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ORIGINAL PAPER

Molecular and morphological characterization of somatic hybrids between *Solanum tuberosum* L. and *S. etuberosum* Lindl.

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Abstract Interspecific potato somatic hybrids between *Solanum tuberosum* L. (di)haploid C-13 and 1 endosperm balance number non-tuberous wild species *S. etuberosum* Lindl. were produced by protoplasts electrofusion. The objective was to transfer virus resistance from this wild species into the cultivated potatoes. Post-fusion products were cultured in VKM medium followed by regeneration of calli in MS_{13} K medium at 20°C under a 16-h photoperiod, and regenerants were multiplied on MS medium. Twenty-one somatic hybrids were confirmed by RAPD, SSR and cytoplasm (chloroplast/mitochondria) type analysis possessing species-specific diagnostic bands of corresponding parents. Tetraploid nature of these somatic hybrids was determined through flow cytometry analysis. Somatic hybrids showed intermediate phenotypes (plant, leaves and floral morphology) to their parents in glasshouse grown plants. All the somatic hybrids were male-fertile. ELISA assay of somatic hybrids after artificial inoculation of Potato virus Y (PVY) infection reveals high PVY resistance.

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Keywords Cytoplasm type · Flow cytometry · RAPD · SSR · PVY resistance

Abbreviations

CRBC	Chicken red blood cell
CTAB	Cetyltrimethylammonium bromide
nDNA	Nuclear DNA
DUS	Distinctness, uniformity and stability
EBN	Endosperm balance number
FC	Flow cytometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PVY	Potato virus Y
RAPD	Random amplified polymorphic DNA
RCBD	Randomized complete block design
SSR	Simple sequence repeat
TPS	True potato seed

Introduction

The wild *Solanum* species of the cultivated potato (*S. tuberosum* L.) are an important source of resistance for various biotic and abiotic stresses (Helgeson and Haberlach 1999). Their introgression into potato using classical breeding methods is time-consuming and may lead to difficulties due to various sorts of sexual incompatibilities and particularly differences in the ploidy level or in the endosperm balance number (EBN) (Johnston et al. 1980; Jackson and Hanneman 1999). Therefore, somatic hybridization is one of the techniques used to overcome these limitations. Somatic hybridization can provide new opportunities for producing pre-breeding materials with increased genetic

variability and transferring desirable agronomic traits into cultivated potatoes. In comparison to other parallel techniques of chromosomal and genetic engineering, it has a unique potential to transfer simultaneously both nuclear and cytoplasmic genes (Zhou et al. 2001). It bypasses the gene segregation and enables the transfer of both mono- and polygenic traits among the sexually incompatible species (Thieme et al. 1997, 2004; Gavrilenko et al. 2003). Symmetric protoplasts fusion between potato and *S. brevidens* was used to integrate virus and aphids resistance (Valkonen et al. 1992a, b; Polgar et al. 1999). In many experiments, somatic hybrids have been produced between cultivated potato and wild species such as *S. tarnii* (Thieme et al. 2008), *S. etuberosum* (Novy and Helgeson 1994a, b; Chavez et al. 1988; Gavrilenko et al. 2003; Novy et al. 2002, 2007; Gillen and Novy 2007; Thompson et al. 2007), *S. pinnatisectum* (Szczerbakowa et al. 2005; Greplova et al. 2008), *S. bulbocastanum* (Szczerbakowa et al. 2001; Greplova et al. 2008), *S. berthaultii* (Bidani et al. 2007), *S. nigrum* (Szczerbakowa et al. 2003; Zimnoch-Guzowska et al. 2003), *S. circaeifolium* (Oberwalder et al. 2000), and *S. acaule* (Rokka et al. 1998) for transferring various biotic and abiotic resistance traits. In future, because of the continuous threat of phytopathogens, the introduction of new resistant germplasm to increase genetic diversity of potato gene pool is of vital need. Therefore, development of more interspecific potato somatic hybrids would provide a broad spectrum of resistant potato lines against major diseases such as late blight and viruses.

The *S. etuberosum* (1 EBN; *Solanum* sect. *Etuberosum*) is characterized as a diploid wild, native to Chile, non-tuberous species having E-genome distinct from A-genome of tuber-bearing cultivated potato *S. tuberosum* (4 EBN; *Solanum* sect. *Petota*) (Spooner and Hijmans 2001). The distinct genomic and taxonomic differences between *S. etuberosum* and cultivated potato have made sexual hybridization difficult. Interestingly, *S. etuberosum* was shown to have high levels of resistance to the potato leaf roll virus (PLRV), potato virus Y (PVY), potato virus X (PVX), and green peach and potato aphid (Valkonen et al. 1992a, b; USDA ARS National Genetic Resources Program 2003). Consequently, the virus resistance trait could be transferred into the cultivated potato gene pool by somatic hybridization.

In the present paper, we report the production of tetraploid interspecific potato somatic hybrids between cultivated potato and *Solanum etuberosum* Lindl. The results of protoplasts electrofusion and regeneration, confirmation of somatic hybrids through random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), cytoplasm (mitochondrial/chloroplast) type, flow cytometry (FC) analysis, phenotypic characterization, male fertility and PVY resistance assessment are discussed here.

Materials and methods

Plant material

The androgenic (di)haploid C-13 ($2n = 2x = 24$) of Indian tetraploid ($2n = 4x = 48$) potato (*Solanum tuberosum* L. subsp. *tuberosum*) cv. Kufri Chipsona 2 was used in protoplast-fusion experiments with 1 EBN non-tuberous wild species *S. etuberosum* (CGN No.: 23066) ($2n = 2x = 24$). The (di)haploid C-13 was developed at the Central Potato Research Institute, Shimla, India (Chanemougasoundharam et al. 2004; Sharma et al. 2010). True potato seeds (TPS) of wild species were obtained from the Centre for Genetic Resources, the Netherlands (CGN), Wageningen University, and Research Centre, Wageningen, the Netherlands. Seeds of wild species were germinated in vitro and subsequently multiplied by sub-culturing leafy nodes on MS (Murashige and Skoog 1962) medium (pH 5.8) supplemented with sucrose (20 g l^{-1}) and solidified with gelrite (2 g l^{-1}). Cultures were grown at 20°C under a 16-h photoperiod (light intensity 50–60 μmol m^{-2} s^{-1}).

