Interspecific somatic hybrids between *Solanum bulbocastanum* and *S. tuberosum* and their haploidization for potato breeding

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Abstract

Protoplast fusion between incongruent *Solanum bulbocastanum* and *S. tuberosum* haploids was accomplished to produce hybrids combining elite traits from both parents. We identified 11 somatic hybrids out of 42 regenerants analyzed through ISSR markers. Some hybrids had loss or gain of fragments compared to the parents, likely due to rearrangements and deletions of chromosome segments after fusion, and/or to somaclonal variation during hybrid regeneration. Increased heterotic vigor for some traits as well as high diversity was observed as the effect of both ploidy and fusion combination. Microsporogenesis analysis indicated the occurrence of multivalent configurations and several meiotic abnormalities, such as chromosomes bridges and various spindle orientations. Since all hybrids were sterile, *in vitro* anther culture was employed for haploidization as a possible strategy to overcome barriers to hybridizations. Haploids were obtained from all the tetraploid *S. bulbocastanum* (+) *S. tuberosum* somatic hybrids tested, although with differences in both the number of embryos per 100 anthers cultured and the number of differentiated green plantlets. This is the first report on the successful production of haploid plants from *S. bulbocastanum* (+) *S. tuberosum* hybrids.

Additional key words: androgenesis, microsporogenesis, molecular markers, somaclonal variation.

Introduction

Among major crops, the tetraploid (2n=4x=48) potato *Solanum tuberosum* has one of the largest gene pools, including about 200 wild relatives (Spooner and Hijmans 2001). Altogether, these species form a polyploid series with x=12 as the basic chromosome set. Typical of wild *Solanum* species is their wide geographical distribution in a broad range of environmental conditions. This has allowed many species to adapt to stress environments and resist pests and diseases. Not surprisingly, therefore, wild *Solanum* represent a rich source of valuable germplasm with traits of interest that can be transferred into the cultivated potato. However, due to incompatibility barriers, several species cannot be crossed with cultivated potato. In *Solanum*, an endosperm balance number (EBN varying from 1 to 4) has been assigned to most species. Successful crosses occur only when a 2:1 maternal to paternal EBN ratio in the hybrid endosperm occurs (Johnston *et al.* 1980).

Somatic hybridization *via* protoplast fusion provides a powerful tool to overcome crossing barriers, allowing the integration of parental nuclear and cytoplasmic genomes, and gene flow across sexual borders. It is a multi-step process involving protoplast isolation and fusion, culture and regeneration of the post-fusion mixture, and identification of somatic hybrids among the regenerated shoots. A review by Orczyk *et al.* (2003) outlines some basic aspects of somatic hybridization and the advantages associated to the use of this breeding approach.

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Abbreviations: AFLP - amplified fragment length polymorphisms; DAPI - 4',6-diamidino-2-phenylindole; DMPD - N,N-dimethyl-*p*-phenylenediamine; ISSR - inter simple sequence repeats; PCA - principal component analysis.
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Interspecific somatic hybridization has been applied to introgress disease resistances from incongruent 2x (1 EBN) *Solanum* species, such as *S. commersonii* (Cardi 2001), *S. brevidens* (Tek *et al.* 2003), and *S. bulbocastanum* (Helgeson *et al.* 1998) into *S. tuberosum* (4 EBN). As a result of interspecific protoplast fusion, many important traits exhibit wide variation both in the somatic hybrids and in their progenies. Due to the presence of wild genome, undesired traits may also appear. Therefore, somatic hybrids produced need thorough characterization for both target and undesired traits prior to use in breeding programs.

In the framework of a potato breeding program to improve quality and resistance traits through the exploitation of wild species, we used somatic hybridization to produce hybrid genotypes between incongruent 2x *S. bulbocastanum* and cultivated *S. tuberosum* haploids. Due to somatic hybrid sterility, we engineered haploidization as a strategy to overcome such a drawback. Indeed, the haploids obtained from anthers arise from meiosis-derived microspores, which have a different genetic asset from that of the parents. This might lead to fertility restoration, as already reported by Yermishin *et al.* (2006) in *S. tuberosum* (+) *S. pinnatisectum* hybrids. We report on the production, identification through ISSR markers and characterization of *Solanum bulbocastanum* (+) *S. tuberosum* somatic hybrids as well as their genome manipulation by *in vitro* anther culture.

