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ORIGINAL PAPER

Novel somatic hybrids (*Solanum tuberosum* L. + *Solanum tarnii*) and their fertile BC_1 progenies express extreme resistance to potato virus Y and late blight

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Abstract *Solanum tarnii*, a wild diploid, tuber-bearing Mexican species belonging to the series *Pinnatisecta* is highly resistant to Potato virus Y (PVY) and Colorado potato beetle and shows a strong hypersensitive reaction to *Phytophthora infestans*. Therefore, it could be a potential source of resistance to pathogens for potato breeders. *S. tarnii* ($2n = 2x = 24$) is reproductively isolated from tetraploid *Solanum tuberosum* and hence difficult to include in potato breeding programmes. In this study, interspecific somatic hybrids were produced for the first time by protoplast electrofusion of the cells of potato cv. Delikat (*Solanum tuberosum* L.) and *Solanum tarnii*. The hybrid nature of the regenerants was confirmed by simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) markers and by morphological analysis and flow cytometry. Selected somatic hybrids were successfully backcrossed with cv. Delikat. Parental lines, primary somatic hybrids and BC_1 progeny were assessed for resistance to PVY by mechanical inoculation, grafting and exposure to viruliferous aphid vectors in the field, and resistance to late blight (*P. infestans*) by detached leaflet and whole tuber tests. The somatic hybrids showed no symptoms of viral infection and most of them displayed high levels of resistance to foliage blight. The BC_1 progenies were highly resistant to PVY and a few were resistant to foliage blight. Selected hybrids and BC_1 clones were evaluated in the field for tuber quality and tuber yield. Some BC_1 clones produced yields of good quality tubers. The results confirm that both the resistance to PVY and to late blight of *S. tarnii* is expressed in somatic hybrids, and PVY resistance is transferred to BC_1 progeny, whereas blight resistance is harder to transfer. Somatic hybridization again proved to be a valuable tool for producing pre-breeding material with increased genetic diversity.

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Introduction

Solanum tarnii, a wild diploid ($2n = 2x = 24$), tuber-bearing Mexican species belonging to the series *Pinnatisecta* is highly resistant to Potato virus Y (PVY) and Colorado potato beetle (Thieme and Thieme 2006), and shows a strong hypersensitive reaction to *Phytophthora infestans*. Sexual or somatic hybrids between *S. tarnii* and common potato are unknown (Jackson and Hanneman 1999). This

1EBN (Endosperm Balance Number) species is reproductively isolated from tetraploid *Solanum tuberosum* and hence difficult to include in classical breeding programmes (Johnston et al. 1980; Jackson and Hanneman 1999). Protoplast fusion allows the transfer of both mono- and polygenic traits between sexually incompatible species. In the past, a number of attempts were made to hybridize different diploid 1EBN wild species to produce somatic hybrids. Symmetric protoplast fusion with *S. brevidens* was used to integrate virus and aphid resistance (Austin et al. 1985; Gibson et al. 1988; Valkonen et al. 1994) and tuber soft rot and early blight resistance into the potato gene pool (Polgar et al. 2000; Tek et al. 2004). Hybrids between *S. etuberosum* + *S. tuberosum* and some of their BC_1 clones show increased resistance to PVY, based on mechanical inoculation (Novy and Helgeson 1994), and extreme PVY resistance, and grafting under greenhouse conditions using an aggressive isolate of PVY^N (Gavrilenko et al. 2003; Thieme et al. 2004). The BC_2 progeny of somatic hybrids (potato × *S. etuberosum* + *S. tuberosum* × *S. berthaultii* hybrids) showed an increased resistance to PVY, *Potato leafroll virus* (PLRV) and aphids in greenhouse and field trials (Novy et al. 2002). The PLRV resistance was transmitted and expressed in the third generation of backcrossing to cultivated potato (Novy et al. 2007). Somatic hybrids between cultivated potato and Mexican, 1EBN wild species *S. pinnatisectum* are also resistant to late blight (Ward et al. 1994; Thieme et al. 1997). Somatic hybrids between *S. bulbocastanum* and *S. tuberosum*, and backcrosses, are resistant to high exposure to late blight in the field (Helgeson et al. 1998). This germplasm was used in sexual crosses to transfer additional resistance into potato breeding lines. Another efficient way of exploiting the potentially durable late blight resistance of wild *Solanum* species is to transfer these genes into existing potato cultivars by transformation. This has been described by Naess et al. (2000) and Song et al. (2003) for RB-genes and is an excellent example of the exploitation of *S. bulbocastanum* germplasm using somatic hybridization by protoplast fusion followed by gene(s) mapping, characterization and transfer by both conventional breeding and transformation.

Here we report the production of fertile somatic hybrids between potato cv. Delikat and *S. tarnii* and their BC_1 clones. This plant material was characterized morphologically and by agronomically important characters, in terms of ploidy level by flow cytometry, by molecular markers (SSR and AFLP) and its reaction to viruses and late blight. It was demonstrated that the *S. tarnii*-derived somatic hybrids and BC_1 clones could be utilized in breeding programmes for the introgression of resistance to PVY and late blight into commercial potato cultivars.

