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Interspecific somatic hybrids between wild potato *Solanum acaule* Bitt. and anther-derived dihaploid potato (*Solanum tuberosum* L.)

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Abstract *Solanum acaule* Bitt. is a disomic tetraploid (4x) wild potato species which is resistant to several potato diseases. Introgression of disease resistance and abiotic stress tolerance to the tetrasomic tetraploid (4x) cultivated potato (*S. tuberosum* L.) gene pool via crossing has been limited due to the difference in the endosperm balance number. In the present study, protoplast fusion was applied to produce hexaploid (6x) somatic hybrids between the parental lines, tetraploid (4x) *S. acaule* and two anther-derived dihaploid (2x) lines of *S. tuberosum* cv. White Lady. One callus (0.4%) of a total of 229 calli obtained regenerated into shoots in the fusion combination *S. acaule* (+) White Lady 15.dh.8.2.2. All the regenerated shoots were confirmed to be interspecific somatic hybrids using species-specific RAPD markers. In another fusion combination, *S. acaule* (+) White Lady 7.dh.23.1.1, fifteen calli (5%) regenerated into a total of sixteen shoots from 289 calli. All the analysed somatic hybrids between *S. acaule* and *S. tuberosum* were hexaploid. The mean DNA content (2C value) of the combination *S. acaule* (+) White Lady 15.dh.8.2.2 somatic hybrids (4.55 pg), was approximately the sum (4.69 pg) of the DNA contents of the parental lines, *S. acaule* (2.95 pg) and *S. tuberosum* (1.74 pg). In the greenhouse, the two somatic hybrids analysed were normal in their morphological characteristics and more vigorous than their parental lines. Most of the morphological characteristics were closer to the tetraploid *S. acaule* than to the dihaploid *S. tuberosum*. The interspecific somatic hybrids are currently being tested for frost tolerance and glycoalkaloid composition.

Key words DNA content · Morphology · Protoplast fusion · RAPD · Somatic hybrid

Abbreviations *EBN* Endosperm balance number · *PVX* Potato virus X

Introduction

Protoplast fusion is an alternative to sexual hybridization for incorporation of genetic material from wild species into cultivated potato (*Solanum tuberosum* L. ssp. *tuberosum*). Using somatic hybridization, several valuable characters have already been transferred from many wild potato species, such as *S. berthaultii* Hawkes (Serraf et al. 1991), *S. brevidens* Phil. (Barsby et al. 1984; Rokka et al. 1994), *S. bulbocastanum* Dun. (Austin et al. 1993), *S. chacoense* Bitt. (Butenko and Kuchko 1979), *S. circaeifolium* Bitt. (Mattheij et al. 1992), *S. commersonii* Dun. (Cardi et al. 1993; Nyman and Waara 1997), and *S. pinnatisectum* Dun. (Ward et al. 1994). Recently, somatic hybrids between *S. tuberosum* and dihaploid *S. acaule* Bitt. have also been reported (Yamada et al. 1997). Some wild species are directly sexually compatible with cultivated potato, but most can only be hybridized via somatic cells or, alternatively, using rescue pollinations and embryo rescue (Watanabe et al. 1995).

S. acaule is a tetraploid (2n=4x=48) South American wild potato species which cannot be directly crossed with *S. tuberosum* without ploidy changes, unreduced gametes or bridge breeding (see Ross 1986), or without compatible second pollination and embryo rescue (Watanabe et al. 1992). This is due to the differences in the endosperm balance number (EBN) (Johnston et al. 1980) which is 2 for tetraploid *S. acaule*, and 4 for tetraploid *S. tuberosum* (Brown and Adiwilaga 1989). *S. acaule* is a disomic tetra-

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ploid species forming bivalents in meiosis, but *S. tuberosum* is a tetrasomic tetraploid forming mostly quadrivalents (Lamm 1945). The genome of *S. acaule* consists of two early hybridized genomes of AA and A^aA^a (Matsubayashi 1982, A_2A_2 and A_3A_3 respectively, in Hawkes 1990). It is not known, however, which species hybridized, but they were probably from the series Megistacroloba and Tuberosa (Hawkes 1990). The two genomes of *S. acaule* are partially but not completely homologous (Matsubayashi 1982).

