Theor Appl Genet (1992) 83:807–812

THEORETICAL AND APPLIED GENETICS

© Springer-Verlag 1992

Tetraploid potato hybrids through protoplast fusions and analysis of their performance in the field

W. M. Mattheij and K. J. Puite*

Department of Cell Biology, Center for Plant Breeding and Reproduction Research CPRO, PO Box 16, NL-6700 AA Wageningen, The Netherlands

Received March 25, 1991; Accepted July 9, 1991
Communicated by Yu. Gleba

Summary. Data on the production of tetraploid hybrid plants after electrofusion of protoplasts from various diploid (2n = 2x = 24) *Solanum tuberosum* and *S. phureja* are reported. Ten different fusion combinations were used. Six hybrids with the tetraploid chromosome number 2n = 4x = 48 were tested under field conditions for their performance in various agronomic traits (tuber type, tuber yield, underwater weight of tubers, numbers of tubers, mean weight per tuber). Tuber yield in five of the six hybrid clones was similar to that of cv Bintje. One hybrid clone (35-4) had three times higher tuber yield than cv Bintje. The mean tuber weight of this hybrid was similar to that of cv Bintje. The results of this study prove that fertile tetraploid somatic hybrids having similar or higher tuber yield than that of cv Bintje can be obtained through somatic hybridization. This technique is now included in commercial potato breeding programs in The Netherlands.

Key words: *Solanum tuberosum* – *Solanum phureja* – Somatic hybrids – Field experiment – Agronomic traits.

Introduction

As potato is an autotretraploid, potato breeding requires extensive selection in large populations of progeny to obtain a high number of desired agronomic traits. Therefore, an analytical breeding system, which involves breeding at the diploid level followed by a return to the tetraploid level ensuring a high degree of heterozygosity, is desirable (Chase 1963). Somatic hybridization using protoplasts from different di(ha)ploid potato clones would be a promising approach to obtain hybrid plants that combine agronomic traits from both parents and express a high level of heterozygosity (Wenzel et al. 1979).

Several authors have carried out investigations on the production of hybrids through protoplast fusion between di(ha)ploid clones of *S. tuberosum*, also including *S. phureja* as one of the fusion parents. Various systems for the selection of fusion products were used, such as difference in performance of parental clones and their hybrids in tissue culture (Austin et al. 1985), hybrid vigor of the fusion products (Debnath and Wenzel 1987; Deimling et al. 1988; Waara et al. 1989), in vitro selection system using different antibiotic resistances (Masson et al. 1989), and early selection of heterokaryons using a micromanipulator (Puite et al. 1986) or flow cytometer (Puite et al. 1988).

In most of these studies fusions were carried out using only one combination of two different parents. Some authors reported the occurrence of a number of tetraploid hybrid plants (Austin et al. 1985; Deimling et al. 1988; Puite et al. 1988). Also, loss of specific chromosomes in the hybrids was observed (Pijnacker et al. 1989). However, so far no field studies have been reported on the performance of somatic hybrid plants with regard to agronomic traits.

The present article reports data on the production of tetraploid hybrids through fusion of protoplasts from di(ha)ploid clones of *S. tuberosum* and *S. phureja*. Ten fusion combinations of *S. tuberosum* and *S. phureja*, involving several breeding clones, were investigated. The hybrid plants with the tetraploid chromosome number 2n = 4x = 48 were grown together with their parents and the cultivar Bintje, used as standard, in experimental plots of the Flevopolder (The Netherlands) to study their performance in various agronomic traits.

* To whom correspondence should be addressed.

Table 1. Details of clones used in the fusion experiments

Clone	Abbreviation	Species	Origin
SH 77-78-1994	SVP1	*S. tuberosum*	SVP*
P 77-1445-224	SVP5	*S. phureja*	SVP
SH 83-81-47	SVP10	*S. tuberosum*	SVP
DH 81-7-1463	SVP11	*S. tuberosum*	SVP
DH 76-1-41	SVP12	*S. tuberosum*	SVP
P 81-1868-435	SVP20	*S. phureja*	SVP
SH 81-128-1579	SVP103	*S. tuberosum*	SVP
SH 81-136-1791	SVP104	*S. tuberosum*	SVP
SY7	SY7	*S. tub.* × *S. phur.*	Peloquin