Materials and methods

Plant material

The potato, *S. tuberosum* L. subsp. *tuberosum* cv. Delikat (Norika, Germany), which is an early maturing variety with large oval tubers and shallow eyes, yellow skin and light yellow flesh, was used in fusion experiments, and as a pollinator and parental cultivar in greenhouse and field trials. Seeds of the diploid Mexican species *S. tarnii* Hawkes et Hjerting (Hawkes et al. 1988), accession GLKS 2870 from IPK Genebank External Branch 'North', Gross Lüsewitz, Germany, were germinated in vitro, and plants from one seedling were used for propagation. The middle to late maturing cv. Sonate (Norika, Germany) was used as a standard in the field trials and as a pollinator for the production of BC_2 progenies.

Protoplast isolation and fusion

Plants of *S. tarnii* and cv. Delikat were micro-propagated in vitro on MS5 medium (Murashige and Skoog 1962), modified by reducing the NH_4NO_3 content to 1.2 g/l. Only one shoot was selected and analyzed for resistance, cloned and used for isolating the protoplasts of both parental species. Shoot apices were transferred each month to MS5 medium. Three to four week old in vitro plants were used for isolating mesophyll protoplasts following the protocol of Möllers et al. (1992). The enzyme solution contained 0.2% macerozyme and 1% cellulase. After purification by sequential centrifugation, parental protoplasts at 1×10^6 pp/ml were mixed in a ratio of 1:1. This mixture was divided into 400 µl aliquots in a lamellar fusion chamber and subjected to electrofusion. The AC field was adjusted to a voltage of 100–200 V/cm and a frequency of 800–1,000 kHz applied for 1–2 min, 2 DC pulses of 1,200 V/cm amplitude and 15 µs duration with a brake of 2 s and a post ramp AC field of 10–20 s. For electrofusion, a CFA 500 device was used (Krüss GmbH, Hamburg). Fusion products and parental non-fused protoplasts were collected and cultured in modified VKM-medium with or without 2 g/l bovine serum albumin at a final density of 1×10^5 pp/ml and maintained at 25°C in the dark. The growing microcalluses were transferred to Cul-medium and kept under a fluorescent light intensity of 55.5 µmol/m^2/s^1, a 16 h photoperiod and 25°C. Newly formed calluses were cultured on regeneration medium RJM and kept under the same conditions. Each month the calluses were transferred onto fresh media until the shoots developed. These shoots were rooted and propagated on MS5-medium. The media used and the corresponding references are as given by Möllers et al. (1992) and Thieme et al. (1997).

Identification of somatic hybrids using flow cytometry and molecular markers

Somatic hybrid clones showing vigorous growth were selected at the callus stage and only the first shoot per callus was cloned in vitro and used for further analysis. The hybrid nature of the shoots was first proved by a flow cytometric determination of ploidy level (Thieme et al. 1997), then SSR assay (Dinu and Thieme 2001) and AFLP analysis.

DNA samples were prepared from leaf tissue of in vitro potato plants. The fast small-scale modified DNA isolation procedure used by Dorokhov et al. (1997) was applied. Briefly, 5 mg of leaf material was vigorously homogenized at room temperature in a mixer-mill disruptor (Retsch GmbH & Co. KG) in extraction buffer. DNA was precipitated with isopropanol, washed with 70% ethanol, air dried and re-dissolved in 100 µl 0.1× TE buffer with RNase A (100 µg/ml). The DNA concentration was measured using a fluorometric assay and a Spectra Max 190 fluorometer (Molecular Devices). Polymerase chain reactions (PCR) were performed in a MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories, Inc.) with cycle parameters described by Provan et al. (1996). PCR was carried out in a total volume of 20 µl and consisted of 30 ng template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 200 mM dNTPs, 1 unit Taq polymerase (Invitek). The amplification products were separated on a 6% polyacrylamide denaturing gel in a Sequi-Gen GT sequencing cell (Bio-Rad Laboratories, Inc.). The DNA fragments were detected using silver-staining. The AFLP analyses were carried out as described by Vos et al. (1995) using enzyme-primer combinations: Pst-AGC/Mse-CTG, Pst-AGC/Mse-AAT, Pst-CTG/Mse-AAT, Pst-CTG/Mse-CAT, Pst-CTG/Mse-CTG, Eco-ACT/Mse-CGA and Eco-ACC/Mse-CCT. The microsatellite-anchor fragment length polymorphisms method (MFLP), which combines the concept of AFLP, and the microsatellite anchor primer technique (Yang et al. 2001), was also used. After digesting a 500 ng DNA sample with MseI restriction enzyme (5 Units), the Mse-adaptor was ligated to the digestion fragments. For the amplification 0.2 µM Mse-primer and anchor primers were used. All Mse-primers contained one or three selective nucleotides at the 3′ ends. The anchor primer MF 51 (0.4 µM) included a trinucleotide SSR motif and was labeled with the fluorescence dye Cy5. The detection of the selective PCR products was performed on an automatic laser fluorescence-sequencing machine (ALFexpress, GE Healthcare).

Crossing experiments

Flowers of greenhouse grown somatic hybrid plants were emasculated at the bud stage and pollinated with pollen of cv. Delikat to produce BC_1 progeny. Cv. Sonate was used as the pollen parent for the generation of BC_2 progeny. Berries were harvested and seeds cultivated in vitro using immature seeds or an embryo rescue technique (Thieme 1991). The seeds with scarified testa were transferred to MS5-medium. In some cases embryos between the torpedo and cotyledon stages were isolated and transferred. The number of seeds plus embryos was recorded.