S. acaule possesses several disease resistance characters and has tolerance to many abiotic stresses (Hawkes 1994). Of the *Solanum* species studied, *S. acaule* is the most tolerant to frost (Chen and Li 1980) and acclimatization to cold temperatures affords it resistance to –9°C (Vayda 1994). Some of the resistance characters, such as extreme resistance to potato virus X (PVX), have already been transferred to cultivated potato via bridge breeding (Ross 1986). However, many polygenic traits are difficult to incorporate from *S. acaule* via sexual breeding, and thus somatic hybridization can enhance the transfer of the valuable genes of *S. acaule* to potato.

Somatic hybrids of potato have been produced by fusion of a diploid wild species with tetraploid *S. tuberosum* (Barsby et al. 1984; Austin et al. 1993; Cardi et al. 1993) or with dihaploid *S. tuberosum* lines (Austin et al. 1985; Cardi et al. 1993; Rokka et al. 1994), or by fusion of hexaploid wild species with dihaploid *S. tuberosum* (Binding et al. 1982) to produce either tetraploid, hexaploid or octoploid somatic hybrids. To further improve the hybrids, they have been used in sexual backcrosses (Ehlenfeldt and Helgeson 1987; Helgeson et al. 1995; Carotenuto et al. 1997) or in somatohaploid production (Rokka et al. 1997). Normally, from four to six sexual backcrosses are needed for breeding a new variety when interspecific crosses are used in potato breeding (Ross 1986).

This paper describes the production of somatic hybrids between tetraploid *S. acaule* and dihaploid anther-derived *S. tuberosum* in order to produce hexaploid hybrids for potato breeding purposes.

Materials and methods

Tetraploid *S. acaule* acc. PI 472655.8 (no.7–8) was kindly provided by Dr. Carol A. Ishimaru, Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, Colo., USA. This *S. acaule* line is resistant to potato leafroll virus (Bamberg et al. 1994) and immune to bacterial ring rot caused by *Clavibacter michiganensis* ssp. *sepedonicus* (Spieck. and Kotth.) Davis et al. (Ishimaru et al. 1994; Kriel et al. 1995). The dihaploid *S. tuberosum* lines, White Lady 15.dh.8.2.2 and White Lady 7.dh.23.1.1, were produced by anther culture of the tetraploid cv. White Lady (Rokka et al. 1996a). The dihaploid White Lady 15.dh.8.2.2 is relatively early maturing and high-yielding in the field. It produces round and shallow-eyed tubers which have light-yellow flesh. Its tuber starch content is higher than in dihaploid lines generally, but it is sensitive to late blight [*Phytophthora infestans* (Mont.) de Bary] and to potato cyst nematode [*Globodera rostochiensis* (Woll.) Behrens]. The dihaploid White Lady 7.dh.23.1.1 is very low yielding, but it is hypersensitive to late blight (J. Kankila, personal communication).

Leaf protoplasts were isolated from in vitro plant material of *S. acaule* and *S. tuberosum* lines after 2 months of culture as described by Rokka et al. (1996b). The protoplasts of *S. acaule*, together with the protoplasts of either White Lady 15.dh.8.2.2 or White Lady 7.dh.23.1.1, were combined in a 1:1 mixture and fused using electrofusion with 125 V cm^{-1} and one to two DC pulses of 1750 V cm^{-1}, cultured and then regenerated into shoots (Rokka et al. 1996b). DNA was extracted from the leaves of regenerated shoots and their fusion parental lines, and RAPD markers were applied to assess the hybridity, comparing the banding patterns of the regenerants with the patterns of the parental lines, as described by Rokka et al. (1995). DNA amplification was carried out with an MJ Research PTC-100 PCR machine using 35 cycles of 30 s at 95°C, 30 s at 35°C and 1 min 10 s at 72°C, after DNA denaturation step of 3 min 30 s at 95°C. The gels were photographed and analysed with Eagle Eye II Still Video System (Stratagene, USA) using Eagle Sight 3.2 Image Capture and Analysis Software. For the protoplast fusion combination *S. acaule* (+) White Lady 15.dh.8.2.2, four primers (OPB-09, OPK-08, OPB-05, OPK-02; Operon, USA) were tested to find a primer which amplified banding patterns that contained specific bands for both fusion partners. Only the primer OPB-05 did not show genetic polymorphism between the lines. Similarly, for the protoplast fusion combination *S. acaule* (+) White Lady 7.dh.23.1.1, six primers (OPB-11, OPD-04, OPB-07, OPD-20, OPC-16, OPK-10) were tested, of which three (OPB-11, OPD-20, OPK-10) showed genetic polymorphism between these potato lines.