SVP* = Foundation for Agricultural Plant Breeding, which is now a part of the Center for Plant Breeding and Reproduction Research CPRO

Materials and methods

Plant genotypes, protoplast isolation, and fusion

Six di(ha)ploid (2n = 2x = 24) clones of *S. tuberosum*, two of *S. phureja*, and one diploid (2n = 2x = 24) desynaptic mutant clone derived from *S. tuberosum* × *S. phureja* were used in the present study. The details of the clones are given in Table 1. All of the genotypes were maintained as shoot cultures on Murashige and Skoog (MS) medium with 3% sucrose. Prior to protoplast isolation, shoots were grown on MS medium with 1% sucrose, or with 3% sucrose supplemented with 0.01 m*M* SAN9789 (norflurazon, Sandoz AG, Basel) for the production of bleached plantlets. The former yielded green mesophyll protoplasts and the latter, white protoplasts with bleached chloroplasts. Protoplasts were isolated according to Puite et al. (1988) with some minor modifications. Fluorescein diacetate (FDA) (final concentration 15 µg/ml) was added directly to the enzyme mixture with bleached leaf material. Protoplasts were collected in 0.38 *M* mannitol layered on top of a 0.43-*M* sucrose solution.

Prior to fusion, green and FDA-stained protoplasts were mixed in a 1:1 or 2:1 ratio in 0.38 *M* mannitol with a total of 2×10^5 and 3×10^5 protoplasts per milliliter, respectively. The 2:1 ratio was also used because the green protoplasts often seemed fragile, with a higher tendency to burst during the fusion process. Electrofusion was performed according to the procedure described earlier (Puite et al. 1986), using a 0.8-ml, four-compartment fusion chamber with an electrode distance of 3 mm. Protoplasts were lined up using an AC field of 100 V/cm at 1 MHz. Optimum conditions for membrane fusion differed between experiments. Parameters that were varied were direct current (DC) pulse height (1300–2300 V/cm), pulse number (1–3), and pulse length (50 or 100 µs).

The fusion chamber, being open underneath, was removed about 1 min after the DC pulse. Further, 0.8 ml of V-KM medium with 0.275 *M* glucose, but without mannitol, was added to the fusion mixture. Thus, the protoplasts were cultured in V-KM medium at half strength with 0.17 *M* mannitol, 0.14 *M* glucose, 0.1 mg/l NAA, 0.5 mg/l zeatin, 500 mg/l casein hydrolysate, and 0.18 mg/ml cefotaxime (pH 5.8), in a growth cabinet at 25 °C under continuous light of about 1,000 lx.

Isolation of fusion products and culture

Fusion products could easily be identified under UV excitation based on simultaneous red and yellow fluorescence from chlorophyll and FDA, respectively. With a micropipette and micromanipulator, 50–150 fusion products were collected from a petri dish 2 or 3 days after fusion and transferred to 200 µl of ½-VKM, supplemented with 0.18 mg/ml of cefotaxime, in the center well of a Falcon dish (no. 3037). The outer ring contained 2 ml of sterile water. In all, 11 separate fusion experiments were carried out. In addition, in one of the experiments (Exp. 11) 5 µl droplets covered with mineral oil (Sigma) (to prevent drying) were positioned in the center well. In this experiment culture medium was present in the outer ring instead of water, because culture medium caused less condensation of droplets on the mineral oil. Three to 23 fusion products were transferred into each of these droplets.

When first divisions appeared, in general after 3 days, 200 µl of fresh ½-VKM medium was added to the 200 µl. The 5 µl droplets were diluted step-wise with 5, 40, and 600 µl during the first weeks. When calli reached a size of 0.1 mm, they were transferred to 35-mm petri dishes containing 0.8 ml liquid culture medium. After further growth, they were placed on callus growth medium (2 weeks), shoot induction medium (3 weeks), and shoot elongation medium. Roots appeared after transfer of excised shoots on MS medium without hormones. For details of the composition of the media and culture conditions used, reference is made to Bokelmann and Roest (1983).