Assessment of resistance to PVY, late blight and agronomic traits

Parental clones, somatic hybrids and BC_1 plants were screened for resistance to PVY by the mechanical inoculation of greenhouse-grown plants (Thieme and Thieme 1998). Depending on the virus isolate, 5–30 plants were assayed per genotype for the presence of PVY in their leaves using an enzyme-linked immunosorbent assay (ELISA). Isolates of all known virus strains were separately included in this test: Amigo-N150/1 (PVY^N, H. Weidemann, BBA, Braunschweig, Germany); Q3 (PVY^C, I. Browning, SASA, Edinburgh, Scotland); Linda (PVY^{NTN}, BAZ, Germany); Wilga O (PVY^NW, M. Chrzanowska, IHAR, Mlochow, Poland), CH605 (PVY^N, P. Gugerli, RAC, Nyon, Switzerland) and 205 (PVY^O, BAZ). For grafting experiments, greenhouse-grown tobacco plants infected with Amigo-N150/1 (PVY-^{N}) and in vitro plants (10–20 replicates per genotype) were used as PVY-infected recipient and scion, respectively. The new shoots that developed on the scions 4 weeks after greenhouse cultivation at 20°C were sampled and subjected to ELISA.

For the field experiments, in vitro plants of selected somatic hybrids, BC_1 clones, parental clones and control varieties were transferred and cultivated in a greenhouse in April/May. At the beginning of June these plants (28 plants per genotype) were transferred to the field in Braunschweig, a region that usually has a high incidence of aphid infestations during summer. The area planted was 12 m × 63 m, with seven plants per genotype (one plot) in four repeats. Virus infected tubers of cv. Linda were planted as three additional rows between the test clones. The occurrence of aphids was scored in June and at the beginning of July by counting the number of alatae, apterae and larvae on two leaves per plant. Additionally, the number of potato colonizing species of aphid on one upper and lower leaf of each of the seven plants of the eleven BC_1 clones and parental lines was recorded.

Evaluated traits of the field-grown plants included survival, habit, tuber number and weight, time and intensity of flowering and maturity of the plants. Tubers were harvested and tuber traits were assessed at the end of September. Tuber weight was determined after combining the tubers of all plants per plot. The yield of an average of four plots was calculated. After a storage period of 3 months, the tubers (14–63 per genotype) were planted in a greenhouse and the

sprouts tested (EBA, excised-bud-assay) in March 2004 for presence of PVY, using ELISA.

Greenhouse-grown plants were assessed for resistance to foliage blight, using the detached leaflet assay method described by Darsow et al. (1988). Leaflets were collected from the middle part of plants that were beginning to flower in June. Five leaflets per clone were single drop-inoculated with 10 µl of a highly aggressive isolate of *P. infestans* with a complex virulence spectrum. The virulence of this isolate was evaluated relative to the widely used Black's differentials carrying R-genes from R1-R11, i.e. v1- v11 (Black et al. 1953). The inoculum density was 5×10^4 zoospores/ml and was prepared by cooling a sporangial suspension of 1.7×10^4 sporangia/ml for 1.5–2 h. Five days after inoculation the intensities of necrosis and sporulation were scored and expressed on a 1–9 scale. Scores for the affected leaf area are given as: 9 = no incidence; 8 = 0.1% with small separate necrotic lesions; 7 = 3–5%; 6 = 15%; 5 = 40%; 4 = 60%; 3 = 75%; 2 = 90% and 1 = 100% with necrotic lesions. Scores for sporulation are also given on 1–9 scale, 9 = no sporulation, 8, 7 = slight; 6, 5 = moderate; 4, 3 = intense sporulation, 2, 1 = intense sporulation over 90–100% of the leaf area. The individual scores for necrosis and sporulation were used to calculate the average scores.

The whole tuber test was used to determine the resistance of tubers to late blight according to Darsow (1983). Field-grown tubers stored at 8°C for 4 months were used for this analysis. The tubers (four tubers per clone) were cut at the stem end and dipped into a solution containing 2.7×10^4 zoospores/ml (9×10^3 sporangia/ml). Six days after inoculation the intensity of necrosis and sporulation were scored after cutting the tubers. A scale of 1 (susceptible) to 9 (resistant) was used according to the percentage of internal infection. The cvs. Adretta and Tevadi were included in these tests as susceptible standards.

Results

Production and characterization of somatic hybrids

Mesophyll protoplast electrofusion produced sufficient fusion products of both multi- and biparental origin. These fusion products thrived in culture, with the first division occurring 3–4 days after fusion and cell colonies 1–2 mm in diameter developing in 3–4 weeks. Vigorous growth of macrocalluses and the morphology of the regenerated shoots were used as criteria for selecting the putative hybrids. In total, in two fusion trials, 3,350 calluses were cultivated (Table 1). Ploidy determination allowed a more precise selection of somatic hybrids—fusion of protoplasts from diploid (*S. tarnii*) and tetraploid (*S. tuberosum* cv. Delikat) clones should produce hexaploid hybrids. In total, 63 hexaploid regenerants were selected. In addition to hexaploid plants, one mixoploid and three octoploid regenerants were also selected. Very slightly deformed or stunted aneuploid or polyploid plants were eliminated at the in vitro stage. Final identification of interspecific somatic hybrids using SSR (not shown), AFLP (Fig. 1) and MFLP (not shown) analyses confirmed the hybrid nature of 67 regenerants, showing an additive pattern of the prominent bands of the parents: the potato cv. Delikat and *S. tarnii* (Table 1; Fig. 1). Somatic hybrids were transferred to a greenhouse, where their morphology was analyzed. The hybrids were intermediate in morphology between parental species (Fig. 2). Slight differences in leaf and flower shape, height, flower colour and tuber shape were observed among the hybrids (Fig. 2a, b, c), but in general all hybrids were more like the *S. tuberosum* parent. First generation somatic hybrids cultivated in the field produced good quality tubers (Fig. 2c).