Materials and methods

Plant material and somatic hybridization: The two *Solanum bulbocastanum* Dunal (blb) accessions were kindly provided by Dr. J. Bamberg, University of Wisconsin, Madison, USA. DEI-23 and V2I-59 were developed at the DiSSPAPA from 4x × 2x crosses between *Solanum tuberosum* L. and a *Solanum phureja* Juz. & Buk. haploid inducer. Three fusion combinations were carried out, namely blb1C(+)DEI-23, blb2C(+)DEI-23 and blb2C(+)V2I-59 and labeled as HF9, HF6 and HF5, respectively. Protoplast isolation, electrofusion and culture were performed as described previously (Cardi *et al.* 1993a,b). The only modification in the protoplast culture procedure was the use of sodium alginate instead of agarose. Briefly, after fusion, the protoplast suspension was mixed with 2.8 % alginate solution (in 0.4 M sorbitol) in a ratio of 1:1 (final protoplast density of 8×10^4 cm^{-3}). The mixture (0.001 cm^3) was layered onto the top of a Petri dish containing setting agar (0.4 M sorbitol, 50 mM $CaCl_2$ and 0.9 % agar). After 2 h in the dark, 0.4 M sorbitol + 50 mM $CaCl_2$ solution was added. Finally, the protoplasts embedded in the 1 cm^3 lenses were cultured as mentioned above. The frequency of protoplast division was estimated 11 d after isolation. Means were calculated from three replications, scoring at least 300 protoplasts per Petri dish. Since preliminary experiments indicated poor regeneration ability of the parental genotypes (data not shown), several regeneration media were tested. Therefore, about 8 to 10 weeks after fusion, green calli were further cultured using one of the following procedures: Bokelmann and Roest (1983), coded P26B-P27B; Austin and Cassells (1983) and Haberlach *et al.* (1985), DH-SP; Haberlach *et al.* (1985) and Sidorov *et al.* (1999), DH-MON; Austin *et al.* (1993) and Austin and Cassells (1983), SA_4-SP; Curry and Cassells (1999), M2-M3; Hunt and Helgeson (1989), SKM. Finally, from each regenerating callus a single shoot was excised and rooted on Murashige and Skoog (1962; MS) medium supplemented with 3 % sucrose and 0.9 % agar.

Identification of somatic hybrids: Somatic hybrids were identified using ISSR markers. DNA from fusion parents and regenerated shoots was extracted from leaves of *in vitro* grown plants, using *Qiagen* (Valentia, CA, USA) *DNeasy* plant mini kit. Sixteen ISSR primers (Matthews *et al.* 1999, Scarano *et al.* 2002) were evaluated on the fusion parents (data not shown). Primers $HVH(TG)_7T$, $BDB(GAT)_5$ and $(AG)_8$ were polymorphic between *S. bulbocastanum* (blb1C/2C) and DEI-23; primers $HVH(TCC)_5$ and $BDB(TCC)_5$ discriminated between blb2C and V2I-59. These primers were therefore chosen to analyze 42 shoots regenerated from the three fusions. PCR parameters were set according to Scarano *et al.* (2002). PCR products were separated on a 1.5 % agarose gel and visualized by ethidium bromide staining under UV radiation. To achieve reproducibility and clear banding patterns, each amplification was repeated at least three times separately. Regenerated shoots showing the specific fragments from both parents were considered somatic hybrids.

