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# Transfer of PLRV resistance from *Solanum verrucosum* Schlechdt to potato (*S. tuberosum* L.) by protoplast electrofusion

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**Summary**

Somatic fusions between an accession of the diploid wild species *Solanum verrucosum* and a dihaploid S. *tuberosum* genotype were produced in order to incorporate resistance to potato leafroll virus (PLRV). In total 15 somatic hybrids out of 16 regenerants were obtained. Identification of hybrids was based on additive RAPD patterns, general morphological characteristics, chromosome numbers and chloroplast counts in stomata guard cells. A field trial was performed with the hybrids, their two parents and the control cultivar Kennebec to assess field performance and phenotypic variability. Yield parameters varied considerably among somatic hybrids. Some of the hybrids gave significantly higher yields, tuber numbers and tuber weights than both parents. Pollen fertility of hybrids ranged from 19 to 77%. Twelve hybrids were found to be resistant to PLRV.

## Introduction

Somatic hybrids between *Solanum tuberosum* germplasm and sexually incompatible *Solanum* wild species can be obtained through protoplast fusions (Bates, 1992). Moreover, the fusion between two diploid genotypes allows a return to the tetraploid level and, if fertile, the hybrid can be used as a parent in crosses within a classical potato-breeding programme. In this way novel germplasm from wild species can be introduced into current breeding material, and disease resistance or other agronomic traits of interest can be transferred. Furthermore, it is possible to increase the genetic variability and the level of heterozygosity in the breeding population.

In potato, the first chemical fusion techniques were applied with *S. tuberosum* and *S. chacoense* (Butenko & Kuchko, 1980) or *S. nigrum* (Binding et al., 1982) as fusion parents, but they did not give any viable hybrids. The first hybrids from chemical fusions were obtained by Austin et al. (1985). The first successful protoplast electrofusion was performed between *S. tuberosum* and *S. phureja* (Puite et al., 1986). Other sexual compatible or incompatible *Solanum* wild species used for somatic hybridizations have been *S. berthaultii* (Serraf et al., 1991), *S. torvum* (Jadari et al., 1992), *S. circaeifolium* (Mattheij et al., 1992), *S. bulbocastanum* (Austin et al., 1993), *S. commersonii* (Cardi et al., 1993), *S. etuberosum* (Novy & Helgeson, 1994), *S. papita*

(Kaendler et al., 1996) and *S. pinnatisectum* (Menke et al., 1996). Phenotypic characterisation revealed a general vegetative hybrid vigour in most combinations. Mattheij & Puite (1992) observed in field trials that several hybrids involving *S. phureja* gave tuber yields as high as or even higher than standard cultivars and that the yield of the hybrids was highly correlated with the yield of the parental clones.

With respect to virus resistance, *S. brevidens*, a non tuberizing species, has been used extensively as a fusion partner (Barsby et al., 1984; Austin et al., 1985, 1986; Fish et al., 1987). This species carries resistance genes against several viral diseases including PLRV (Jones, 1979), PVY and PVX (Gibson et al., 1990). The transfer of these traits to somatic hybrids is well-documented (Helgeson et al., 1986). A novel source for PLRV resistance, the tuber-bearing species *S. verrucosum*, has been described recently (Ruiz de Galarreta et al., 1998). The use of tuber bearing species as fusion partners should improve tuber characteristics and somatic hybrids could have a more direct use in potato breeding.

Different methods have been applied for the identification of somatic hybrids. With the advent of molecular biology, DNA-based markers like RAPDs have been used successfully to identify somatic hybrids resulting from protoplast fusions (Baird et al., 1992; Xu et al., 1993; Rasmussen & Rasmussen, 1995).

We report in this paper the production, identification and characterisation of somatic hybrids between *S. tuberosum* and *S. verrucosum*, the transfer of PLRV resistance to the hybrids and describe their field performance and male fertility.