Assessment of PVY and late blight resistance of the somatic hybrids

None of the greenhouse grown somatic hybrids tested became infected with PVY after mechanical inoculation with six different isolates of five PVY strains (Table 2). The extreme resistance of *S. tarnii* and the somatic hybrids to PVY was confirmed by grafting and field experiments (Table 2). Plants of potato cv. Delikat became infected in the greenhouse and field trials (80–100 and 25–95%, respectively, Table 2). Somatic hybrids that showed no evidence of PVY after artificial infection were used for foliage and tuber blight assessment.

Table 1 Results of the protoplast fusion experiments between potato (*tbr*) cv. Delikat and *S. tarnii* (*trn*)(mix–mixoploid)

Fusion, date	Total no of calluses (*n*)	Re-generation of the first shoot (months)	No of shoots (=calluses) transferred (*n*)	Results based on an analysis using flow cytometry and molecular markers			
				No of Plants analysed	No of hybrid shoots (ploidy)	No of Parental regenerants *tbr*	No of Parental regenerants *trn*
I 08/1999	950	4	49	34	17 (6*x*) 3 (8*x*)	14	0
II 06/2000	2400	6	155	131	46 (6*x*) 1 (mix)	84	0

Fig. 1 Identification of the somatic hybrids (*H*) between *S. tuberosum* cv. Delikat (*D*) and *S. tarnii* (*W*) using AFLP analyses with following enzyme-primer combinations: **a** Pst-AGC/Mse-CTG, **b** Pst-AGC/Mse-AAT, **c** Pst-CTG/Mse-AAT, **d** Pst-CTG/Mse-CAT, **e** Pst-CTG/Mse-CTG, **f** Eco-ACT/Mse-CGA, **g** Eco-ACC/Mse-CCT

In the detached leaflet assay, the resistant parent *S. tarnii* scored as 8.7 and the susceptible cv. Delikat as 2.8. Of 31 hybrids, 24 were resistant and the remaining 7 were susceptible to foliage blight (score ≤6.9). Four of the ten somatic hybrids subjected to further study expressed the same high level of resistance to late blight as *S. tarnii* (Table 3). The other six hybrids had scores from 7.3 to 8.4, indicating a high level of resistance to foliage blight. The whole tuber test gave scores of 3.7–6.1 for the hybrids, which were better than the score of 3.2 for cv. Delikat, but lower than the level of resistance shown by the wild parent, *S. tarnii*, which had a score of 7.0 (Table 3).

Production of the BC_1 and BC_2 progeny

Eight hexaploid hybrids, all extremely resistant to PVY and highly resistant to foliage blight, were used in backcross experiments (Tables 2, 3). With the exception of one, all these hybrids produced flowers (Fig. 2a). The backcrossing was expected to reduce the ploidy level of the hexaploid somatic hybrids and improve their agronomic traits. After nearly 140 pollinations of seven hybrids, 87 berries developed (Table 3). Fifteen weeks after pollination, immature seeds or in some cases embryos were rescued from berries of three hybrids and a total of 227 BC_1 clones were regenerated (Table 3). BC_1 clones were propagated in vitro and used in further tests. In 2006, eight-selected BC_1 clones that originated from the somatic hybrids 838/2 and 838/7 were successfully backcrossed with PVY susceptible cv. Sonate. More than 40 berries were harvested and the seed was used