Protoplast isolation and electrofusion

Protoplasts were isolated from 3-week-old in vitro plants following highly optimized protocols: in vitro plants were grown in the dark (covered with dark-black muslin cloths) in a culture room at 20°C for 48 h under 16-h photoperiod to integrate cell cycles (Binding et al. 1978). The enzymatic mixtures of 1% cellulose 'Onozuka' RS (Yakult Pharmaceuticals, Tokyo) and 0.5% macerozyme R 10 (Yakult) solutions were used for cell wall degradation. Young leaf tissues were minced in 90 × 15 mm Petri dish containing protoplast digestion solution (PDS) (10 ml PDS g^{-1} tissue) followed by incubation in the dark at 25°C for 16 h without shaking. After incubation, 0.3 M KCl (sterile) was added in a 1:1 ratio after gentle shaking of PDS containing released protoplast. Subsequently, suspension was filtered through a 60-μm nylon sieve and collected in centrifuge tubes. Purification of protoplasts was performed as: filtrates were centrifuged at 50 RCF for 5 min and then resuspended the pellets in 9 ml of 0.6 M sucrose (sterile) followed by 1 ml of 0.3 M KCl layer onto it and centrifuged as above. Protoplasts were recovered from sucrose: KCl interface, diluted with 5 ml of 0.3 M KCl, centrifuged as above and finally suspended in 0.5 M Mannitol to a final density of 1×10^6 protoplast ml^{-1}. The protoplasts were electrofused in a 3.0-mm microslide using the BTX Electro Cell Manipulator ECM 2001 (Harvard Apparatus, Mass., USA). An AC field of 100–120 V cm^{-1} and high frequency (1 MHz) was first applied for 20–30 s to align the protoplasts. Two square DC pulses 800–1,250 V cm^{-1}

were applied for 40–60 μs to achieve protoplast fusion, with a 10-s post-fusion AC field for compacting the homokaryons/heterokaryons.

Protoplast culture and plant regeneration

Fused protoplasts were cultured in VKM liquid medium (Binding and Nehls 1977) supplemented with glucose (90 mg ml^{-1}) at 20°C in the dark. Following the cell wall development, regenerating cells were cultured on MS_{13} K medium (Behnke 1975) at 20°C under a 16-h photoperiod for the calli development. Newly regenerated shoots from the calli were cultured in vitro on MS medium for subsequent growth and multiplication. Identification of hybrid regenerants was based on culture of entire fusion products. Subsequently, first shoot per callus was regenerated and multiplied for further studies.

DNA analysis

The hybrid nature of the somatic hybrids was confirmed by RAPD and SSR analysis. Further, cytoplasm type (mitochondrial and chloroplast genome) was also analyzed using mitochondrial and chloroplast specific primers. Total genomic DNA was isolated from in vitro leaf samples following a modified CTAB-dichloromethane protocol of Saghai-Maroof et al. (1984). Leaf samples (~300 mg) were vigorously homogenized in CTAB extraction buffer (1 ml) at room temperature. DNA was precipitated with isopropanol, washed with 70% ethanol, air dried and diluted in 100 μl TE buffer (pH 8.0) after RNase A (100 μg ml^{-1}) treatments.

RAPD analysis was performed using 15 random decamer primers, viz., OPAC-06, OPAC-09, OPAC-13, OPAC-14, OPAQ-02, OPAQ-14, OPAQ-15, OPAQ-16, OAPQ-20, OPAT-03, OPAT-06, OPAT-09, OPD-03, OPG-09 and OPK-06 (Operon Technology), for the confirmation of somatic hybrids. The polymerase chain reaction (PCR) was carried out in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) in a total volume of 25 μl and consisted of 50 ng DNA templates in 1× PCR buffer, 2.5 mM $MgCl_2$, 200 mM dNTP, 0.2 μM of primer, 1 Unit Taq Polymerase (Qiagen). The PCR procedure included: one cycle of 4 min at 94°C, 1 min at 36°C and 2 min at 72°C followed by 43 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C, with a final extension of 8 min at 72°C. The amplified RAPD products were separated by electrophoresis on 1.6% agarose gel stained with ethidium bromide (0.5 μg ml^{-1}) in 0.5× TBE buffer (Tris–borate-EDTA, pH 8) using horizontal gel electrophoresis system Sub-Cell GT (Bio-Rad, USA) at room temperature. The gels were visualized under Gel Doc System (Alpha Innotech, San Leandro, CA, USA) and compared with 100 bp DNA ladder (Fermentas, Burlington, Canada).

Twenty-five potato SSR markers, viz., STG0001, STG0010, STG0016, STG0025, STI0001, STI0003, STI0004, STI0012, STI0014, STI0030, STI0032, STI0033, STM0019a, STM0019b, STM0031, STM0037, STM1052, STM1053, STM1064, STM1104, STM1106, STM5114, STM5121, STM5127 and STPoAc58 (Ghislain et al. 2009), and 11 tomato SSR markers, viz. SSR 22, SSR 31, SSR 67, SSR 76, SSR 111, SSR 128, SSR 136, SSR 146, SSR 188, SSR 310 and SSR 350 (Van der Hoeven et al. 2001), were used for the confirmation of somatic hybrids. Annealing temperature (Ta) was decided using Mastercycler Gradient (Eppendorf) and PCR was performed as described by Ghislain et al. (2009). Amplified products were resolved on 4% high resolution agarose (Sigma–Aldrich, St Louis, MO, USA) and 8% polyacrylamide gel electrophoresis (PAGE) followed by gel documentation as above.