## Materials and methods

*Plant material.* The *S. verrucosum* accession (ver1340, 2n=2x=24) was provided by the Commonwealth Potato Collection (SCRI, Dundee, Scotland). Previous experiments revealed resistance to PLRV in this accession (Ruiz de Galarreta et al., 1998). The dihaploid *S. tuberosum* clone R3064 was obtained from the Max-Planck Institut für Züchtungsforschung (Köln, Germany). Plants were maintained in vitro and propagated by nodal subculture on MS medium (Murashige & Skoog, 1962) in a culture chamber under 16 h photoperiod (38 $\mu E\ m^{-2}\ s^{-1}$ light intensity) at 23±1 °C.

*Protoplast isolation, fusion and regeneration.* For protoplast fusions leaves were taken from four to five week-old in vitro plants. Protoplasts were prepared according to Sihachakr et al. (1988) and applying 1% (w/v) cellulase R-10 (Yakult Co., Japan) and 0.2% (w/v) macerozyme R-10 (Yakult Co., Japan) for digestion of the cellular walls. The purified protoplasts were suspended in 0.5 M mannitol solution at a density of $3.5 \times 10^5$/ml. Protoplasts of *S. verrucosum* and *S. tuberosum* were mixed 1:1 and 0.7 ml aliquots were pipetted into Petri dishes (60 mm Ø). The fusion chamber consisted of a movable multi-electrode system made of 12 parallel gold plated copper strips spaced 2 mm apart. The fusion chamber was connected to an in-house developed power supply described by Kramer et al. (1987). The movable electrodes were placed into the suspension. Fusion was achieved by applying first an AC- field of 160 to 190 V/cm at 1 MHz during 15 s for aligning the protoplasts, followed by two to

four DC square pulses of 1.2 kV/cm, each during 20 μs, for producing the fusion.

Immediately afterwards, 6 ml of modified V-KM medium (Boekelmann & Roest, 1983) was added to the fused protoplast suspension. In this medium the original composition of the microelements had been substituted with those of the MS medium. The cultures were kept in a culture chamber at 23±1 °C in the dark during the first seven days and were then progressively exposed to 16 h/day illumination (38 μE $m^{-2}$ $s^{-1}$). After four weeks of culture, calli were transferred onto CM medium for shoot regeneration. This medium was composed of MS salts, vitamins (0.5 mg/l pyridoxine, 0.1 mg/l thiamine and 0.5 mg/l nicotinic acid), 100 mg/l inositol, 2 mg/l glycine, 3% (w/v) glucose, 2 mg/l zeatin riboside, 0.02 mg/l naftalenacetic acid, 0.02 mg/l giberellic acid and 0.7% agar. Shoots were excised from the callus and rooted on MS medium. Rooted plants were transferred to pots and grown in the greenhouse.

*Identification and characterisation of somatic hybrids. RAPD analysis.* The hybrid nature of regenerants was assessed by RAPD analysis. DNA was isolated from the leaf tissue of in vitro plants as described by Edwards et al. (1991). The DNA pellet was dissolved in 400 μl 1 × TE buffer.

PCR reaction mixtures had a total volume of 25 μl. The mixture contained 0.75 units of Taq DNA Polymerase (Pharmacia), 0.32 μM primer, 200 μM dNTPs, 0.5 mM MgCl2, the appropriate dilution of the reaction buffer prepared by the company supplying the polymerase (500 mM KCl, 15 mM $MgCl_2$ and 100 mM Tris-HCl pH 8.3), and approximately 30 ng of template DNA. Reaction mixtures were overlayed with 30 μl mineral oil, before being placed in a Robocycler Gradient 96 (Stratagene). The PCR programme had an initial cycle of 94 °C for 5 minutes. The subsequent 45 cycles had a denaturation step of 94 °C for one minute, an annealing temperature of 35 °C for one minute and an elongation step of two minutes at 72 °C. A final elongation step at 72 °C for 10 minutes followed. Eleven 10-mer primers of arbitrary sequence (AL10, AU20, AV20, AW09, AW19, AW20, B10, B20, K10, K20, N20; Operon Technologies, Alameda, California) were used for PCR amplifications. The amplification products were visualised on 1% agarose gels after staining with ethidium bromide (Sambrook et al., 1989). PCR reactions were repeated twice and only reproducible bands were considered. Primers were initially tested to detect species specific amplification products and only those primers, which revealed unique amplification patterns in each parental clone were used further to identify somatic hybrids.

