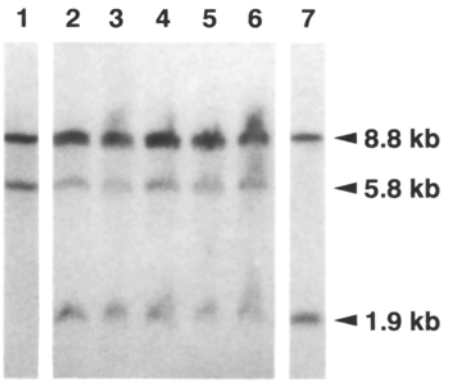

Fig. 2. Restriction fragment length polymorphism pattern of the parental clones *Solanum tuberosum* (lane *1*) and *S. circaeifolium* (lane *7*), and four tetraploid (lanes *2, 3, 4, 6*) and one hexaploid (lane *5*) somatic hybrids. DNA was restricted with *Hin*dIII. Probe KG12 was used for hybridization

characteristics, such as the corolla form and fruit shape (Fig. 1 B), also showed clearly intermediate features. These intermediate morphological features indicate that all regenerants, with the exception of clone 117-10A, were hybrids.

Analysis of ploidy level and number of chromosomes

The number of chloroplasts in the stomatal guard cells gives an indication of the ploidy level (Schreiter et al. 1989). These could be determined in eight of the nine plants transferred to the greenhouse (Table 2). The severely wrinkled leaves of hybrid 117-3A made it impossible to count the chloroplasts in the stomatal guard cells. Determining ploidy level using the flow cytometer gave similar results to those obtained through the chloroplast counts (Table 2).

These data show that only five of nine putative hybrid plants were near tetraploid. As one of these five plants (117-10A) showed a parental (*tbr*) phenotype, it was not analyzed any further. Also presented in Table 2 are the results of the chromosome counts. Three hybrids were found to be aneuploid (with only 47 chromosomes) and one had the expected 48 chromosomes.

Table 2. Ploidy level of fusion products, the fusion parents *Solanum circaeifolium* (BGRC 27058) and *S. tuberosum* (DH81-7-1463), and a tetraploid control 35-4-1a1 (*S. tuberosum*; 2n = 48), as determined by chloroplast counts in stomatal guard cells, flow cytometric (FCM) analysis of nuclear DNA content, and counting the number of chromosomes

Clone number	Chloroplast analysis		FCM analysis ploidy level	Chromosome analysis No. of chromosomes
	No. of chloroplasts	Estimated ploidy level		
35-4-1a1	12.01 ± 1.15	4x	4x	48
BGRC 27058	6.97 ± 0.94	2x	2x	24
DH81-7-1463	7.47 ± 1.02	2x	2x	24
117-1A	13.93 ± 2.37	4x	4x	48
117-2A	17.17 ± 2.51	6x	6x	ND[a]
117-4A	12.19 ± 1.15	4x	4x	47
117-5A	12.10 ± 1.14	4x	4x	47
117-7A	17.05 ± 2.26	6x	6x	ND
117-9A	11.51 ± 1.40	4x	4x	47
117-10A	13.57 ± 1.99	4x	4x	ND
117-14A	23.91 ± 2.88	8x	8x	ND
117-3A[b]	ND	ND	5x–8x	ND

[a] ND: not determined
[b] Wrinkled leaf made counting of chloroplasts impossible

Molecular analysis

DNA was extracted only from those plants that had a hybrid morphology. Figure 2 shows the RFLP patterns of four tetraploid (117-1A, 117-4A, 117-5A, and 117-9A) and one hexaploid (117-7A) putative somatic hybrid. All of the tested putative hybrids had a hybrid RFLP pattern.