Two somatic hybrids of the combination *S. acaule* (+) White Lady 15.dh.8.2.2 were planted in soil in plastic pots in the greenhouse and plant morphology was assessed at the full flowering stage. Somatic hybrid male fertility was measured by staining pollen grains with 1% acetocarmine (1% carmine in 45% acetic acid).

Chromosome numbers of the somatic hybrid combination *S. acaule* (+) White Lady 15.dh.8.2.2 were counted from in-vitro-grown root tip cells (Rokka et al. 1996a). The DNA contents of the parental lines and the somatic hybrids were analysed from greenhouse-grown plantlets using flow cytometry (FACSort, Becton-Dickinson, USA) (Rokka et al. 1995). In each flow cytometric sample, chicken red blood cells were used as an internal DNA standard, having a DNA content of 2.33 pg (Galbraith et al. 1983).

Results

One week after plating the fused protoplasts, there were several divided cells in the protoplast culture media of the two fusion combinations, *S. acaule* (+) White Lady 15.dh.8.2.2 and *S. acaule* (+) White Lady 7.dh.23.1.1. Two weeks later, the first microcalli were observed. After 2 months culture, 229 calli derived from the combination *S. acaule* (+) White Lady 15.dh.8.2.2, and 289 calli derived from the combination *S. acaule* (+) White Lady 7.dh.23.1.1 were transferred onto the shoot regeneration medium D. One callus (0.4%) regenerated into shoots from the fusion combination *S. acaule* (+) White Lady 15.dh.8.2.2 and it formed a total of 18 shoots. The first 2 shoots regenerated 5 months after transferring to medium D, i.e. 8 months after the protoplast fusion and plating. The fused protoplasts of the combination *S. acaule* (+) White Lady 7.dh.23.1.1 regenerated into shoots faster –4 months after the protoplast fusion. Fifteen calli (5%) regenerated into a total of 16 shoots in this combination.

The RAPD analysis was carried out for five regenerated plants derived from the same callus of the combination *S. acaule* (+) White Lady 15.dh.8.2.2. The RAPD markers

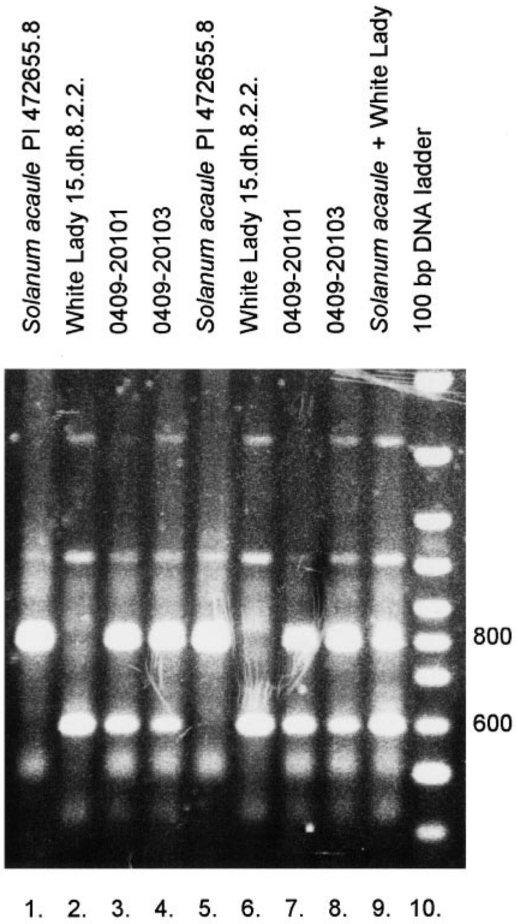

Fig. 1 RAPD analysis of regenerants derived from the protoplast fusion combination *Solanum acaule* (+) *S. tuberosum* White Lady 15.dh.8.2.2. Somatic hybrids (*lanes 3, 4, 7, 8*) and the mixed DNA sample (*lane 9*) had the combination of the banding patterns of the fusion partners, *S. acaule* (*lanes 1, 5*) and *S. tuberosum* (*lanes 2, 6*). The PCR amplification was done using the primer OPK-02