Phenotyping: The somatic hybrids produced were morphologically evaluated in experimental field in Camigliatello Silano, Southern Italy (1300 m a.s.l.) during the summer of 2006. Ten tubers of each hybrid were planted at the end of May. Planting distances were 70 cm between rows and 35 cm on the row. At harvest, at the beginning of October, the tubers of each plant were weighed and counted. Specific gravity of tubers was determined using the mass-in-air/mass-in-water method. Antioxidant activity in lyophilized aqueous extract was determined with the N,N-dimethyl-p-phenylenediamine (DMPD) method (Fogliano *et al.* 1999). Absorbance was measured at 505 nm and compared with that obtained from a standard ascorbic acid solution. Total soluble proteins were estimated according to Bradford (1976) and the content was measured at 595 nm.

Anther culture: The anthers of five tetraploid somatic hybrids (HF5A, FH6A, HF6E, HF9I, and HF9AP) were cultured on the medium described by Rokka *et al.* (1995). After 8 weeks, anthers either developed embryos or produced calli and both were transferred onto regene-

ration medium (Wenzel *et al.* 1981) for additional 8 weeks. On this medium embryos evolved in green plantlets (direct embryogenesis) or never sprouted, and calli started to regenerate shoots (indirect androgenesis). Shoots obtained were sub-cultured on MS medium supplemented with 3 % sucrose, 0.9 % agar (pH 5.8). Plantlets were cultivated in a growth chamber at temperature of 24 ºC and 16-h photoperiod with irradiance 60 μmol m^{-2} s^{-1}. The number of embryos per anther and per callus was recorded after 8 and 16 weeks. Amplified fragment length polymorphisms (AFLP; Vos *et al.* 1995) was carried out on the regenerated shoots and the hybrids they derived from. The commercially available AFLP kit and protocol (*Gibco-BRL* AFLP analysis system I, *Life Technologies*, Gaithersburg, MD, USA) was used. For selective amplification, two combinations of primers were employed: E-ACT/M-CTA; E-ACT/M-CAG. Both EcoRI primers were 5′-labeled with *FAM* dye. The PCR products of selective amplifications were separated by capillary electrophoresis on an *ABI Prism 310* genetic analyzer and detected by fluorescence. An internal size marker, *GeneScan 500-Rox* (*Applied Biosystems*, Foster City, CA, USA) was added. Technical and biological replicates were performed twice and only reproducible banding patterns were considered for analysis.

Cytological assessment: Chromosome counts in root tip cells were performed to assess both the ploidy level of the somatic hybrids and the embryo-derived plants. Root tips about 1 - 2 cm long were collected from young *in vitro* plants and treated as already described (Dong *et al.* 2000). Chromosome counts were based on five well-spread metaphases, using DAPI to stain the chromosomes. Three somatic hybrids (HF6A, HF6E and HF9M) were used for the meiotic analysis. The procedure for meiotic chromosome preparation was essentially the same as that used for mitotic chromosomes from root tips (Dong *et al.* 2000) with the following modification: anthers were digested in the enzyme mixture (4 % cellulase, 2 % pectinase) for 2 h at 37 ºC. Meiotic stages were stained with DAPI and observed under UV fluorescent microscope. Hybrids were also checked for pollen production and pollen staining by acetocarmine, counting at least 200 pollen grains.

Data analysis: Data on phenotypic characterization were analyzed by principal component analysis (PCA). Statistical analyses were performed using *SPSS* software *v. 11.5* (*SPSS* Inc., Chicago, USA). In particular, mean values of five variables, average tuber yield per plant, average number of tubers per plant, tuber specific gravity, soluble protein and antioxidant content were processed by a PCA model to graphically discriminate hybrids based on extracted factors. AFLP marker data were scored into 1/0 binary matrices. Genetic similarity between clones was calculated by Nei and Li standard (1979) using *SPSS* software.