*Analysis of ploidy level.* The ploidy level was determined by chloroplast counts in the stomatal guard cells in leaves from well-developed plants. The lower epidermis was removed, transferred to 96% alcohol for one hour and then placed in a drop of a diluted iodine-potassium iodide solution (Lugol's solution). The number of chloroplasts in 20 stomata guard cells was counted using light microscopy. Chromosome numbers were determined in root tips from greenhouse-grown plants as described by Mattheij et al. (1992).

*Field trial.* During the summer 1998 a field trial with the tetraploid hybrids, their parents, and the commercial cultivar Kennebec was performed in the experimental plots of the CIMA, Vitoria (Spain). The experimental design consisted of a completely randomised block design with three blocks and four plants per genotype in a plot. Plants were spaced at a distance of 35 cm within rows and 75 cm between rows. Tubers were planted at the end of May and harvested during the second half of September. During the growing period, maturity was scored and pollen fertility was determined by staining with 1% acetocarmine. After harvest tuber yield, tuber number and average tuber weight were recorded. Analysis of variance was performed using the SAS (version 6.11) procedure PROC GLM on a Microvax.

*Virus testing.* Greenhouse plants were inoculated with PLRV by Myzus persicae (20 viruliferous aphids per plant), which were fed previously for 48 h on PLRV infected plants. The aphids were maintained for 48 h on the experimental clones. The PLRV strain used (K-5) had been isolated from naturally infected plants of cv. Kennebec in a local potato field. The presence of PLRV was determined in the youngest expanded leaf tissue 6 weeks after inoculation by ELISA (Clark & Adams, 1977), using a PLRV test kit (Boehringer, Mannheim). Uninoculated parental plants were used as healthy controls. Each test was repeated twice. Twice the mean of the absorbance values of the healthy control was chosen as limit for significant positives for PLRV infection.

## Results

*Protoplast fusion and regeneration of plants.* On average, $4.8 \times 10^6$ protoplasts per gram of foliar mesophyl were obtained from the wild species *S. verrucosum* and $5.7 \times 10^6$ protoplasts from *S. tuberosum* R3064. Cell divisions started seven days after fusion and microcalli became visible after 10 days of culture. Seven weeks after fusion the first shoots appeared. From a total of 141 calli obtained, 26 (18.4%) showed shoot formation. On average 7.6 shoots per callus were observed. One shoot of each callus was rooted and maintained separately to constitute a single clone. Finally, only 16 clones could be established, since some of the calli did not produce functional shoots.

*Identification of hybrids.* There was no difficulty in finding useful polymorphisms between the parental clones, since four out of eleven random primers amplified at least one polymorphic fragment between parents. Banding patterns obtained with primer B20 (5'-AAGGCCCCTG-3) are shown as example in Fig. 1. The results of RAPD analysis confirmed the hybrid character of 15 out of the 16 regenerated clones, since putative fusion products showed a combination of the specific bands of each parent. The other clone showed banding patterns of *S. tuberosum* R3064.

The observed number of chloroplasts in both diploid fusion parents R3064 and *S. verrucosum* were 12 to 16. However, 22 to 26 chloroplasts were found in the stomata guard cells of all hybrids previously identified by RAPD analysis. According to Frandsen (1968), these are the expected numbers for tetraploid individuals. Moreover, 48 chromosomes were found in the cells of the root tips of all analysed

clones. The regenerant that did not show additive RAPD patterns was shown in these analyses to be tetraploid. This 4x regenerant may descend either from an autofusion of two R3064 protoplasts, or from spontaneous chromosome doubling during regeneration.