showed that all of the regenerants were somatic hybrids between *S. acaule* and *S. tuberosum* (Fig. 1). The primer OPK-02 (5′GTC TCC GCA A3′) amplified a *S. acaule*-specific band of 800 bp which also appeared in the protoplast fusion regenerants. The same primer amplified a dihaploid White Lady 15.dh.8.2.2 specific band of 600 bp and it also appeared in the fusion regenerants (Fig. 1) confirming that the shoots were somatic hybrids. In testing the hybridity in the combination *S. acaule* (+) White Lady 7.dh.23.1.1, the primer OPD-20 (5′ACC CGG TCA C3′) was used. It produced a *S. acaule*-specific band of 1000 bp and a White Lady 7.dh23.1.1-specific band of 500 bp. Each of the three plants analysed regenerated from a separate callus. Two plants had both the bands, confirming hybridity, and one plant had a similar banding pattern to *S. acaule*.

Chromosome numbers and flow cytometric analysis of five somatic hybrids of the combination *S. acaule* (+) White Lady 15.dh.8.2.2 showed that they were hexaploid. The 2 C value of 4x *S. acaule* determined by flow cytometry was 2.95 pg, and the 2C value of 2x *S. tuberosum* White Lady 15.dh.8.2.2 was 1.74 pg. The somatic hybrids had the 2C values varying from 4.35 pg (hybrid 0409–20109) to 4.76 pg (hybrid 0409–20101) which approximated to the sum of 2C values of the parental lines (4.69 pg) (Table 1). The chromosome numbers proved that the somatic hybrids were euploid or very close to euploid at the hexaploid level (Table 1). Similarly, using flow cytometry, the somatic hybrids of the combination *S. acaule* (+) White Lady 7.dh.23.1.1 were also hexaploid.

In the greenhouse, the morphology of the two somatic hybrids of *S. acaule* (+) White Lady 15.dh.8.2.2 was partly intermediate between the fusion partner lines, but most of the characters assessed were closer to *S. acaule* than to *S. tuberosum* (Table 2). *S. acaule* had a spreading growth habit and anthocyanin-pigmented stems. It had mid-green leaves and mottled pigmentation on leaf axils. The flowers were purple with a short style. Dihaploid *S. tuberosum* had an erect growth habit and no anthocyanin pigmentation. Its leaves were light green. Dihaploid *S. tuberosum* White Lady 15.dh.8.2.2 did not produce flowers in the greenhouse or in the field. The two somatic hybrids characterized had a spreading growth habit resembling *S. acaule*, but were taller. Their internode distance was longer than in the parental lines. The leaves were mid-green with a light gloss similar to *S. acaule*. The number of secondary leaflets was higher than in *S. acaule*, but not as high as in *S. tuberosum*. The surface of the leaflets was rough, similar to *S. acaule*, but the leaflets were larger than in the parental lines. Anthocyanin pigmentation was similar to

Table 1 Results of the cytological and flow cytometric analyses of the somatic hybrids between 4x *Solanum acaule* and 2x *S. tuberosum*. 2C values are the mean of three flow cytometric measurements

	Chromosome number ploidy level	2C value (pg)	SE
Protoplast fusion parental lines			
S. acaule PI 472655.8	48/4x	2.95	0.02
S. tuberosum White Lady 15.dh.8.2.2	24/2x	1.74	0.03
Somatic hybrids			
0409-20101	70/6x	4.76	0.12
0409-20103	72/6x	4.63	0.06
0409-20105	68/6x	4.63	0.10
0409-20107	72/6x	4.36	0.08
0409-20109	69/6x	4.35	0.04