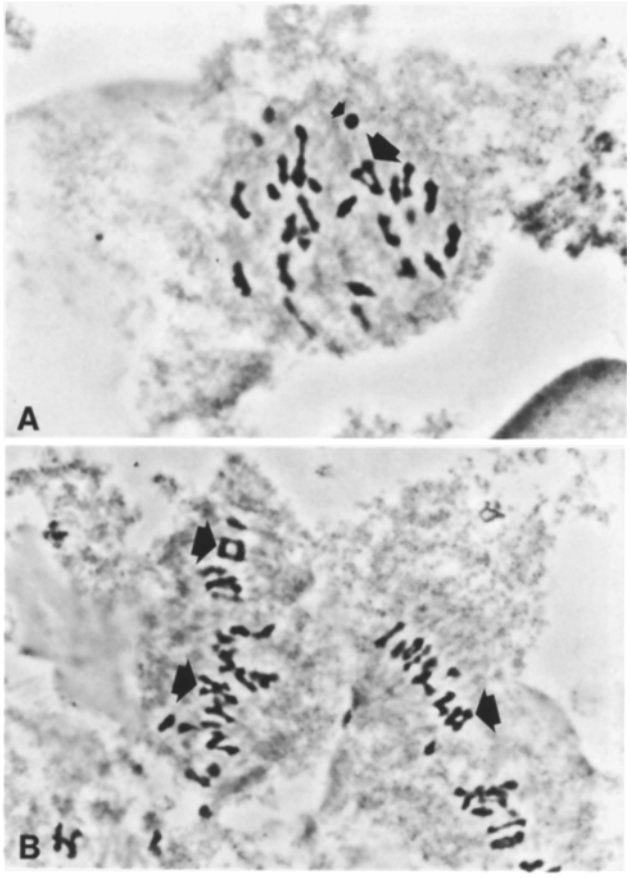

Fig. 3 A and B. Meiotic configuration of the somatic hybrid 117-4A showing multivalent formation. **A** Diakinesis showing a trivalent (*large arrow*) and univalent (*small arrow*). **B** Two metaphase I showing different quadrivalents (*large arrows*)

Meiotic analysis and fertility

In meiosis, all tetraploid hybrid plants showed multivalent chromosome pairing. In Fig. 3 two different examples of meiosis of the hypotetraploid hybrid 117-4A with 47 chromosomes are presented, one at diakinesis with a trivalent, and the other two meiotic metaphases I having quadrivalents. Almost every cell that was studied showed at least one multivalent. In the somatic hybrids with $2n = 47$ some univalents were found, even more multivalents, but mainly there were bivalents. The flowering of the hybrid plants was abundant, but male fertility was very low or even complete sterility occurred, while the parental clones had 10% (*tbr*) and 64% (*crc1*) vital pollen. The tetraploid hybrids produced little pollen, which sometimes showed staining, but also had an aberrant morphology. One of the hexaploid regenerants flowered sparingly but produced a high number of pollen grains, of which about 0.03% was stainable pollen, which was still more than the tetraploid hybrids had.

The tetraploid hybrids, when used as female parents, proved to be fertile. It was possible to obtain seeds from crosses with tetraploid genotypes, the desynaptic diploid (88% success after pollination and 3.9 seeds/berry), or a diploid clone that did not produce 2n-pollen (76% success after pollination and 4.0 seeds/berry). From these seeds, germinated in vitro, plants could be obtained.

Table 3. Resistance of four tetraploid somatic hybrids, their parents *Solanum tuberosum* (*tbr*) and *S. circaeifolium* (*crc1*), and some standards to *Phytophthora infestans* and *Globodera pallida*. The degree or resistance to *P. infestans* is expressed as the number of inoculation sites showing infection on leaves after inoculation with a zoospore suspension of the physiological race 1.2.3.4.5.6.7.10.11., and the degree of resistance to *G. pallida* pathotypes 2 and 3 (Pa2 and Pa3) is expressed as the mean number of cysts on the roots of individual plants after inoculation with 25–30 cysts

	Phytophthora infestans	*Globodera pallida*	
	No. of inoculation sites showing infection	Mean no. of cyst on the roots of individual plants	
		Pa2	Pa3
Standards			
'Pimpernel'	9/18		
'Bildtstar'	12/18		
'Maritta'		204.3	311.3
62-33-2		0.3	
78-3778			0.0
Parents			
tbr	18/18	100.3	54.0
crc1	0/18	0.0	0.0
Somatic hybrids			
117-1A	0/18	1.0	2.0
117-4A	0/18	0.3	0.0
117-5A	0/18	2.7	1.0
117-9A	4/18	5.3	0.7