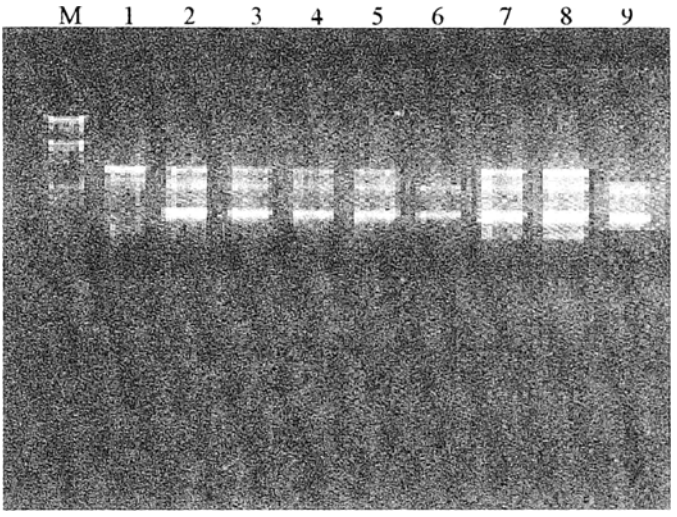


Fig. 1. RAPD fragments obtained with primer B20 in the parental clones *S. tuberosum* (R3064) and *S. verrucosum* (ver 1340) and in their regenerants after somatic hybridization. Lane 1: parental line ver 1340, Lane 9: parental line R3064, Lanes 2 to 5, 7 and 8: RAPD patterns of somatic hybrids, Lane 6: tetraploid regenerant of R3064. Lane M: Molecular weight marker (λ digested with HindIII and EcoRI).

*Field performance and fertility*. In the field, tetraploid hybrid plants were taller than the diploid parents and showed increased growth vigour. Results of the field trial and analyses of fertilities are presented in Table 1. With respect to the vegetative cycle measured on a scale 9 (very early) to 1 (very late), all hybrids were found to be semi-late (between 4.5 and 5.5). Four of them were intermediate with respect to the parents and the others had similar cycles to the *S. tuberosum* parent R3064.

Tuber yield and its components differed widely among hybrids, although average yield and tuber weight of all hybrids were much higher than those of R3064, while average tuber number was about the same. Based on the results of the statistical analyses (Table 1), eight hybrids had significantly higher yields than parent R3064 and seven gave similar yields. As expected, the wild species produced a few small tubers and had the lowest yield. Hybrid VR9 produced the significantly highest tuber number and hybrids VR11 and VR7 showed similar values to the wild parent. The somatic hybrid VR4 gave significantly the highest yield which was nearly 78% of that from the commercial cv. Kennebec. Average tuber weight was less variable among hybrids and both fusion parents exhibited the lowest tuber weights. Cv. Kennebec

gave significantly the highest value for this trait.

Fig. 2 shows the morphology of leaves, flowers and tubers of one somatic hybrid and its parents. The somatic hybrids produced a somewhat broader leaf when compared with R3064 and *S. verrucosum*, which both have narrow leaves. This selected hybrid plant also showed flowers and tubers larger than both parental genotypes. The colour of the flowers and tubers resembled those of *S. verrucosum* and despite some minor variability, these observations can be generalised to all the hybrids obtained.

The average value of pollen fertility was 41.6%. The maximum value of nearly 77% was observed in hybrid VR10 and the minimum value of 19% was found in hybrid VR4.