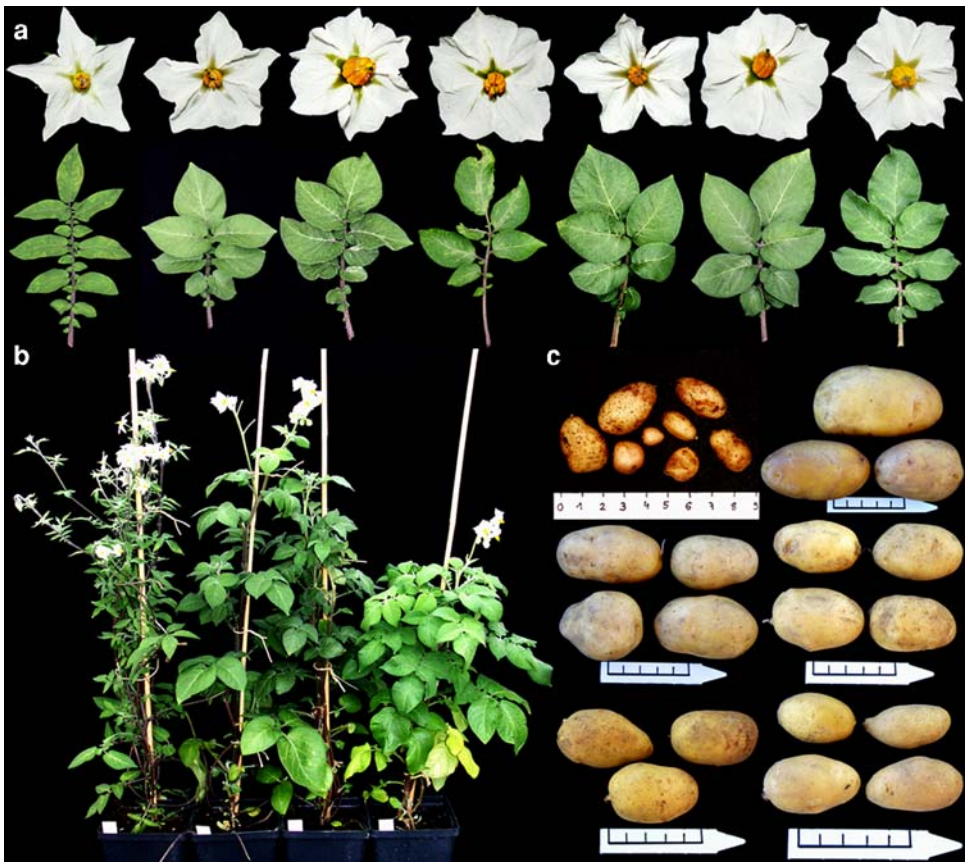

Fig. 2 Morphological traits of the somatic hybrids compared with that of their parents. **a** Flowers and leaves of *S. tarnii*, the hexaploid hybrids 838/2, 838/7, 849/4, 849/5, 838/11 and potato cv. Delikat (from left to right, respectively). **b** Greenhouse-grown plants of *S. tarnii*, the hexaploid hybrids 838/2; 838/7 and potato cv. Delikat (*from left to right*, respectively). **c** Tubers of *S. tarnii* and potato cv. Delikat, (*top*), the somatic hybrids 838/2; 838/7 (*middle row*) and BC_1 clones 838/7/24 and 838/7/38 (*bottom*) grown in the field (scale: 5 cm)

Table 2 The assessment of resistance to single PVY strains of somatic hybrids (*H*) between potato cv. Delikat + *S. tarnii*: PVY incidence following mechanical inoculation and grafting in a greenhouse and in field trials (2003–2006) using the following strains (of isolates): PVY^N (Amigo-N150/1)–**N***, PVY^N (CH605)–**N**, PVY^O (205)–**O**, PVY^C (Q3)–**C**, PVY^{NTN} (Linda)–**NTN**, PVY^NW (Wilga O)–**W**, (number of plants tested/number of plants infected; nt–not tested)

Genotype	Virus tests										
	Mechanical inoculation						Grafting field				
								2003	2004	2005	2006
	N*	**N**	**O**	**C**	**NTN**	**W**	**N***	**N***	**N***	**N***	**N***
cv. Delikat	20/20	5/5	5/5	5/5	5/5	5/5	15/12	68/17	26/18	58/36	20/19
S. tarnii	20/0	5/0	5/0	5/0	5/0	5/0	13/0	nt	nt	nt	nt
H 838/2	20/0	5/0	5/0	5/0	5/0	5/0	15/0	62/0	25/0	30/0	39/0
H 838/7	19/0	5/0	5/0	5/0	5/0	5/0	15/0	61/0	23/0	40/0	56/0
H 838/11	20/0	5/0	5/0	5/0	5/0	5/0	15/0	58/0	nt	nt	nt
H 848/5	20/0	nt	nt	nt	nt	nt	12/0	58/0	nt	nt	nt
H 849/4	20/0	5/0	5/0	5/0	5/0	5/0	14/0	63/0	nt	nt	nt
H 849/5	20/0	5/0	5/0	5/0	5/0	5/0	15/0	54/0	nt	nt	nt
H 851/2	20/0	5/0	5/0	5/0	5/0	5/0	9/0	64/0	nt	nt	nt
H 852/5	20/0	5/0	5/0	5/0	5/0	5/0	nt	nt	nt	nt	nt
H 857/3	20/0	5/0	5/0	5/0	5/0	5/0	nt	nt	nt	nt	nt
H 857/5	20/0	5/0	5/0	5/0	5/0	5/0	15/0	63/0	nt	nt	nt

Tubers from the field trials were tested using the excised-bud-assay and ELISA

Table 3 The assessment of ploidy, resistance to late blight (*Phytophthora infestans*) and fertility of somatic hybrids (H) between potato cv. Delikat + *S. tarnii*: resistance to foliage (detached leaflet assay) and tuber blight (whole tuber lab test) is recorded on a 1–9 scale (1–susceptible, 9–resistant); the numbers of crosses, berries, immature seeds (rescued embryos or incised seeds in vitro); BC_1 and BC_2 clones resulting from crosses between somatic hybrids and potato cv. Delikat and cv

Genotype	Ploidy	Scores for late blight test		Number of			
		Foliage blight (mean ± SD)	Tuber blight (mean ± SD)	Pollinations/ berries	Berries/ immature seeds	BC_1 clones	BC_2 clones
cv. Delikat	4*x*	2.8 ± 1.2	3.2 ± 2.1	–	–	–	–
S. tarnii	2*x*	8.7 ± 0.5	7.0 ± 1.8	–	–	–	–
H 838/2	6*x*	9.0 ± 0.0	5.0 ± 1.3	32/16	4/145	122	130
H 838/7	6*x*	7.1 ± 0.9	5.0 ± 1.6	10/5	4/92	62	320
H 838/11	6*x*	9.0 ± 0.0	3.7 ± 0.5	6/2	2/67	43	nt
H 848/5	6*x*	7.3 ± 3.3	5.1 ± 1.1	nf	–	–	–
H 849/4	6*x*	9.0 ± 0.0	6.1 ± 1.6	22/16	nt	nt	nt
H 849/5	6*x*	7.9 ± 2.3	5.3 ± 0.8	30/23	nt	nt	nt
H 851/2	6*x*	9.0 ± 0.0	5.0 ± 0.0	14/7	nt	nt	nt
H 852/5	Mix	7.7 ± 2.1	nt	nt	nt	nt	nt
H 857/3	6*x*	7.3 ± 2.6	nt	nt	nt	nt	nt
H 857/5	6*x*	8.4 ± 1.7	5.1 ± 0.7	27/18	nt	nt	nt

Sonate, respectively, are also indicated (*nt* not tested, determined, *nf* no flowers)

to produce 450 BC_2 clones (Table 3). Additionally, backcrosses of two other somatic hybrids with the cv. Sonate produced more than 20 berries.