Cytoplasm type was analyzed by using mitochondrial specific primers pair ALM4/ALM5 and ALM 6/ALM7, and chloroplast type was determined using primer pair ALCP1/ALCP3. The PCR condition was followed at optimized annealing temperatures (Ta = 55°C for mitochondrial specific primers and Ta = 45°C for chloroplast specific primers) as described by Lössl et al. (2000). Amplified products were analyzed by electrophoresis on 1.6% agarose gel in 0.5× TBE buffer and stained with ethidium bromide (0.5 μg ml^{-1}) as above. As a result of RAPD, SSR and mitochondrial DNA analysis, somatic hybrids were confirmed on the basis of presence/absence of species-specific diagnostic bands of the corresponding parents in the hybrids profiles.

Flow cytometry analysis

The hybrid nature of somatic hybrids was also proven by ploidy determination through flow cytometric analysis following the protocol described by Arumuganathan and Earle (1991b), and Sharma et al. (2010). Fresh leaf samples (~100 mg) from 3- to 4-week-old in vitro plants were macerated in 1 ml nuclei isolation buffer [$MgSO_4$ buffer ($MgSO_4$•$7H_2O$, 10 mM; KCl, 50 mM; HEPES, 5 mM), 14.325 ml; Dithiothretol (15 mg); Triton X (375 μl)] on ice. Macerates were filtered through a 41-μm nylon sieve followed by centrifugation at 5,000*g* for 5 min. White pellets (nuclei) were resuspended in $MgSO_4$ buffer and treated with 2 μl RNAse at 37°C for 15 min. Propidium iodide-stained nuclei were used to measure relative fluorescence in a FACSCalibur flow cytometer (Becton–Dickinson, San Jose, USA) using CRBC (chicken red blood cell) (2C value of DNA = 2.33 pg; Galbraith et al. 1983) as an internal standard. The materials were analyzed for forward (FSC) versus side (SSC) scatter signals for at least 10,000 nuclei in each sample. The peak corresponding to the CRBC nuclei was adjusted to around channel 250 set

on a linear scale of fluorescence intensity. The nuclear DNA amount (2C value in pg) was estimated by direct comparison of the mean position of nuclear peak of somatic hybrids to that of CRBC (Arumuganathan and Earle 1991a). For each sample, there were three independent replicated measurements.

Potato virus Y (PVY) resistance assay

In vitro raised plants were mechanically inoculated using PVY^{o} isolates maintained in tobacco (*Nicotiana glutinosa* L. and *N. tabacum* L.) plants at CPRI, Shimla. Infected fresh tobacco leaf tissue (~2.5 g) was ground in 25 ml of cold 1 mM potassium phosphate (pH 8) buffer. Two to three young leaves of each plant were dusted with carborandrum powder and then lightly rubbed with cheesecloth dipped in virus inoculum. Fresh tissue from the third compound leaf (from the top) of artificially inoculated plants was collected and ground in grinding buffer using a hand crushing machine. Plants were tested by das-ELISA at 20 days after PVY inoculation using the method described by International Potato Centre, Lima-Peru (Anonymous 2007). Two negative and two positive controls maintained at CPRI, Shimla were used in each 96-well ELISA plate. A popular and PVY susceptible variety Kufri Chandramukhi was used as standard check in PVY assay. Absorbance values were measured at 405 nm (A_{405} nm) using an automated ELISA reader (BioTek, Winooski, VT, USA). The absorbance threshold for susceptible plants was set at absorbance levels two times greater than means of negative controls (Sutula et al. 1986). The antibody (1 μg/μl) used for the coating on the 96-well ELISA plates was produced at CPRI, Shimla.

Phenotypic characterization

The morphology of in vitro regenerated somatic hybrids was assessed in the earthen pot-grown plant. Somatic hybrids and parents were planted in the glass-house in the summer season (May–August) at Shimla (31.06°N, 77.10°E, 2,202 m above msl), as potato plants typically flower only under long-day conditions of Shimla hills (mid-Himalayas) in northern India. The in vitro plants (5 plants per clone) were planted in earthen pots in a randomized complete block design (RCBD) with three replications. Fertilizer schedules and cultural practices were followed using a recommended package of practices. Morphological characters for general canopy characteristics: plant vigor, plant height, foliage structure, foliage color and foliage gloss; stem morphology: solidity, predominant coloration, secondary coloration and wings type; leaf morphology: leaf structure, anthocyanin coloration of rachis, length, width, shape and waviness of margin were recorded at 50 days after planting. Observations on days to first flowering, inflorescence size, corolla size, corolla color, anthocyanin coloration of bud, outer and inner side of corolla, anther color, stylar length and stigma shape were recorded after initiation of flowering. Plant maturity type was recorded at 90 days after planting whereas tuber characters: shape, skin color, skin type, flesh color and depth of eyes, were recorded after harvesting (at 115 days after planting). All the non-parametric categories of the traits were taken from the DUS characterization of potato crop as described by Gopal et al. (2008).

Male fertility determination

Male fertility of the somatic hybrids was determined by acetocarmine (1%) staining of pollens collected from the glass-house grown plants. Stained viable and non-viable pollen was examined through an inverted florescence microscope (Olympus, Tokyo). Counts were recorded from at least ten different flowers from five different locations on slide and mean value was estimated.

Statistical analysis

Prior to univariate ANOVA analysis, homogeneity and normality assumption were tested for the independent variables. Accordingly, data were analyzed followed by all pairwise multiple comparisons of mean values using the post-hoc Tukey's honestly significant difference (HSD) test (MSTAT-C; Michigan State University, Michigan, USA). The term significant has been used to indicate differences for which $P \leq 0.05$. Verification ability of flow cytometry and DNA analysis (RAPD, SSR and mitochondrial DNA) was tested by McNemar Chi-square test to assess the null hypothesis (H_0) that no difference exists among flow cytometry, DNA analysis for the hybridity.