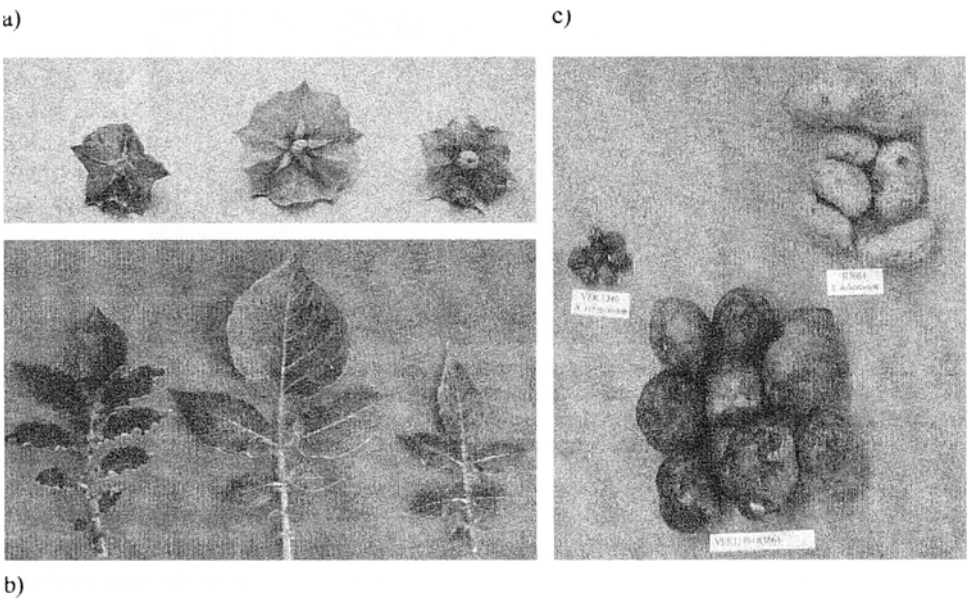


Fig. 2. Morphological characteristics of parental lines *S. verrucosum* (ver 1340), *S. tuberosum* (R3064), and their somatic hybrid VR2. (a) Flowers of *S. verrucosum* (left), VR2 (centre) and *S. tuberosum* (right), (b) leaves of *S. tuberosum* (left), VR2 (centre) and *S. verrucosum* (right) and (c) tubers of *S. verrucosum* (left), VR2 (centre) and *S. tuberosum* (right).

*Virus testing*. Results of PLRV resistance evaluation in the somatic hybrids are indicated in Table 2 and twelve of the fifteen hybrids showed resistance to PLRV based on the ELISA reading values. For the other clones VR7, VR8 and VR14, these values were higher than those of the resistant parent *S. verrucosum*. Therefore these hybrids were considered as susceptible. Nevertheless their ELISA values were lower than those of the susceptible parental line and the control cv. Kennebec.

Table 1. Field performance and fertility of parental lines *S. verrucosum* (ver1340), *S. tuberosum* (R3064), their somatic hybrids (VRi) and the commercial cultivar Kennebec.