Results

Protoplasts from each genotype started to divide within 5 - 7 d after the fusion. As for parental genotypes, both *S. bulbocastanum* clones displayed high division frequencies, whereas protoplasts from DEI-23 and V2I-59 divided at much slower rates (data not shown). Parental genotypes blb 1C, blb 2C and V2I59 did not regenerate or had a very low regeneration frequency, whereas regeneration frequency of DEI 23 ranged from 0 to 17.4 %. As for fusion combinations, frequency of division ranged from 14 % in blb1C(+)DEI-23 to 38 % in blb2C(+)DEI-23 (Table 1). A total of 6962 hybrid calli were mass-cultured. Overall, 49 regenerating calli were obtained, with regeneration frequency of 0.1 % for blb2C(+)V2I-59 and 0.9 % for blb1C/2C (+) DEI-23. The best regeneration performance was obtained using DH-MON, with regeneration frequencies of 1.7 % in blb1C(+)DEI-23, 3.7 % in blb2C(+)DEI-23 and 0.5 % in blb2C(+)V2I59. Out of 49 shoots, 42 were further propagated, while the remaining seven were discarded because of poor growth and/or growth abnormalities.

Table 1. Regeneration frequencies of electrofused *Solanum tuberosum* (+) *S. bulbocastanum* protoplast-derived calli using different media. The number of calli transferred onto each medium is reported in parentheses (M2mod - hormones as in M2, other ingredients as in DH, - not determined)

Plants	p26B-P27B	DH-SP	DH-MON	SA-SP	M2-M3	M2mod-M3	SKM	Regenerated calli [%]
Parents								
blb 1C	0 (309)	0 (259)	0.5 (375)	-	0 (80)	0 (120)	0 (360)	2 (0.1)
blb 2C	0 (30)	0 (147)	0 (171)	0 (148)	0 (209)	0 (300)	-	0 (0)
DEI 23	17.4 (23)	2.5 (80)	15.8 (133)	0 (63)	0 (89)	3.0 (65)	-	29 (6.4)
V2I59	0 (30)	-	0 (12)	-	0 (19)	-	-	0 (0)
Hybrids								
blb 1C (+) DEI 23	0.8 (524)	0.5 (586)	1.7 (976)	1.4 (142)	0.3 (297)	0.3 (630)	0.8 (600)	35 (0.9)
blb 2C (+) DEI 23	0 (188)	0.3 (306)	3.7 (243)	-	0.4 (275)	0.2 (568)	-	12 (0.8)
blb 2C(+) V2I 59	0 (86)	0 (212)	0.5 (407)	-	0 (323)	0 (599)	-	2 (0.1)

Out of 35 blb1C(+)DEI-23 regenerated shoots, 30 were analyzed with ISSR markers. Of these, seven proved to be somatic hybrids (coded HF9B, HF9I, HF9M, HF9R, HF9AI, HF9AP, HF9AQ), showing the specific fragments from both parents; the remaining 23 plantlets resembled the electrophoretic pattern of DEI-23 (data not shown). Hybrid HF9B lost the 0.85 kb band from the blb1C parent, whereas HF9M lost two bands (1.5 and 0.65 kb) from blb1C and two (1.4 kb and 0.9 kb) from DEI-23 (Fig. 1). Out of 12 shoots regenerated from blb2C(+)DEI-23, 10 were analyzed. Three (HF6A, HF6E, HF6G) were somatic hybrids, and seven originated from DEI-23. As for blb2C(+)V2I-59, out of two shoots obtained, one was a somatic hybrid (HF5A) and the other resembled the banding pattern of blb2C.

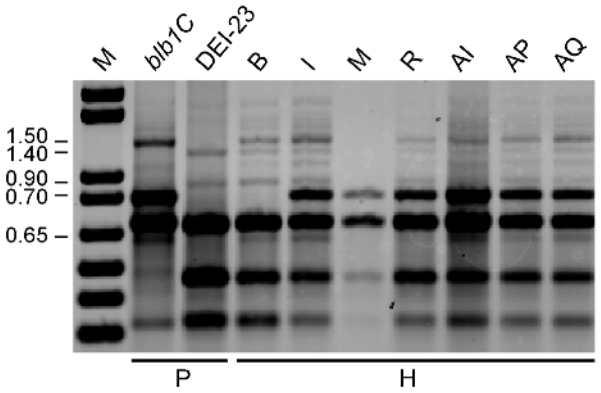

Fig. 1. Identification of somatic hybrids between *S. tuberosum* (DEI-23) and *S. bulbocastanum* (blb1C) using ISSR primer $(AC)_8$. Sizes of lost parental bands in HF9B and HF9M are reported. M - DNA size marker; P - parents; H - somatic hybrids.