Table 2 Plant morphological characteristics of the somatic hybrids *S. acaule* (+) *S. tuberosum* White Lady 15.dh.8.2.2 compared with their parental lines

Characteristic	*S. acaule*	*S. tuberosum* White Lady 15.dh.8.2.2	Somatic hybrid 0409-20101	Somatic hybrid 0409-20103
General and canopy category				
General vigour	Good	Good	Good	Good
Plant height (cm)	30	20	50	40
Plant habit	Spreading	Erect	Spreading	Spreading
Internode distance (cm)	1	1	3	4
Foliage colour	Mid-green	Light green	Mid-green	Mid-green
Foliage gloss	Light gloss	Medium gloss	Light gloss	Light gloss
Leaf and stem morphology				
Days to senescence	100	100	120	110
Secondary leaflets (*n*)	2	8	6	7
Lateral leaflet surface	Rough	Smooth	Rough	Rough
Terminal leaflet size (cm^2)	11	22	24	28
Lateral leaflet size (cm^2)	6	11	15	14
Anthocyanin on stem	General	Absent	General	General
Anthocyanin on leaf axil	Mottling	Absent	Mottling	Mottling
Floral characteristics				
Days to flowering	49	–[a]	49	61
Days to 50% of flowers opening in the first cluster	53		53	67
Buds/flowers per cluster	8		12	7
Flower colour	Purple		Purple	Purple
Anther colour	Yellow		Yellow	Yellow
Anther morphology	Regular		Regular	Regular
Protrusion of style	Short, 1–3 mm		Average, 3–5 mm	Average, 3–5 mm
Petal diameter (mm)	26		29	28
Length of anther (mm)	3.0		4.5	5.0
Colour of stigma	Light green		Dark green	Dark green
Tuber characteristics				
Tuber shape	Round[b]	Short oval	Short oval	Short oval
Tubers/plant	29	20	24	24
Total tuber weight/plant (g)	17	150	22	39
Eyes/tuber	1–3	4–5	3	3
Tuber skin colour	White	White	White	White
Tuber flesh colour	White	Light yellow	White	White

[a] Completely sterile without flower production

[b] Tubers of *S. acaule* produced in a growth chamber at 15 °C in a 12-h photoperiod

S. acaule. The flower characteristics of the somatic hybrids were also very similar to those of *S. acaule*, but their flowers were a little bigger and the anthers and styles longer than those of *S. acaule* (Table 2, Fig. 2). *S. acaule* did not tuberize in the greenhouse in a 16-h photoperiod at 18–22°C, but under a short day (12 h) and cool conditions (15°C) in a growth chamber it produced tubers. This contrasted with the dihaploid White Lady and the two somatic hybrids, all of which produced tubers in the greenhouse. The two somatic hybrids were both male fertile as determined by acetocarmine staining of pollen grains. The hybrid 0409-20101 also produced berries with seeds, demonstrating its female fertility.

Discussion

Interspecific somatic hybridization between cultivated potato and its wild relatives has already demonstrated its potential in potato breeding. Potato is a species where fusion technology has been very successful (Waara and Glimelius 1995). While primary somatic hybrids are often low yielding, the backcross populations derived from them have introgressed genomes from wild potato species such as *S. brevidens* (Helgeson et al. 1993), *S. bulbocastanum* (Helgeson et al. 1995) and *S. commersonii* (Carotenuto et al. 1997) into the cultivated potato gene pool and improved the agronomic and resistance traits of potato. Some of these traits, such as soft rot (*Erwinia* sp.) resistance (Austin et al. 1988), and late and early blight (*Alternaria solani* Sorauer) resistances (Helgeson et al. 1995) expressed in the somatic hybrids were heritable and segregated freely in the backcross populations (Austin et al. 1988; Helgeson et al. 1993, 1995). An alternative approach to incorporate resistance characters from wild potato species would be repeated cycles of anther culture and somatic hybridization as described by Rokka et al. (1995).