Results

Protoplast isolation, electrofusion, culture and regeneration

Protoplasts were isolated from the mesophyll tissue of the parents and 257 electrofusion attempts were made followed by successful culture of post-fusion products. Formation of cell wall was observed for a minimum of 21 days in the fused products; this varies from product to product taking more than a year for final regeneration into plantlets. As a result, 142 calli were recovered from the post-fused products. Subsequently, 84 in vitro somatic hybrids plants were regenerated and the most vigorously growing one was multiplied, while the rest were discarded because of growth

Table 1 Number of protoplast fusion attempted, regenerated calli, microplants grown and identified true somatic hybrids

Fusion combination	Number				
	Fusion attempted	Regenerated calli	Regenerated in vitro shoots	In vitro plants grown	Identified somatic hybrids
S. tuberosum (di)haploid C-13 (+) *S. etuberosum*	257	142	84	40	21

abnormalities. Finally, 40 in vitro somatic hybrids plants were grown successfully for the molecular, cytogenetic and phenotypic studies (Table 1).

Identification of somatic hybrids

RAPD analysis

Somatic hybrids were confirmed by using 15 RAPD decamer primers. All primers showed high polymorphic bands in the somatic hybrids and their parents. The presence of species-specific RAPD bands of their corresponding parents in the somatic hybrids confirmed the hybridity. Figures 1 and 2 show RAPD profiles of somatic hybrids and parents generated by primer OPAC-13, where somatic hybrids showed four diagnostic bands, two of each parent: 630 and 996 bp of C-13, and 1,138 and 1,605 bp of *S. etuberosum*. Similar trends of highly polymorphic bands of species-specific diagnostic bands were revealed by the other 14 RAPD primers and confirmed the 21 clones, namely E 1 to E 12, E 18 to E 20, E 22, E 24 to E 26, E 32 and E 33, as somatic hybrids (Table 2). Thus, based on the RAPD fingerprinting patterns, these 21 somatic hybrids were confirmed, whereas 19 somatic hybrids, E 13 to E17, E 21, E 23, E 27, E 28, E 29 to E 31, E 34 to E 40, showed only *S. etuberosum* bands, and therefore they failed as somatic hybrids.

SSR analysis

Analysis of somatic hybrids' plant DNA was assayed by 36 SSR primers out of which only 4 SSR, viz., STG0016, SSR

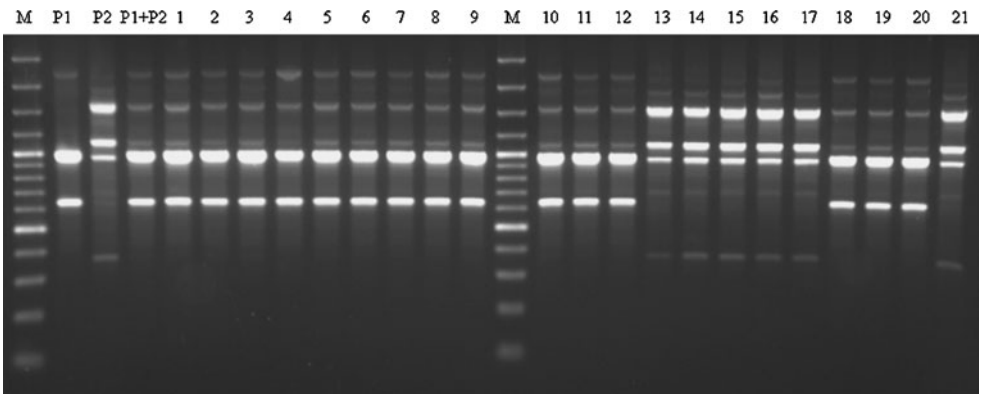

Fig. 1 RAPD profiles generated by primer OPAC-13 on 1.6% agarose gel. *M* = 100-bp ladder, P_1 (Parent 1) = C-13, P_2 (Parent 2) *S. etuberosum* $P_1 + P_2$ = pooled parental DNA, 15 clones (nos. *1–12* and *18–20*) were confirmed as somatic hybrids, whereas 6 clones (nos. *13–17* and *21*) were not somatic hybrids (continued on Fig. 2)

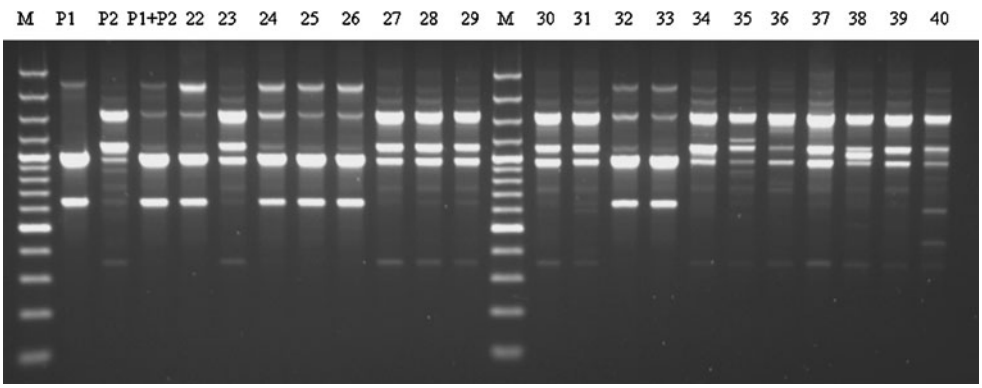

Fig. 2 RAPD profiles generated by primer OPAC-13 on 1.6% agarose gel. *M* = 100-bp ladder, P_1 (Parent 1) = C-13, P_2 (Parent 2) *S. etuberosum* $P_1 + P_2$ = pooled parental DNA, 6 clones (nos. *22, 24–26, 32, 33*) were confirmed as somatic hybrids, whereas 13 clones (nos. *23, 27–31, 34–40*) were not somatic hybrids

Table 2 Species-specific diagnostic DNA bands (bp) of the parents in the somatic hybrids *S. tuberosum* (di)haploid C-13 (+) *S. etuberosum* generated by 15 RAPD decamer primers