Mitotic analysis revealed the occurrence of seven tetraploid (2n=48), three hexaploid (2n=72; HF9B, HF9R, HF9AI) and one aneuploid (HF9AP; 2n=49) hybrid. Ten out of eleven hybrids produced tubers under long day conditions like the *S. tuberosum* parents. Tubers were much larger than those of the parents. Exceptions were HF9AQ and HF9B, which set very small tubers. Most hybrids presented oblong tubers and shallow eyes. Tuber production ranged from 80 (HF9B) to 972 g plant^{-1} (HF9B), whereas the specific gravity of tubers varied from 1.066 (HF9R) to 1.086 (HF6G). Antioxidant activity is expressed in μg of ascorbic acid per cm^3 of lyophilized aqueous extract. The contents of ascorbic acid in the wild parents were too low to be detected, whereas in the parents DEI-23 and V2I-59 they were 0.4 and 5.5 μg cm^{-3}, respectively and in the somatic hybrids ranged from 1.3 μg cm^{-3} (HF6A) to 5.8 μg cm^{-3} (HF9AP).

To highlight differences among hybrids and identify those performing best, mean values obtained for measured parameters were analyzed by PCA and two factors were extracted explaining comprehensively 80 % of overall variability. PCA analysis emphasized the high diversity of somatic hybrids produced and suggests that this variation is related both to ploidy and to fusion combination (data not shown). Indeed, Group 1 clustered all the hexaploid hybrids (HF9R, HF9AI and HF9B) deriving from blb1C(+)DEI-23 fusion combination. By contrast, Group 2 clustered hybrids HF6A, HF6E and HF6G, all obtained from blb2C(+)DEI-23 fusion combination.

Meiotic analysis of three hybrids (HF6A, HF6E and HF9M) was difficult, since the chromosomes tended to clump together. However, at pachytene stage, it was possible to observe that several chromosomes paired as bivalent (Fig. 2*A*). Multivalent configurations were detected as well, at both pachytene (sporadically) and at diplotene-diakinesis (Fig. 2*B*, *E*). The three hybrids analyzed had complete meiotic division with several anomalies, including putative loops at pachytene stage, dicentric chromatid bridges at anaphase I and II, and various spindle orientations at metaphase II-anaphase II (Fig. 2*C*,*D*,*F-I*). Dyads, triads and tetrads with and without micronuclei were recovered at telophase II (Fig. 2*J-M*). Several tetraploid hybrids (HF5A, HF6E, HF9I, HF9AP) shed stainable pollen. Hexaploids HF9B and HF9R and tetraploid HF6A had rare and collapsed pollen grains. Intra/inter-ploidy and intra/inter-EBN crosses with *Solanum* genotypes failed.

Anther-derived plants were obtained by direct androgenesis (Fig. 3*A-D*) as well as by indirect androgenesis (Fig. 3*E*,*F*). All hybrids underwent androgenesis. As for direct androgenesis, 27 embryos from four hybrids

Table 2. Results from *in vitro* anther culture of tetraploid somatic hybrids between *S. bulbocastanum* (*blb1C* and *blb2C*) and *S. tuberosum* haploids (V2I-59 and DEI-23). For each somatic hybrid, the number of anthers cultivated, the total number of embryos produced, the number of green plantlets differentiated by direct and indirect embryogenesis are reported (the percentage in parentheses).