AFLP marker analysis showed that the BC_1 genotypes tested contained bands specific to the wild species *S. tarnii* (Figs. 1, 3).

Assessment of agronomic characteristics of BC_1 field-grown plants

Parental clones, two somatic hybrids and their 29 BC_1 clones were assessed for yield per plot (Table 4), maturity and for tuber characters. The somatic hybrids and the

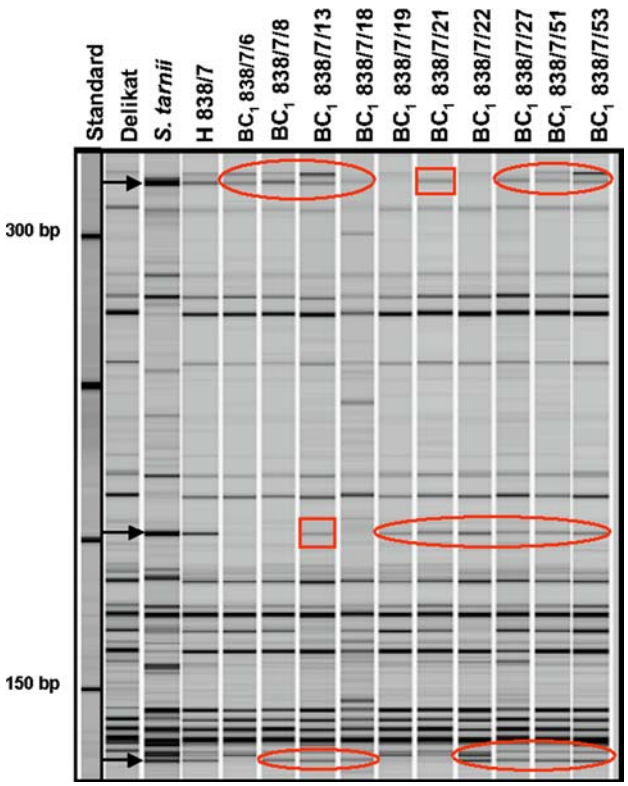

Fig. 3 An example of AFLP analysis of the somatic hybrid H 838/7, ten BC_1 clones, and the fusion partners potato cv. Delikat and *S. tarnii*, using primer: Pst-AGC/Mse-CTG

majority of the BC_1 clones were late maturing, similar to the relatively late maturing cv. Sonate, but six BC_1 clones were earlier than cv. Delikat. In ten of the BC_1 clones green vines and flowers were present 3 months after planting. All clones produced tubers; in general the tuber shape was oval to long-oval with shallow eyes and slight to more intense yellow flesh. There were differences in the yield among the BC_1 clones (Table 4), but only slight differences in their tuber shape (Fig. 2c). No tubers were harvested from plants of the wild species *S. tarnii* grown in the field, but tubers were harvested from plants grown in a climatic chamber and compared with those produced by somatic hybrids and BC_1 clones grown in the field (Table 4).

Resistance to PVY and aphids of BC_1 clones

The results of the mechanical inoculation tests are shown in Table 4. It can be seen that *S. tarnii*, the somatic hybrids and the BC_1 clones are all resistant to all isolates of PVY, whereas, cv. Delikat is susceptible to all except C. The resistance was confirmed in the field. The aphid species *Myzus persicae, Aphis frangulae, A. nasturtii*, *Aulacorthum solani*, and *Macrosiphum euphorbiae*, which are known to settle on potatoes and act as virus vectors, were identified and the total number is presented in Table 4. An average of 0.7–5.1 aphids per plant were recorded on each genotype on 1 July 2004, indicating a potential for PVY infection by these vectors.

Late blight resistance of BC_1 clones

Results of the detached leaflet assay of 139 BC_1 clones derived from two of the somatic hybrids are given in the Table 5. Only three of the clones expressed a high level of resistance to foliage blight similar to that of the wild species *S. tarnii*, which shows a hypersensitive response resulting in small necrotic spots. Nine clones expressed medium resistance but most were susceptible (Table 5). All of the 30 BC_1 clones tested were susceptible to tuber late blight.

Discussion

The aim of this research was to enrich the cultivated potato gene pool by incorporating genes from a new exotic wild species, in order to enhance resistance to aphid transmitted PVY and late blight caused by *P. infestans*.