Primer	Parents (bands in bp)		Somatic hybrids (combined parental bands in bp)	
	C-13	*S. etuberosum*	E 1–12, 18–20, 22, 24–26, 32 and 33	E 13–17, 21, 23, 27, 28, 29–31 and 34–40
OPAC-06	840:1472	1051:1366	840:1051:1366:1472	1051:1366
OPAC-09	556:1025:1631	734:1168	556:734:1025:1168:1631	734:1168
OPAC-13	630:996	1138:1605	630:996:1138:1605	1138:1605
OPAC-14	958:1075:1556	1293:1916	958:1075:1293:1556:1916	1293:1916
OPAT-03	740:1036	817:976	740:817:976:1036	817:976
OPAT-06	481:567:741	1052:1293:1556	481:567:741:1052:1293:1556	1052:1293:1556
OPAT-09	541:955	1169	541:955:1169	1169
OPAQ-02	1128:1322:1548	1897:2173	1128:1322:1548:1897:2173	1897:2173
OPAQ-14	1119	1223	1119:1223	1223
OPAQ-15	1846	1241:1462:2225	1241:1462:1846:2225	1241:1462:2225
OPAQ-16	855:2086	1389:1539:1773	855:1389:1539:1773:2086	1389:1539:1773
OPAQ-20	553:828:1196:1437	653:942:1091	553:653:828:942:1091:1196:1437	653:942:1091
OPG-09	1510:2220:2590	685:1047:1425	685:1047:1425:1510:2220:2590	685:1047:1425
OPD-03	814:920:1469:1778	1020:1172:1630	814:920:1020:1172:1469:1778:1630	1020:1172:1630
OPK-06	1042:1221:2061:2473:2900	1464	1042:1221:1464:2061:2473:2900	1464

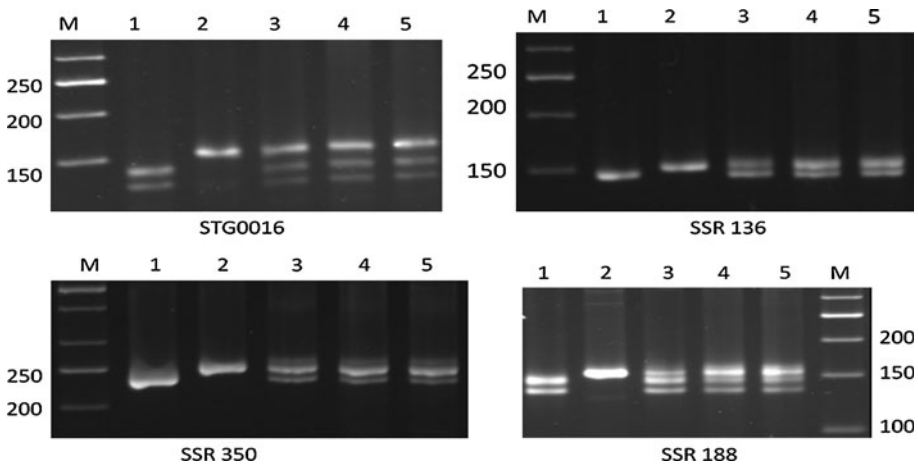

Fig. 3 Amplification products using different SSR primers *STG0016*, *SSR 136*, *SSR 350* and *SSR 188*. Nos. *1* and *2* denote C-13 and *S. etuberosum*, respectively, and *3–5* show somatic hybrids

136, SSR 188 and SSR 350, confirmed the somatic hybrids nature. Since, they possessed species-specific diagnostic bands of their corresponding parents. For example, primer STG0016 showed distinct amplification at Ta = 63.1°C whereas the other three, SSR 136, SSR 188 and SSR 350, amplified at Ta = 55°C. Primer STG0016 produced amplification products of 137 and 148 bp in parent C-13, and 160 bp in parent *S. etuberosum* as described by Ghislain et al. (2009). In addition, primer SSR 136 amplified at 147 and 158 bp in C-13 and *S. etuberosum*, respectively; SSR 350 produced 242 bp and 262 products in C-13 and *S. etuberosum*, respectively; whereas the profile obtained by SSR 188 was 146 and 157 bp in C-13 and 168 bp in *S. etuberosum*; expected sizes as described by Gillen and Novy (2007). As a result, combined amplification of their corresponding parents was produced in the somatic hybrids' profile (Fig. 3). Other primers showed the amplification with some common bands among the parental lines (data not shown) which could not be characterized as species-specific diagnostic bands for the confirmation of hybridity of plants.

Cytoplasm type analysis

The cytoplasmic genome (mitochondrial and chloroplast DNA) analysis was based on the primers described by Lössl et al. (2000). Amplification with mitochondrial primers ALM4/ALM5 and ALM6/ALM7 and chloroplast primer ALCP1/ALCP3 showed a distinct polymorphism between parental profiles and consequently confirmed the

hybridity. The amplification profile obtained by ALM4/ALM5 primers showed a band of 2,400 bp in C-13, a unique band of 780 bp in *S. etuberosum* and, as a result, both parental bands (780 and 2,400 bp) were produced in the somatic hybrids E 2-3 and E 2-6 profile while all other showed only a 780-bp band. The use of primer ALM6/ALM7 also revealed a polymorphism between parental lines, and somatic hybrids led to profiles comprised of their parental PCR products: 470 bp of C-13 and 2,400 bp of *S. etuberosum*. Somatic hybrids E 2-1, E 2-2, E 2-8, E 6-1, E 6-2, E 6-3, E 8 and E 10 showed products of 2,400 bp while all others produced 2,000-bp products by ALM6/ALM7. The chloroplast primer ALCP1/ALCP3 produced amplicon of 622 bp in both the parents while an additional band of 815 bp was also obtained in *S. etuberosum*. Consequently, all the somatic hybrids produced only product size of 622 bp. Data suggest that the somatic hybrids having amplification products of 2,400 bp using ALM4/ALM5 and ALM6/ALM7, and 622 bp by ALCP1/ALCP3 possess W/α type cytoplasm (W: mitochondria type and α: chloroplast type) (Lössl et al. 2000) while all others differed from these amplified products have unique and undetermined cytoplasm type (Figs. 4, 5).