| Genotype | Tuber yield (g) | Tuber number | Mean tuber weight (g) | Maturity[a] | Fertility (%) |
|---|---|---|---|---|---|
| Kennebec | 3760 a | 27.2 de | 138.2 a | 7.0 | 91.0 a |
| R3064 | 1000 hij | 27.2 de | 36.8 cd | 5.5 | 91.5 a |
| *S. verrucosum* | 172 k | 13.2 jk | 13.3 d | 3.5 | 65.0 c |
| VR4 | 2920 b | 31.2 c | 91.4 b | 5.5 | 19.0 i |
| VR3 | 2290 c | 41.2 b | 55.7 c | 5.5 | 66.1 b |
| VR12 | 1880 d | 39.6 b | 47.4 cd | 5.5 | 32.5 fgh |
| VR9 | 1800 de | 46.0 a | 39.2 cd | 5.0 | 65.5 bc |
| VR6 | 1560 de | 38.8 b | 40.2 cd | 5.5 | 35.3 fg |
| VR5 | 1400 ef | 23.2 fgh | 60.4 bc | 5.5 | 25.2 ghi |
| VR2 | 1330 efg | 20.8 i | 64.7 bc | 5.5 | 55.6 cd |
| VR17 | 1260 efgh | 28.8 cd | 43.9 cd | 5.5 | 21.7 hi |
| VR10 | 1170 fghi | 26.0 def | 45.6 cd | 5.5 | 76.8 b |
| VR16 | 1120 fghi | 25.2 ef | 44.3 cd | 4.5 | 32.6 fg |
| VR14 | 1090 fghi | 24.4 efg | 45.3 cd | 4.5 | 25.8 ghi |
| VR8 | 1030 ghij | 21.6 ghi | 47.8 cd | 5.5 | 42.2 ef |
| VR11 | 980 hij | 15.2 j | 65.0 bc | 5.5 | 45.0 de |
| VR1 | 890 ij | 20.8 hi | 43.1 cd | 5.5 | 56.4 cd |
| VR7 | 710 j | 10.8 k | 62.5 bc | 4.5 | 25.1 ghi |
| *VR (mean)* | *1429* | *27.6* | *53.1* | *5.3* | *41.6* |
| *VR (CV)* | *41.0* | *36.3* | *26.1* | *7.9* | *44.1* |

*VR (mean)* = mean value for all somatic hybrids. *VR (CV)* = coefficient of variation of all somatic hybrids. Data represent average values per plant. Means followed by the same letter(s) are not significantly different at $P$=0.05 (Fisher's LSD).
[a] 1=very late; 9=very early.

## Discussion

One notable fact was the short period before the appearance of the first shoots after fusion. This can be considered as very favourable since the risk of developing somaclonal variation would be reduced and therefore the whole regeneration process can be shortened. One possible reason for this behaviour could be the use of the modified V-KM medium since, in comparable assays, formation of the first cell colonies did not occur when using the original V-KM microelement solution (results not shown). Also the elimination of $NH_4NO_3$ from the macroelements according to the culture medium of Boekelmann & Roest (1983) seemed to favour cellular divisions. Upadhya (1975) as well as Shepard & Totten (1977) observed that ammonia had a negative effect on the survival of potato protoplasts. As suggested by Sihachakr et al. (1992) 250 mg/l of PEG 6000 were added to both culture media. We can confirm that viability of the protoplasts was improved, probably due to the antioxidant properties of PEG.

Table 2. Results of ELISA tests for PLRV infection in somatic hybrids and their parents *S. tuberosum* (R3064) and *S. verrucosum* (ver 1340).

| Clone | $A_{405nm}$[a] | Response |
|---|---|---|
| R3064 | 0.5675±0.03[b] | Susceptible |
| *S. verrucosum* (ver 1340) | 0.154±0.005 | Resistant |
| VR1 | 0.142±0.001 | Resistant |
| VR2 | 0.149±0.002 | Resistant |
| VR3 | 0.133±0.002 | Resistant |
| VR4 | 0.146±0.005 | Resistant |
| VR5 | 0.136±0.001 | Resistant |
| VR6 | 0.153±0.001 | Resistant |
| VR7 | 0.287±0.003 | Susceptible |
| VR8 | 0.309±0.002 | Susceptible |
| VR9 | 0.146±0.001 | Resistant |
| VR10 | 0.130±0.001 | Resistant |
| VR11 | 0.143±0.002 | Resistant |
| VR12 | 0.138±0.001 | Resistant |
| VR14 | 0.376±0.005 | Susceptible |
| VR16 | 0.140±0.001 | Resistant |
| VR17 | 0.136±0.001 | Resistant |
| Kennebec | 0.689±0.011 | Susceptible |
| Uninoculated control | 0.115±0.001 | ------- |

[a] ELISA absorbance readings (405nm) after 2 h with air blank
[b] Mean of two readings ± standard error of the mean.