Sexual hybridization of diploid wild potato species from Mexico and common potato is limited because of the differences in ploidy levels and EBN (Johnston et al. 1980; Jackson and Hanneman 1999). Thus, protoplast fusion is the only way to introgress valuable resistant genes into the *S. tuberosum* gene pool, as it bypasses sexual incompatibility and gene segregation (Millam et al. 1995; Thieme et al. 1997, 2004; Gavrilenko et al. 2003). Therefore, somatic hybridization was used to obtain hybrids between a potato cultivar and *S. tarnii*. For this electrofusion protocol, the procedure adopted here for plant regeneration and identification of hybrid plants proved efficient. Fusion of two parental protoplasts should result in the production of hexaploid somatic hybrids, which consist of four genomes of the susceptible tetraploid potato parent (AAAA genome) and two genomes from the diploid resistant wild species *S. tarnii* (BB genome). Hexaploid hybrids (AAAABB genome) were more similar in leaf and flower morphology, tuber colour and shape to *S. tuberosum*, cv. Delikat, as expected, based on the genome dosage effect. However, the genome dosage effect did not influence the expression of viral resistance in somatic hybrids, which were extremely resistant to PVY. In the previous research, hexaploid somatic hybrids (genome composition AAAAEE) between potato and the extremely PVY resistant diploid wild species *S. etuberosum* (genome EE) were highly susceptible to viral infection (Gavrilenko et al. 2003). Sexual crosses between somatic hybrids, *S. tuberosum* + *S. tarnii* (AAAABB genome) and tetraploid potato (AAAA genome) should result in the formation of pentaploid BC_1 progeny (AAAAB genome), which possess one haploid genome of the wild species.

Table 4 The assessment of yield (tuber weight per plot) and PVY resistance of BC_1 clones, cv. Delikat + *S. tarnii* somatic hybrids (H), parental genotypes and a standard cultivar (cv. Sonate) in 2004: PVY^N (CH605)–**N**, PVY^O (205)–**O**, PVY^C (Q3)–**C**, PVY^{NTN} (Linda)–**NTN**, PVY^NW (Wilga O)–**W,** PVY^N (Amigo-N150/1)–**N***

Genotype	Tuber weight (kg/plot ±SD)	Virus tests						Aphids (*n*)
		Mechanical inoculation					Field	
		N	**O**	**C**	**NTN**	**W**	**N***	
cv. Sonate	3.47 ± 0.78	nt	nt	nt	nt	nt	28/20	nt
cv. Delikat	1.74 ± 0.33	5/5	5/5	5/0	5/5	5/5	26/16	84
*S. tarnii***	0.02	5/0	5/0	5/0	5/0	5/0	nt	20
H 838/2	3.25 ± 0.35	5/0	5/0	5/0	5/0	5/0	25/0	71
BC_1 838/2/2	1.07 ± 0.67	nt	nt	nt	nt	nt	26/0	52
BC_1 838/2/8	1.60 ± 0.26	nt	nt	nt	nt	nt	22/0	55
BC_1 838/2/14	2.31 ± 0.41	nt	nt	nt	nt	nt	25/0	142
BC_1 838/2/17	2.43 ± 0.15	nt	nt	nt	nt	nt	22/0	93
BC_1 838/2/25	1.62 ± 0.13	5/0	5/0	5/0	5/0	5/0	14/0	51
BC_1 838/2/30	1.25 ± 0.09	nt	nt	nt	nt	nt	17/0	34
BC_1 838/2/38	2.34 ± 0.27	nt	nt	nt	nt	nt	24/0	38
BC_1 838/2/44	1.77 ± 0.12	nt	nt	nt	nt	nt	21/0	59
BC_1 838/2/54	4.48 ± 0.26	nt	nt	nt	nt	nt	21/0	127
BC_1 838/2/56	2.36 ± 0.14	nt	nt	nt	nt	nt	26/0	84
BC_1 838/2/68	0.14 ± 0.01	nt	nt	nt	nt	nt	25/0	39
BC_1 838/2/77	0.31 ± 0.02	nt	nt	nt	nt	nt	20/0	35
BC_1 838/2/87	4.55 ± 0.23	5/0	5/0	5/0	5/0	5/0	27/0	34
BC_1 838/2/113	1.45 ± 0.07	nt	nt	nt	nt	nt	24/0	111
BC_1 838/2/115	1.28 ± 0.28	5/0	5/0	5/0	5/0	5/0	16/0	70
H 838/7	2.61 ± 0.22	5/0	5/0	5/0	5/0	5/0	23/0	65
BC_1 838/7/1	2.04 ± 0.15	5/0	5/0	5/0	5/0	5/0	28/0	89
BC_1 838/7/6	1.26 ± 0.07	5/0	5/0	5/0	5/0	5/0	27/0	114
BC_1 838/7/8	0.85 ± 0.08	5/0	5/0	5/0	5/0	5/0	23/0	43
BC_1 838/7/13	0.54 ± 0.05	5/0	5/0	5/0	5/0	5/0	28/0	29
BC_1 838/7/18	3.13 ± 0.29	5/0	5/0	5/0	5/0	5/0	28/0	48
BC_1 838/7/19	2.21 ± 0.31	5/0	5/0	5/0	5/0	5/0	18/0	82
BC_1 838/7/21	0.60 ± 0.08	5/0	5/0	5/0	5/0	5/0	28/0	96
BC_1 838/7/22	0.13 ± 0.01	5/0	5/0	5/0	5/0	5/0	32/0	47
BC_1 838/7/23	0.93 ± 0.22	5/0	5/0	5/0	5/0	5/0	26/0	66
BC_1 838/7/24	1.70 ± 0.17	5/0	5/0	5/0	5/0	5/0	25/0	42
BC_1 838/7/27	2.46 ± 0.24	5/0	5/0	5/0	5/0	5/0	27/0	82
BC_1 838/7/28	2.23 ± 0.23	5/0	5/0	5/0	5/0	5/0	26/0	101
BC_1 838/7/38	1.49 ± 0.10	5/0	5/0	5/0	5/0	5/0	24/0	56
BC_1 838/7/39	1.07 ± 0.14	5/0	5/0	5/0	5/0	5/0	23/0	62