Flow cytometry analysis

All regenerated somatic hybrids of C-13 (+) *S. etuberosum* were tested for ploidy level by FC analysis. It revealed the co-efficient of variation of the nDNA content means varied between 1.51 and 3.82% among the somatic hybrids (Fig. 6). The nDNA content of somatic hybrid plants ranged from 1.61 to 3.82 pg. For the tetraploid somatic hybrids, the value ranged between 3.34 and 3.82 pg, whereas for diploid clones it ranged between 1.61 and 2.03 pg. Based on nDNA content, 21 somatic hybrids (E1 to E12, E18 to E20, E22, E24 to E26, E32 and E33) were observed as tetraploid, whereas 19 somatic hybrids (E 13 to E17, E21, E23, E27, E28, E29 to E31, E34 to E40) were found to be diploid (Table 3). A highly significant difference in nDNA content was estimated in multiple pairwise comparisons between diploid and tetraploid, according to Tukey's HSD test at $P \leq 0.01$, whereas an insignificant difference was observed in the paired comparison of either diploid–diploid or tetraploid–tetraploid following the same test.

PVY resistance assessment

Somatic hybrids showed vigor as they derived from a PVY-resistant line (*S. etuberosum*), and the effects of virus infection of these lines was investigated. It was also performed for hybrids coming from two contrasting parental lines. Hybrid plants and their parents were planted in the glass-house and mechanically inoculated with PVY^{o}. However, one parent, C-13, displayed sensitivity to the virus with clear symptoms of progressive leaf discoloration and decrease in plant growth and symptom appearance. The hybrids showed resistance to PVY compared to parents. They displayed resistance to PVY consisting of the absence of virus multiplication and symptoms compared to parent C-13. Twenty-one somatic hybrids showed resistance to PVY as a result of das-ELISA. The ELISA value (A_{405} nm) of susceptible line C-13 was more than double (>0.06) that of the negative healthy control (0.03). In all the somatic hybrids, the das-ELISA value was lower than the threshold value of healthy control (0.03); consequently, they were confirmed as somatic hybrids. Other plants which failed to be somatic hybrids were also found to have PVY resistance as they possess the wild component *S. etuberosum* as revealed by molecular data.

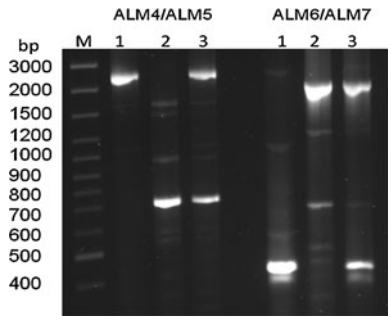

Fig. 4 Amplification products using mitochondrial primers *ALM4/ALM5* and *ALM6/ALM7*. Nos. *1*, *2* and *3* denote C-13 *S. etuberosum* and somatic hybrids, respectively

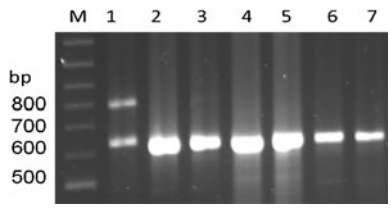

Fig. 5 Amplification products using chloroplast primers ALCP1/ALCP3. Nos. *1*, *2* and *3* denote C-13, *S. etuberosum* and somatic hybrids, respectively

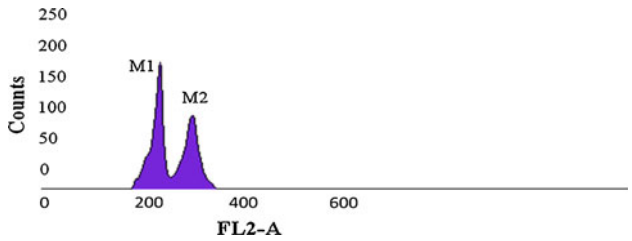

Fig. 6 Fluorescence intensities associated with nuclear DNA of somatic hybrid dihaploid C-13 *S. etuberosum*. Peak *M1* internal standard (CRBC), *M2* somatic hybrid clone

Table 3 Flow-cytometric nDNA content (pg/2C), chromosome number and ploidy level of somatic hybrids of *S. tuberosum* (di)haploid C-13 (+) *S. etuberosum* and their corresponding parents

Somatic hybrids[a]	nDNA content (pg/2C)	Chromosome no.	Ploidy level	Somatic hybrids[a]	nDNA content (pg/2C)	Chromosome no.	Ploidy level
E1	3.34	48	4*x*	E21	1.64	24	2*x*
E2	3.48	48	4*x*	E22	3.37	48	4*x*
E3	3.57	48	4*x*	E23	1.87	24	2*x*
E4	3.61	48	4*x*	E24	3.71	48	4*x*
E5	3.44	48	4*x*	E25	3.63	48	4*x*
E6	3.51	48	4*x*	E26	3.41	48	4*x*
E7	3.78	48	4*x*	E27	1.68	24	2*x*
E8	3.59	48	4*x*	E28	1.74	24	2*x*
E9	3.61	48	4*x*	E29	1.72	24	2*x*
E10	3.41	48	4*x*	E30	1.67	24	2*x*
E11	3.58	48	4*x*	E31	1.66	24	2*x*
E12	3.79	48	4*x*	E32	3.82	48	4*x*
E13	2.02	24	2*x*	E33	3.54	48	4*x*
E14	1.71	24	2*x*	E34	1.74	24	2*x*
E15	1.87	24	2*x*	E35	1.68	24	2*x*
E16	1.79	24	2*x*	E36	1.72	24	2*x*
E17	1.61	24	2*x*	E37	2.03	24	2*x*
E18	3.47	48	4*x*	E38	1.61	24	2*x*
E19	3.69	48	4*x*	E39	1.95	24	2*x*
E20	3.41	48	4*x*	E40	1.89	24	2*x*
LSD ($P \leq 0.05$) = 1.23							

nDNA content of parents C-13 and *S. etuberosum* were 1.71 and 1.67 pg/2C, respectively, and are diploid ($2n = 2x = 24$) as estimated by flow cytometry analysis

[a] Univariate *F* test for nDNA content of the somatic hybrids was not significant. In pairwise multiple comparisons statistics of means of nDNA content between the same ploidy level, either tetraploid–tetraploid or diploid–diploid, Tukey's honestly significant difference (HSD) tests were not significant at $P \leq 0.05$. In the paired comparison between diploid–tetraploid, HSD test was highly significant at $P \leq 0.01$. Null hypothesis (H_0) was rejected according to McNemar Chi-square test that no difference exists between flow cytometry and DNA analysis for the confirmation of somatic hybrids