Hybrid	Combination	Anthers	Embryos direct	Embryos indirect	Plantlets direct	Plantlets indirect
HF5A	*blb2C* (+) V2I-59	100	7 (7.0)	83 (83.0)	1 (14.3)	83 (100)
HF6A	*blb2C* (+) DEI-23	440	16 (3.6)	30 (6.8)	6 (37.5)	30 (100)
HF6E	*blb2C* (+) DEI-23	200	0	1 (0.5)	0 (0)	1 (100)
HF9I	*blb1C* (+) DEI-23	740	1 (0.1)	3 (0.4)	1 (100)	3 (100)
HF9AP	*blb1C* (+) DEI-23	1000	3 (0.3)	3 (0.3)	2 (75)	3 (100)

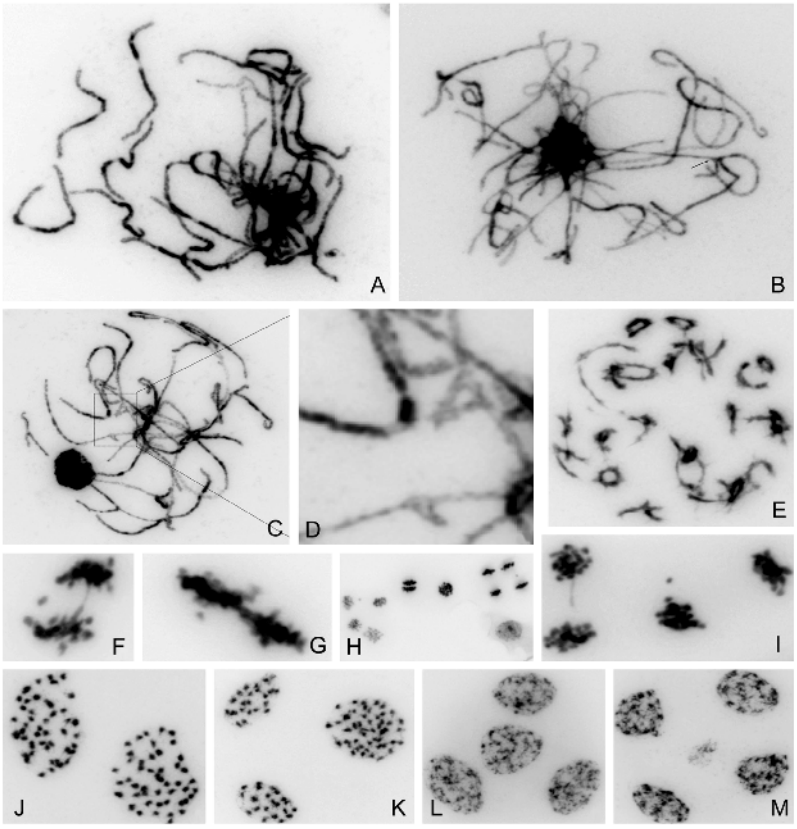

Fig. 2. Meiotic analysis of tetraploid *S. tuberosum* (+) *S. bulbocastanum* somatic hybrids. *A* - Chromosomes at pachytene stage paired as bivalent. *B*, *E* - Putative multivalent configurations detected at pachytene (sporadically) and at diplotene-diakinesis. *C*,*D*,*F-M* - Several meiotic anomalies (*C*,*D* - putative loops at pachytene stage (close-up in *D*), *F*,*I* - dicentric chromatid bridges at anaphase I and II, *G*,*H* - various spindle orientations at metaphase II-anaphase II, *J-M* - dyads, triads and tetrads with and without micronuclei at telophase II).