We have used RAPD markers for the identification of somatic hybrids. Other authors have applied different techniques for this purpose such as morphological analysis (Austin et al., 1985), isoenzymatic analysis (Serraf et al., 1994), RFLP markers (Novy & Helgeson, 1994), primers which amplify simple sequence repeats (Stadler et al., 1995; Menke et al., 1996) and PCR with oligonucleotides which amplify specifically the 5S rDNA spacer region (Zanke et al., 1995). The problems of RAPD markers with respect to reproducibility of results under the specific conditions in different laboratories, or with respect to equal-sized markers which may not be sequence homologous are well known (Black, 1993). Despite these problems we believe that for this application and, given that several primers are used for analysis in the same laboratory, RAPD markers offer a quick and cheap method for the identification of hybrids. Furthermore, a high degree of polymorphism can usually be obtained and only small amounts of genomic DNA are needed and no DNA sequence information is required.

With respect to fertility, flowering plants have been obtained for all hybrids. In fusions between *S. nigrum* and *S. tuberosum*, Horsman et al. (1997) obtained flowers in only three out of 16 generated fusion genotypes. Pollen viability varied considerably between our hybrids. Genotype VR4 was found to be practically androsterile. As previously suggested by Cardi et al. (1993) and Rokka et al. (1994), this hybrid could be used as female in crossings. Androsterile hybrids between

*S. tuberosum* and *S. bulbocastanum* were also used by Austin et al. (1993) as female parents and in 11% of the crossings an average of 28.7 seeds were obtained per berry.

Compared with the commercial cv. Kennebec, the relative high yield obtained by some hybrids such as VR4 and VR3 is remarkable. However, considerable variation was observed among the different hybrids. Such phenotypic differences have been also described previously for interspecific fusions (Austin et al., 1986; Pehu et al., 1989; Jacobsen et al., 1993), and could be due to somaclonal variation (Larkin & Scowcroft, 1981) or to interactions between nuclear and cytoplasmatic genes of the parents (Preiszner et al., 1991).

From a review of literature, different character expressions of somatic hybrids relative to their parents can be generally observed. Austin et al. (1993) reported phenotypically intermediate hybrids between *S. tuberosum* and *S. bulbocastanum* with respect to some morphological characters. Menke et al. (1996) found that tuber characters of hybrids resembled more the *S. tuberosum* parent, while Cardi et al. (1993) observed in the hybrids tuber characteristics similar to the wild parent *S. commersonii*. Except for tuber and flower colour, our somatic hybrids were closer to the *S. tuberosum* parent. With respect to yield and its components they were mostly superior due to the effects of the tetraploid genome.

During the present work we obtained somatic hybrids showing resistance to PLRV. This confirms previous results of Ruiz de Galarreta et al. (1998) with respect to the virus resistance detected in *S. verrucosum*. Hybrids were classified as susceptible if absorbance values were larger than twice the mean value of the uninoculated control. According to Swiezynski et al. (1988) absorbance values larger than 0.2 should be considered as positive. Following both criteria, the incorporation of resistance to PLRV was not achieved in all our hybrids. Variability in virus resistance in somatic hybrids as observed in our experiments has also been described previously (Gibson et al., 1988; Pehu et al., 1990). Possible explanations for somaclonal variation include an increase in DNA methylation (Brown & Lörz, 1986), new interactions between nucleus and cytoplasm which are inappropriate for the expression of the resistance (Kemble et al., 1986) or aneuploidy (Fish et al., 1988).

More advanced studies are necessary to define the type of resistance mechanisms as well as the monogenic or polygenic nature of the resistance in the hybrids. Depending on the type of resistance, some of the resistant somatic hybrids with high yields could be used in potato breeding programmes for backcrossing with *S. tuberosum*. This work confirms the usefulness of protoplast fusion as a possible strategy for transferring resistance and other favourable characters present in wild species to obtain improved commercial potato cultivars.

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