Phenotypic assessment

A distinct phenotypic variation was observed in the somatic hybrids of C-13 (+) *S. etuberosum* and parental genotypes in the glass-house under Shimla conditions. The recorded data are presented in the Table 4. All the somatic hybrids displayed intermediate phenotypes of their parents (Figs. 7, 8 and 9), except 19 hybrids (E13 to E17, E21, E23, E27, E28, E29 to E31, E34 to E40) which showed wild parental type phenotypes. Interestingly, leaf shape of the somatic hybrids was ovate-lanceolate type with weak margins in contrast to ovate-lanceolate type and strong margins of C-13, and ovate type and weak margins of *S. etuberosum*. Intermediate type leaf structure was appeared in the somatic hybrids in contrary to closed type in both the parents. Phenotype of the wild parent was dominant in the somatic hybrids particularly with respect to foliage color (mid-green), foliage gloss (medium), secondary stem color (purple), flower intensity (low), presence of anthocyanin color in rachis, bud and corolla, anther color (yellow), stigma shape (round), and non-tuberous characters. Parent C-13 was dominant in the somatic hybrids for foliage structure (semi-compact). The corolla size of somatic hybrids was medium (3.56 cm) compared to the small size of C-13 (2.38 cm) and *S. etuberosum* (2.84 cm). Flowers of somatic hybrids were white with purple shade while C-13 had white with yellow stripes. All the somatic hybrids including parents were of late maturing type (>120 days). There was no tuber formation in the 1 EBN wild parent *S. etuberosum* and somatic hybrids.

Male fertility determination

Flower intensity was high in somatic hybrids of C-13 (+) *S. etuberosum* grown in the glass-house under Shimla conditions in the months August–September. The mean value of the pollen fertility of the somatic hybrids was estimated to be about 81.24%. In the parents C-13 and

Table 4 Phenotypic assessment of interspecific potato somatic hybrids *S. tuberosum* (di)haploid C-13 (+) *S. etuberosum* and their corresponding parents

Characters[a]	C-13	*S. etuberosum*[b]	C-13 (+) *S. etuberosum*[b]
General and canopy characteristics			
Plant vigour	Good	Good	Good
Plant height (cm)	Short (53.41)	Short (35.71)	Medium (76.83)
Foliage structure	Semi-compact	Compact	Semi-compact
Foliage colour	Light-green	Mid-green	Mid-green
Foliage gloss	Light	Medium	Medium
Maturity (days)	Late (>120)	Late (>120)	Late (>120)
Stem morphology			
Stem solidity	Hollow	Solid	Hollow
Predominant color	Green	Green	Green
Secondary color	Green	Purple	Purple
Wings type	Wavy	Wavy	Wavy
Leaf morphology			
Leaf structure	Close	Close	Intermediate
Anthocyanin coloration of rachis	Absent	Present	Present
Leaf length (cm)	Small (4.16)	Small (7.32)	Small (12.72)
Leaf width (cm)	Small (2.01)	Small (1.21)	Small (2.23)
Leaf shape	Ovate-lanceolate	Ovate	Ovate-lanceolate
Waviness of margin	Strong	Weak	Weak
Floral characteristics			
Days to first flowering	54	69	67
Flower intensity	Medium	Low	Low
Inflorescence size	1–2 branch	1–2 branch	1–2 branch
Corolla size (dia. in cm)	Small (2.38)	Small (3.12)	Small (3.76)
Corolla colour	White	White	White
Anthocyanin coloration of bud	Absent	Present	Present
Anthocyanin coloration of corolla on outer side	Absent	Present	Present
Anthocyanin coloration of corolla on inner side	Present	Present	Present
Anther colour	Orange	Yellow	Yellow
Stylar length (in comparison to stamen)	Longer	Longer	Longer
Stigma shape	Lobed	Round	Round
Tuber characteristics			
Tuber shape	Oblong	–	–
Tuber skin color	Brown-green	–	–
Tuber skin type	Rough	–	–
Tuber flesh color	White	–	–
Depth of eyes	Shallow	–	–
Male fertility (%)	81.24	75.42	78.54

[a] Non-parametric class of the trait was derived from DUS criteria

[b] There was no tuber formation in the 1 EBN wild species *S. etuberosum* and the somatic hybrids under the glass-house experiment

S. etuberosum, pollen fertility was estimated at 75.42 and 78.54%, respectively.

Discussion

The results reported here show that the fusion protocol used was highly efficient, and as a result, 21 somatic hybrids were produced with an average frequency of 15% success over regenerated calli. Similar report has been documented on electrofusion which is a widely used technique associated with a relatively high frequency of heterokaryons and somatic hybrids (Fish et al. 1988). It even more effective than chemical fusion (Pehu et al. 1989). In our experiment, somatic hybrids C-13 (+) *S. etuberosum* were confirmed on the different criteria, as in earlier reports, such as RAPD (Rokka et al. 1994; Thieme et al. 1997; Naess et al. 2001; Barone et al. 2002; Szczerbakowa et al. 2003; Greplova et al. 2008), SSR (Nouri-Ellouza et al. 2006; Gillen and Novy 2007; Thieme