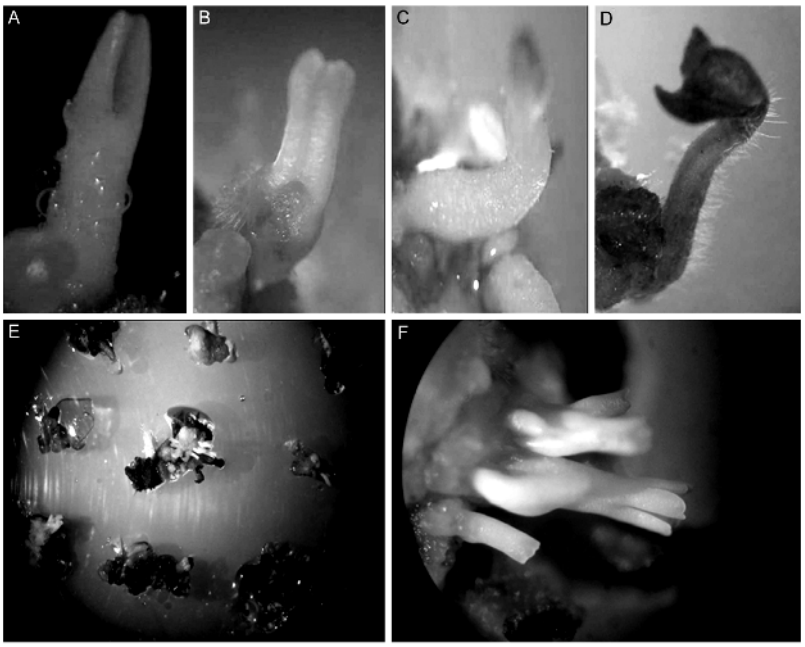

Fig. 3. Direct (*A-D*) and indirect (*E*,*F*) *in vitro* haploid induction by blb (+) tbr anther culture (*A* - embryos 6 d, B - 12 d, C - 3 weeks, *D* - 4 weeks after culture initiation, *E* - embryogenetic calli, *F* - magnification of embryos sprouting from anther-callus).

were obtained. Of these, 59 % (16) were produced by HF6A. The best androgenic ability was shown by HF5A, which produced seven embryos from 100 cultured anthers. Ten embryos developed into green plantlets (37 %): six from HF6A, two from HF9AP and one from HF5A and HF9I. The highest shoot regeneration capacity was shown by HF9I. As for indirect embryogenesis, 120 embryos were obtained from anther-derived calli. The highest regeneration frequency was observed in HF5A (83 %); the lowest in HF9AP (0.3 %) (Table 2). All embryos evolved into shoots, but only 14 survived under *in vitro* culture (six from HF5A, three from HF9I and HF9AP, one from HF6A and HF6E, data not shown). All embryo-derived plants were haploids with 24 chromosomes, which substantiated that they derived from reduced (n=2x=24) microspores. AFLP analysis on regenerated shoots was performed to confirm the androgenetic origin of the regenerated shoots (data not shown). We compared the AFLP profile of each regenerated plant with its parent. Overall, out of 199 loci analyzed an average polymorphism of 62 % was observed. The similarity coefficient ranged from 0.51 (between HF5A and AS5A1) to 0.70 (between HF9AP and AS9AP5), highlighting the occurrence of segregation of AFLP alleles present in the parental plants. Such evidence confirmed the gametophytic origin of our haploids.

Discussion

The fusion of *S. bulbocastanum* with *S. tuberosum* haploids was carried out to overcome crossing barriers and to produce hybrids combining elite traits from both parents. A high rate of cell division and callus formation was observed, probably due to the choice of fusion parents with different tissue culture response. By contrast, regeneration frequency was very low, confirming results on regeneration from somatic hybrid calli already reported in the literature (Cardi *et al.* 1993a,b, Taski-Ajdukovic *et al.* 2006). The analysis of putative fusion products is an essential step to confirm hybrid status. Although this can be performed with morphological, biochemical, and cytological analysis, molecular markers represent the most efficient, informative and rapid tool for such a purpose. Hybridity of potato plants from heterokaryons has often been confirmed using various types of molecular markers (Orczyk *et al.* 2003). In our study ISSRs proved to be a swift, highly reproducible and economical method to generate informative genetic markers and to identify 11 somatic hybrids. ISSR analysis also indicated the loss or gain of fragments in the somatic hybrids compared to the parental patterns. This may well be due to rearrangements and deletions of chromosome segments after fusion, and/or to somaclonal variation during hybrid regeneration.