Disease resistance and steroidal glycoalkaloid content

Table 3 presents the results of the *P. infestans* resistance test. In total, nine leaves were inoculated in duplo with the zoospore suspension. At 7 days after inoculation, the *tbr* parent showed sporulating spots on all 18 inoculation sites, while both the field-resistant cultivar 'Pimpernel' and the susceptible 'Bildtstar,' used as standards, showed spots on 9 and 12 of the inoculation sites, respectively. In contrast, *crc1* showed complete resistance. Three out of the four tetraploid hybrids tested were also completely resistant. Hybrid 177-9A showed infected spots, but no sporulating colonies were formed.

Also shown in Table 3 are the results of the *G. pallida* resistance test. The standard cultivar 'Maritta' (sensitive) and the parent *tbr* showed a high degree of susceptibility to both *G. pallida* pathotypes. The standards 62-33-3 (resistant to Pa2) and 78-3778 (resistant to Pa3), clone *crc1*, and all four tetraploid somatic hybrids showed a high degree of resistance.

Table 4. Glycoalkaloid content of potato tubers in three somatic hybrids and their parents *Solanum tuberosum* (*tbr*) and *S. circaeifolium* (*crc1*)

Clone	Glycoalkaloid content (class 0 to 5[a])				
	Solanidine glycoside	Solasodine glycoside	Tomatidenol glycoside	Tomatidine glycoside	Demissidine glycoside
Parents					
tbr	5	0	0	0	0
crc1	0	0	2	5	0
Somatic hybrids					
117-2A	3	0	0	2	2
117-4A[b]	4	0	0	2	1
117-4A	4	0	0	3	3
117-5A	2	0	1	5	1

[a] The different classes are 0 (0 mg/kg), 1 (1–50 mg/kg), 2 (51–100 mg/kg), 3 (101–150 mg/kg), 4 (151–200 mg/kg), and 5 (>200 mg/kg)
[b] The tubers of hybrid 117-4A were harvested on two different dates, and therefore the analysis was performed twice

Because not enough tuber material was available from every hybrid, only the tetraploid somatic hybrids 117-4A and 117-5A and the hexaploid hybrid 117-2A could be analyzed for steroidal glycoalkaloid content. Data on the steroidal glycoalkaloid content expressed in six different concentration classes of the three hybrids are shown in Table 4. One tetraploid hybrid, 117-4A, was tested twice. The tubers of this hybrids were harvested on two different dates and show some variation in the steroidal glycoalkaloid content. In addition to components found in the parents and the hybrids, the new component demissidine glycoside was observed. The total steroidal glycoalkaloid content of the hybrids is comparable to the mean content of the parents.

Discussion

The selection of hybrid fusion products based on callus morphology was described previously by Deimling et al. (1988) in fusion between different *S. tuberosum* clones, and in *S. melongena* and *S. torvum* fusion by Guri and Sink (1988). The present results indicate that selection of hybrids based on callus morphology in fusions of *crc1* and *tbr* was also very successful. From previous experience with other *S. tuberosum* as well as *S. phureja* clones, we know that a large number formed bright green calli with the method used. Therefore, we feel that the hybrid selection criterium used in this study can be applied in the combination of *crc1* and a number of these *S. tuberosum* and *S. phureja* clones.

With one exception, all of the plants transferred to the greenhouse could be identified as hybrids, showing several morphological traits intermediate to both of the parents, such as corolla and fruit shape, leaf morphology, and the presence of anthocyanin formation in the stem. Of these greenhouse-grown plants, four were found to be tetraploid, the others being polyploid or mixoploid. Ploidy analysis using chloroplast counts and flow cytometry gave similar results. As flow cytometric analysis of the hybrids is a sophisticated and expensive technique, counting chloroplasts in the stomatal guard cells might have an advantage over flow cytometric analysis in practice. In those cases where periclinal chimaera occur, flow cytometric analysis is preferred.