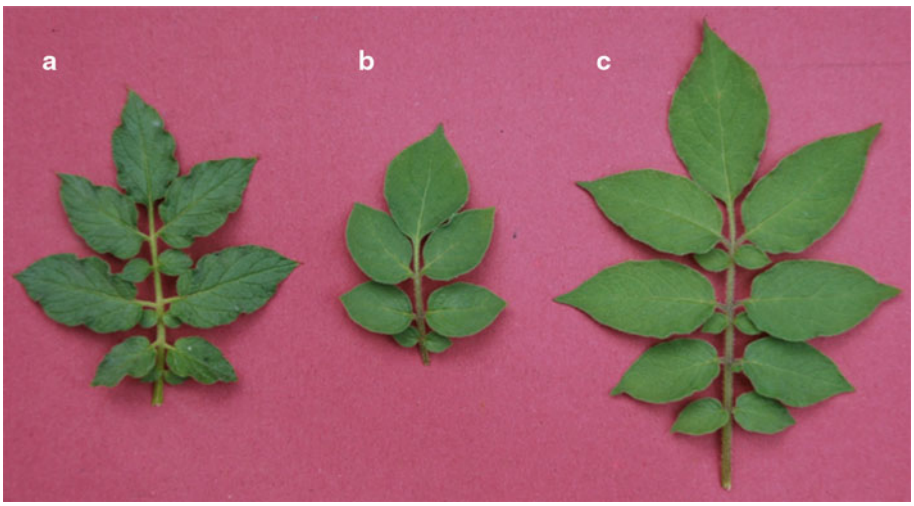

Fig. 7 Leaf of **a** parent *S. tuberosum* dihaploid C-13, **b** parent *S. etuberosum* and **c** somatic hybrid clone C-13 (+) *S. etuberosum*

Fig. 8 Flower of **a** parent *S. tuberosum* dihaploid C-13, **b** parent *S. etuberosum* and **c** somatic hybrid clone C-13 (+) *S. etuberosum*

Fig. 9 Plant of **a** parent *S. tuberosum* dihaploid C-13, **b** parent *S. etuberosum* and **c** somatic hybrid clone C-13 (+) *S. etuberosum*

et al. 2008), flow cytometric nDNA content (Thieme et al. 1997; Maciejewska et al. 1999; Oberwalder et al. 2000; Horsman et al. 2001; Szczerbakowa et al. 2003; Greplova et al. 2008), and phenotypic assessment (Novy and Helgeson 1994a; Thieme et al. 2008; Szczerbakowa et al. 2003; Greplova et al. 2008). Confirmation of somatic hybrids was revealed by species-specific diagnostic bands (RAPD and SSR) from both the parental components. Based on RAPD and SSR data, we confirmed 21 interspecific potato somatic hybrids. However, the most common problem with RAPD is its low reproducibility (Devos and Gale 1992). To check the minor differences in amplified products, it can be optimized with PCR reaction to obtain reproducible and interpretable results (Caetano-Anolles et al. 1992). The use of RAPD for routine screening of somatic hybrids could be insufficient if the amount of DNA introgressed from one of the fusion partner is too low (i.e., below 8%; Oberwalder et al. 1998). We confirmed the hybridity by standardization of PCR cycles which resulted in distinct 'species-specific diagnostic bands'. Additionally, the use of SSR primers also made it possible to confirm the hybridity and validate the results of RAPD. Nevertheless, out of 36 SSR primers, only 4 validated the RAPD findings which further suggests that somatic hybrid plants are the result of recombination between C-13 and *S. etuberosum* genomes. Our findings of

STG0016 agree with Ghislain et al. (2009), while SSR 136, SSR 188 and SSR 350 also concur with Gillen and Novy (2007) by producing expected PCR products. Other SSRs did not confirm the hybridity due to some shared bands between the parents which could not be characterized in the hybrids from which they derived. Cytoplasm type data suggest that the somatic hybrids possess W/α type cytoplasm (Lössl et al. 2000) while other hybrids which did not show specific bands have undetermined cytoplasm type. These cytoplasmic type data confirmed the somatic hybrids and suggest that the unique hybrids may likely be the result of different recombination within the parental genome, and are potential materials for future study.

Flow cytometry analysis for ploidy estimation reveals the tetraploid nature of somatic hybrids and has also been successfully used earlier. The nDNA content also gives preliminary information on genome size of the somatic hybrids. Nevertheless, cytogenetic and chromosomal counts enable estimation of completeness and proportion of parental genomes in the somatic hybrids. Phenotypic evaluation of somatic hybrids validated the RAPD, SSR and FC results. The somatic hybrids are possible because of good cytoplasmic and nuclear fusion in heterokaryon. Probably, the 19 somatic hybrids failed due to the high regeneration potential of individual calli and shoot development thereof from one parent only. Finally, studies on male fertility assessment of somatic hybrids revealed relatively higher fertility, thus indicating their wider application for transferring virus resistance to the cultivated potatoes. In the past, researchers have used the fertile pollen and transferred the virus resistance characters from *S. etuberosum*-derived somatic hybrids into cultivated potatoes and their subsequent progenies (Gavrilenko et al. 2003; Novy et al. 2002, 2007; Gillen and Novy 2007; Thompson et al. 2007).

Taking into account the intermediate phenotypes, the ploidy level, and the analysis of nuclear and cytoplasmic (mitochondrial/chloroplast) genomes, we conclude that the 21 plants are somatic hybrids. It suggests that both parental genomes expressed well at molecular, cytogenetic and morphological levels in the somatic hybrids. The response of the hybrids to inoculation with PVY showed resistance to virus in comparison to parent C-13. These data clearly show that interspecific protoplast fusion can lead to an improved resistance to PVY in the resulting regenerated plants, whereas parent C-13 is highly sensitive and the other one, *S. etuberosum*, is resistant. Valkonen and Rokka (1998) reported a loss of parental resistance characters in the interspecific hybrids resulting from protoplast fusion between *S. brevidens* and *S. tuberosum*. Other studies (Gibson et al. 1988; Valkonen et al. 1994) described symmetric somatic hybrids with intermediate titer of PVY compared to parental lines. These results suggest that parental genome recombination and somaclonal variation events (Polgar et al. 1999) may lead to somatic hybrid lines harboring different kinds of responses to phytopathogens. Resistance genes can be entirely or partially inactivated or lost, as they can be activated by recombination of parental genomes.

These encouraging results demonstrate that interspecific somatic hybridization could be considered as an alternative and effective means of creating resistant cultivated potatoes. It has the potential to supplement conventional breeding methods for potato improvement. However, its inheritance and durability require more detailed assessment for their stability in subsequent progenies. In addition, such somatic hybrids would be of particular interest for more analysis at the molecular level for genomic compositions of the E- and A-genomes. Additionally, combining resistance genes into the cultivated potatoes and their validation though molecular markers are a future need.

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