Compared to previous reports on *S. bulbocastanum* (+) *S. tuberosum* somatic hybrids (Helgeson *et al.* 1998, Bołtowicz *et al.* 2005), ours were mostly tetraploid. Phenotypically, the hybrids showed vigor for several traits, including tuber production. Increased vigor after intra- and inter-specific fusions in potato has been reported elsewhere (Polgar *et al.* 1993, Cardi 2001). Increased vigor is generally due to the expression of heterosis, but also to the return to the tetraploid level of the cultivated potato and to the transmission of useful linkages. Particularly attractive is also their resistance to late blight, and the loss of undesired glycoalkaloids from the wild parent (Savarese 2007). The phenotypic variability found may be partly due to differences in the ploidy level and in the genomic constitution of the somatic hybrids. Recently, multicolor GISH analysis demonstrated that our tetraploid hybrids had a 2:2 wild:cultivated genomic ratio. By contrast, 6x somatic hybrids had diverse genomic constitution, with either a 4:2 or a 2:4 wild:cultivated ratio (Iovene *et al.* 2007). These authors also provided evidence that most of the hybrids inherited the plastidial and mitochondrial DNA of the cultivated parent. A few hybrids with a rearranged mitochondrial genome were also identified.

Somatic hybridization can be fully exploited if pairing and genetic recombination occur between homeologous chromosomes, and if the hybrids are fertile to be backcrossed with *S. tuberosum*. In our study, observation of multivalent configurations confirmed molecular data by Helgeson *et al.* (1998) and suggests the occurrence of intergenomic chromosome pairing between these two species. Some of the meiotic irregularities we observed might also indicate intergenomic recombination between homeologous chromosomes. All attempts to use these hybrids in crosses with *Solanum* genotypes failed although they exhibited complete meiotic division and meiotic defects were indistinctly observed both in the "pollen-less" phenotypes and in those shedding stainable pollen. This confirmed that sterility of hybrids is a common bottleneck of somatic hybridization (Novy *et al.* 1994, Rokka *et al*. 1995) and forced us to produce androgenic haploids to overcome hybrid sterility.

We obtained 24-chromosome haploids from all the tetraploid *S. bulbocastanum* (+) *S. tuberosum* hybrids tested, although with different androgenic capacity in terms of both number of embryos per 100 anthers cultured and number of differentiated green plantlets. The key role played by the genotype in androgenesis is well known not only in potato (Kopecký and Vagera 2005), but also in many other species (Datta 2005, Bal and Abak 2007). It relies on the evidence that anther culture response is genetically determined and the genes controlling this trait can be differentially inherited. Anther culture performed with blb1C and blb 2C revealed that these genotypes were totally recalcitrant (data not shown). Hence we presume that the androgenic capacity in our hybrids was inherited from *S. tuberosum*.

This is the first report of successful anther culture involving somatic hybrids between *S. bulbocastanum* and *S. tuberosum*. Previous attempts to produce haploids from this species combination failed (Yermishin *et al.* 2006). Androgenic haploids from somatic hybrids were previously obtained only from *S. acaule* (+) *S. tuberosum* (Rokka *et al.* 2005) and *S. brevidens* (+) *S. tuberosum* (Rokka *et al.* 1995) hybrids. Interestingly, only hexaploid hybrids displayed androgenic ability, producing triploid anther-derived genotypes. Pret'ová *et al.* (2006) outlined the importance of haploids in gamete and embryo biology, breeding, genetics, cytogenetics, mutation and transformation studies. We believe that our *S. bulbocastanum* (+) *S. tuberosum* haploids are promising material for both basic research and applied breeding. Analysis of their glycoalkaloid content provided useful information on inheritance of parental glycolakaloids (Savarese 2007). Haploids should theoretically have an EBN between 1 and 2. Indeed, Ehlenfeldt and Hanneman (1988) reported that three unlinked loci with additive effects and with equal EBN gene value within a species control the EBN. Under this additive model, genetic recombination in the somatic hybrids may produce haploids with various EBN gene values. Therefore, crosses with 2x (2EBN) *S. tuberosum* and other genotypes with known EBN will be performed to determine their EBN. Most importantly, from the practical standpoint, controlled pollinations will be used to continue breeding efforts.

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