The generation of different ploid levels is not an uncommon feature, as multiple fusion events and somaclonal variation (Sree Ramulu et al. 1989) often occur after fusion and plant regeneration. Detailed cytological analysis showed that, of the four hybrids identified as tetraploids by flow cytometric analysis, only one clone had the exact tetraploid chromosome number of 48; the others lack one chromosome. Loss of one or more chromosomes was also found by Pijnacker et al. (1989) in somatic hybrids of *S. tuberosum* and *S. phureja*. In our hybrids this did not interfere with female fertility, as crosses with different pollinators gave fruit set and seeds that proved to be viable. From these seeds, plants could be grown in vitro. The successful pollination of the hybrids with pollen from the tetraploid cultivars 'Amsel' and 'Désirée' and the diploids KW 84-19-2471 and SY7 shows that there were no crossing barriers between the somatic hybrid and haploid pollen from KW 84-19-2471, or diploid pollen from SY7 and the cultivars. However, male fertility was very low. This was not due to chromosome loss, as the fertility of the tetraploid hybrid carrying 48 chromosomes was found to be similarly low. A reason for this could be the specific parental combination used in our experiments; perhaps another combination will lead to male-fertile hybrids.

The fact that the somatic hybrids could be crossed with tetraploid clones makes them useful for further breeding, but only if introgression of *crc1* DNA into *tbr* takes place. Meiotic configurations such as the formation

of multivalents, also found in tetraploid and hexaploid somatic hybrids of *S. brevidens* and *S. tuberosum* by Ehlenfeldt and Helgeson (1987), indicate a possible chromosome exchange between the genomes of *crc1* and *tbr*.

The results presented here show that a high degree of resistance to both pathogens tested was still present in the hybrids. Only clone 117-9A (2n=47) manifested some sensitivity after infection with the complex race of *Phytophthora infestans*. This might be explained by the loss of one chromosome, which may carry some resistance genes.

The glycoalkaloid composition of the hybrids differed from that of the parents, showing a novel glycoalkaloid (i.e., demissidine glycoside). The total glycoalkaloid content (253–405 mg/kg) had not changed compared to that of the parents (*tbr* with 264 mg/kg and *crc1* with 319 mg/kg). Similar results were found by Roddick and Melchers (1985) for the total glycoalkaloid level in leaves of somatic hybrids of potato and tomato. They observed an increased glycoalkaloid level in the tubers, with tomatine as the major (60–70%) alkaloid present, contributed by the tomato parent. Backcrossing with cultivated *tbr* will be necessary to reduce the total glycoalkaloid content (See Ross 1986).

Further crossing is required to introduce the valuable traits of these somatic hybrids in potato breeding programs. As the tetraploid somatic hybrids show chromosome pairing between the different genomes and can be crossed with other tetraploid genotypes, it will be possible to incorporate the resistances of *crc1* in new potato cultivars. This study again demonstrates the usefulness of somatic hybridization of wild species with the cultivated potato to transfer valuable traits.

Acknowledgements. The help of the following people is gratefully acknowledged: H.J. Vinke (*Globodera resistance* testing), L.T. Colon and D.J. Budding (*Phytophthora resistance* testing), H.H. Jonker (steroidal glycoalkaloid analyses), and Plant Cytometry Services, Schijndel, The Netherlands (flow cytometric analyses). The critical reading of the manuscript by C.M. Colijn-Hooymans, F.A. Krens, K.J. Puite, K. Sree Ramulu, and J. Hoogendoorn is highly appreciated.