S. acaule possesses broad disease resistance and tolerance to many abiotic stresses (Hawkes 1994). There has

Fig. 2 Plant morphology of *S. acaule* (*left*), dihaploid *S. tuberosum* White Lady 15.dh.8.2.2, and their somatic hybrids 0409-20103 and 0409-20101 (*right*)

already been some success in introgression of the *S. acaule* genome into the potato gene pool (see Ross 1986). However, direct hybridization between *S. tuberosum* and *S. acaule* is difficult because of the EBN differences. In addition to sexual crossing, somatic hybridization could enhance the introgression of the valuable polygenic traits of *S. acaule*. Some high-yielding relatively frost tolerant potato lines have also been produced using sexual hybrids of *S. acaule* and *S. tuberosum* (Mastenbroek 1956), but according to Vayda (1994) all *S. tuberosum* cultivars were still frost sensitive. *S. acaule* is valuable for potato breeding because it is also able to cold harden (Chen and Li 1980). However, the glycoalkaloid content is quite high in many Andean frost-resistant species (Burton 1989) which may become a bottleneck for cultivar development. Thus, our ongoing work is monitoring both frost tolerance and glycoalkaloid composition in the interspecific hybrids we produce. The monogenic PVX resistance and also the wart [*Synchytrium endobioticum* (Schilb.) Perc.] resistance derived from *S. acaule* exist in many cultivars (see Ross 1986). The expression of immunity to bacterial ring rot, which has been earlier determined in our *S. acaule* PI 472655.8 clone (Ishimaru et al. 1994; Kriel et al. 1995), is also an interesting character to follow according to its expression after several hybridization cycles.

The ploidy level of the somatic hybrids between tetraploid *S. acaule* and dihaploid *S. tuberosum* was hexaploid as expected. The 2 C values of two somatic hybrids analysed with flow cytometry were approximately the sum of the 2 C values of the parental species. The chromosome counts also proved that the somatic hybrids were euploid or close to euploid at the hexaploid level. It seems that the general morphology of the hybrids is related to their parental genome doses. Morphologically, our somatic hybrids were closer to the 4x *S. acaule* than to the 2x *S. tuberosum*, as was also found in the work of Yamada et al. (1997) where the hybrids resembled more 4x *S. tuberosum* than 2x *S. acaule* parental species. Because *S. acaule* is an allotetraploid species derived via interspecific hybridization of the Tuberosa series (AA) and the Megistacroloba (A^aA^a) series (Matsubayashi 1982; Hawkes 1990), the produced somatic hybrids ($AAAAA^aA^a$) should contain more genetic material (2/3) from the Tuberosa series. In terms of genetic composition, our somatic hybrids would be comparable to other hexaploid somatic hybrids between tetraploid *S. tuberosum* cultivars and diploid wild potato species (Austin et al. 1986; Cardi et al. 1993). Hexaploid somatic hybrids often cross better with tetraploid pollinators than their tetraploid counterparts (Ehlenfeldt and Helgeson 1987). Similarly, our interspecific hexaploid hybrids produced flowers of normal morphology and had both male and female fertility, even though the meiosis of hexaploid somatic hybrids is generally more abnormal than that of tetraploids (Ehlenfeldt and Helgeson 1987). More seeds have also been obtained by backcrosses using hexaploid hybrids rather than tetraploid hybrids as parents (Ehlenfeldt and Helgeson 1987). In this light, our hexaploid hybrids may have potential in introgression of useful traits of *S. acaule* to the gene pool of cultivated potato.

Previously, hexaploid somatic hybrids between tetraploid *S. tuberosum* and dihaploid *S. acaule* (Yamada et al. 1997) have been produced, as have intergeneric somatic hybrids between *Lycopersicon esculentum* and *S. acaule* (Schweizer et al. 1988). From the genetic point of view, it would also be interesting to produce tetraploid somatic hybrids between dihaploid *S. acaule* (Rokka et al., in press) and dihaploid *S. tuberosum* lines to follow the recombination of homoeologous chromosomes and expression of *S. acaule* traits in the cultivated potato genome.

The present work showed the production of rare hexaploid somatic hybrids between tetraploid wild potato species *S. acaule* and a dihaploid *S. tuberosum* line. These somatic hybrids will be incorporated into potato breeding programmes in the future.

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