Tubers from the field trials were tested using excised-bud-assay and ELISA, number of aphids was recorded on 28 plants (number of plants tested/number of plants infected; *nt* not tested, ** determined, 2 plants from a climatic chamber)

Table 5 Results of the detached leaflet assay of 139 BC_1 clones from two somatic hybrids, 838/2 and 838/7, between potato cv. Delikat and *S. tarnii*

Genotype Number	Number of clones tested	Number of clones with resistance scores of between		
		7.0–9.0 Resistant	5.0–6.9 Medium	1.0–4.9 Susceptible
BC_1 838/2/1-117	99	3	8	88
BC_1 838/7/1-62	40	0	1	39

When the R-genes, which control extreme resistance to PVY in *S. tarnii*, are in the homozygous state, resistance should segregate in BC_2. In the present study, somatic hybrids as well as their BC_1 progeny showed no incidence of PVY after mechanical inoculation, grafting and field assessment. This indicates the homozygotic dominant state of the R-genes, which control extreme resistance to PVY in *S. tarnii* and can be transferred to hybrids and BC_1 progeny. Additionally, ten somatic hybrids and three of the BC_1 clones showed a similar level of resistance to foliage blight

as *S. tarnii*, and nine clones medium resistance. The results of the detached leaflet assay presented in this paper for the somatic hybrids and BC_1 clones were, as expected, rather variable because of the quantitative nature of this trait, whose inheritance and expression are more complicated than that of the PVY resistance.

Very often somatic hybrids or their progenies produce sterile flowers, which hamper their utilization and integration into breeding schemes. This was not the case for the somatic hybrids *S. tuberosum* + *S. tarnii* and their BC_1 progeny. The hybrids were highly fertile possibly because of their cytoplasmic composition. A total of 47 of the 52 hybrids had new mitochondrial genomes—revealed by amplification of fragments from both potato and *S. tarnii* parents (Gavrilenko et al. 2005). These new combinations of nuclear and cytoplasmic genes cannot be achieved by sexual crosses. Therefore, it is advantageous to use somatic hybridization to increase the genetic diversity available for breeding potato. Some of the hexaploid somatic hybrids as well as BC clones produced good quality tubers in the field. Further field experiments using seed tubers produced by somatic hybrids, BC_1 and BC_2 clones are in progress to confirm and to broaden these results.

Mexican wild species of the series *Pinnatisecta* are known for their resistance to both pathogens (Hawkes 1990). But, most diploid *Solanum* species are represented by genetically variable populations maintained as gene bank accessions. Average resistant scores to pathogens do not reveal the genetic variation within these populations (Smilde et al. 2005). A screening of individual plants within an accession is important (Douches et al. 2001). Therefore, plants of *S. tarnii* gene bank accessions with high resistance traits needed to be identified. If the accessions are stored as seeds, plants from separate seeds have to be tested for resistance to pathogens. Only one *S. tarnii* seedling was chosen in our study for propagation, which was cloned in vitro to ensure a uniform source of resistance.

Plants with extreme resistance to PVY either show no symptoms or limited necrosis, which prevents virus multiplication. R-genes with monogenic dominant type of inheritance and comprehensive action were the obvious choice for incorporation into new cultivars. Plants of *S. tarnii* did not show any symptoms of PVY after grafting and mechanical infection, using different virus strains and isolates. In the field after colonization by virus transmitting aphids, *S. tarnii*, somatic hybrids and their BC progeny showed no symptoms of PVY infection, indicating extreme resistance to PVY. The aphids were viruliferous because the parental cv. Delikat and standard cv. Sonate were infected with PVY. Therefore, this wild species is a particularly valuable source of genetic resistance to PVY, which should complement that already found in *S. stoloniferum* and *S. hougasii* (Solomon-Blackburn and Barker 2001).

The *S. tarnii* accession used possessed a high level of resistance to foliage blight. After drop inoculation the leaves reacted by developing necrotic spots at the inoculation site, which indicated a strong hypersensitive reaction. This species could therefore provide a new source of resistance to late blight, but its inheritance and durability require more detailed assessment.

Because of the great threat of virus and aphid infestations as well as late blight to potato cultivation, and the adaptability of pathogens, the introduction of "exotic" germplasm with novel types of resistance from wild species is a promising method of increasing genetic diversity for potato breeding. Interspecific somatic hybrids and their progenies should provide new breeding clones with greater resistance to virus (Polgar et al. 2000; Novy et al. 2002). In the future, combinations of major resistance genes are likely to provide another example of somatic hybrid material and molecular markers improving the integration of more resistant traits into the potato gene pool. Marker-assisted selection using PCR-based diagnostic assays was used by Gebhardt et al. (2006) to select clones with multiple, monogenic resistant traits (PVY, PVX, root cyst nematode and potato wart).

Our results suggest that stable resistance to PVY and foliage blight can be transferred by somatic hybridization, and that it persists in BC_1 clones with some segregation for resistance to foliage late blight occurring in the BC_1 population. Recently, the hybrids and BC_1 clones exhibiting resistance to PVY and foliage blight were used to introgress these sources of resistance into potato cultivars. BC_1 clones were backcrossed and the resultant BC_2 clones had fewer chromosomes as a result of the backcrossing. However, their resistance to PVY and *P. infestans* remains to be demonstrated. Such material should be of particular interest for the molecular analyses, identification and characterization of the genetic background of the resistance to both these pathogens.

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