References

Austin S, Baer M, Ehlenfeld M, Kazmierczak PJ, Helgeson JP (1985) Intraspecific fusions in *Solanum tuberosum*. Theor Appl Genet 71:172–175

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

Binding H, Jain SM, Finger J, Mordhorst G, Nehls R, Gressel J (1982) Somatic hybridization of an atrazine resistant biotype of *Solanum nigrum* with *Solanum tuberosum*. Theor Appl Genet 63:273–277

Binding H, Zuba M, Rudnik J, Mordhorst G (1988) Protoplast gel fusion. J Plant Physiol 133:409–413

Butenko R, Kuchko A, Komarnitsky I (1982) Some features of somatic hybrids between *Solanum tuberosum* and *S. chacoense* and its F_1 sexual progeny. In: Fujiwara A (ed) Plant tissue culture. Proc 5th Int Congr Plant Tiss Cell Cult, Tokyo, pp 643–644

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

Dellaporta SJ, Wood J, Hicks JB (1983) A plant DNA minipreparation II. Plant Mol Biol Rep 1:19–21

Ehlenfeldt MK, Helgeson JP (1987) Fertility of somatic hybrids from protoplast fusion of *Solanum brevidens* and *S. tuberosum*. Theor Appl Genet 73:395–402

Ferreira DI, Zelcer A (1989) Advances in protoplast research. Int Rev Cytol 115:1–65

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

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051

Guri A, Sink KC (1988) Interspecific somatic hybrid plants between eggplant (*S. melongena*) and *S. torvum*. Theor Appl Genet 74:490–496

Hawkes JG, Hjerting JP (1989) The potatoes of Bolivia. Their breeding value and evolutionary relationships. Clarendon Press, Oxford, 472 pp

Helgeson JP (1989) Somatic hybridization of wild *Solanum* species with potato: a potential source of diversity for breeders. In: Louwes KM, Toussaint HAJM, Dellaert LMW (eds) Parental line breeding and selection in potato breeding. Pudoc, Wageningen, pp 87–94

Hermsen JGTh (1977) Incorporation of new germ plasm: wild species. Report of the planning conference on utilization of the genetic resources of the potato. International Potato Center, Lima, pp 91–100

Huaman Z, Ross RW (1985) Updated listing of potato species names, abbreviations, and taxonomic status. Am Potato J 62:629–641

Kreike CM, Koning JRA de, Krens FA (1990) Non-radioactive detection of single-copy DNA-DNA hybrids. Plant Mol Rep 8(3):172–179

Laat AMM de, Blaas J (1984) Flow-cytometric characterization and sorting of plant chromosomes. Theor Appl Genet 67:463–467

Louwes KM, Hoekstra R, Mattheij WM (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. 2. Sexual hybrids. Theor Appl Genet

Masson J, Lancelin D, Bellini C, Lecerf M, Gerche P, Pelletier G (1989) Selection of somatic hybrids between diploid clones of potato (*Solanum tuberosum* L.) transformed by direct gene transfer. Theor Appl Genet 78:153–159

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

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

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

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

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

Roddick JG, Melchers G (1985) Steroidal glycoalkaloid content of potato, tomato and their somatic hybrids. Theor Appl Genet 70:655–660

Ross H (1986) Potato breeding – problems and perspectives. Paul Parey, Berlin Hamburg, 132 pp

Schreiter J, Munzert B, Moll A (1989) Bestimmung des Ploidiegrades durch Chloroplastenzählungen in Stomata bei In-vitro-Kallusregeneraten von Kartoffeln. Arch Zuechtungsforsch 19:69–73

Snow R (1963) Alcoholic hydrochloric acid-carmine as a stain for chromosomes in squash preparations. Stain Technol 38:9–13

Sree Ramulu K, Dijkhuis P, Roest S (1989) Patterns of phenotypic and chromosome variation in plants derived from protoplast cultures of monohaploid, dihaploid and diploid genotypes and in somatic hybrids of potato. Plant Sci 60:101–110

Uhrig H (1981) Regeneration of protoplasts of dihaploid potato plants bleached by a herbicide (SAN 6706). Mol Gen Genet 181:403–405

Vries SE de, Jacobsen E, Jones MGK, Loonen AEHM, Tempelaar MJ, Wijbrandi J, Feenstra WJ (1987) Somatic hybridization of amino acid analogue-resistant cell lines of potato (*S. tuberosum* L.) by electrofusion. Theor Appl Genet 73:451–458

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