Measurement of nuclear DNA content by flow cytometry

Small pieces of leaf tissue were chopped in a buffer solution on ice. The composition of the buffer was according to Blumenthal et al. (1979). After filtration through a 15-µm nylon filter and centrifugation (260 g), the pellet was resuspended in the buffer medium and stained with 50 µg/ml ethidium bromide. Samples kept on ice were analyzed by flow cytometry using the 488 nm excitation of the argon laser of the FACS IV Cell Sorter (Becton Dickinson, USA).

Molecular analysis of regenerants

DNA was extracted from 4 g of leaf material of putative somatic hybrid plants and their parents, according to the procedure described by Dellaporta et al. (1983). About 10 µg DNA per sample was digested to completion with the restriction endonucleases *Eco*RI or *Hin*dIII (2–3 units/µg) for 2 h at 37 °C. After digestion, the fragments were separated by electrophoresis on a 0.8% agarose (Ultrapure) gel and subsequently transferred onto a nitrocellulose filter (Southern 1975). Isolated DNA inserts from clones IE10, C5, A10, and C7 from a Bintje leaf cDNA library were labeled with biotin and hybridized against the digested plant DNA (Jacobs et al. 1990).

Chromosome counts

Roots from fast-growing plants were treated in a saturated solution of α-mono-bromo-naphthalene for 1.5 h at room temperature. Afterwards, roots were thoroughly washed in running tap water and fixed in absolute alcohol: glacial acetic acid (3:1, v/v) for 24 h. Roots were washed for 10 min in demineralized water and hydrolyzed in 1 *N* HCl for 8 min at 60 °C, followed by a second washing in demineralized water for 10 min. The surplus water was removed and the roots were transferred to Feulgen reagent. Only meristematic, purple-colored roots were washed in demineralized water and treated with a 5% pectinase (SERVA, Heidelberg) solution for 14 min at 40 °C, followed by washing in demineralized water for 10 min. Tips of about 1–1.5 mm were removed from the roots and squashed in a drop of 1.5% acetocarmine.

Field experiment

During the summer of 1990, a field experiment with the tetraploid hybrids, their parents, and the cultivar Bintje was per-

formed in the experimental plots of the Flevopolder (The Netherlands). The trial consisted of three blocks, each containing randomly distributed plots of eight plants per tested clone. Tubers were harvested during the middle of September. Analysis of various agronomic traits included the development of the foliage at harvest, tuber type, general impression of the tubers, tuber yield, number of tubers, and underwater weight per 5 kg of tubers, being a measure of the starch content of the tubers.

Results

The results of 11 separate fusion experiments are given in Table 2. In all, ten combinations of fusions between di(ha)ploid *S. tuberosum* and *S. phureja*, involving six genotypes of the former, two of the latter, and one diploid hybrid derived from *S. tuberosum* × *S. phureja* (SY7), were studied. The fusion combination of the two parents involved is given in the second column; bleached protoplasts from the first parent were fused with the green protoplasts from the second parent.

The ploidy level of the 82 regenerated plants was determined by flow cytometric measurement of the nuclear DNA content. The results showed that 15 were diploid, 54 were tetraploid, and that 28 had a higher ploidy level. As can be seen from Table 2, regenerants obtained from fusions of protoplasts between SVP103 or SVP104 and those of SY7 were mainly tetraploid. As only the 4x plants are of interest, these were further checked for the hybrid character using RFLP analysis (Fig. 1). Of the 54 tetraploid plants, 9 were found to be hybrids.

Further, the RFLP analysis revealed that with only one exception, all the tetraploid plants obtained in the fusion combination of SVP103 or SVP104 with SY7 were of the SY7 type. Therefore, a second series of SVP103 (+) SY7 fusions was carried out (Table 2, Exp. 11). Fusion products were cultured as usual in 200 µl of culture medium, as well as in 5 µl droplets of this medium. The latter procedure resulted in higher percentages of microcalli (Table 3). From the second series of SVP103 (+) SY7 fusions six regenerants were obtained, among which there were three hybrid plants, one being a 4x hybrid.

All the tetraploid hybrid plants were transferred to the greenhouse and propagated via cuttings. All hybrids were male fertile, as determined from observations on pollen stainability using lactophenol fuchsin.

Field experiment

Six hybrid clones with the tetraploid chromosome number ($2n = 4x = 48$) were tested in the field plot, together with the parental clones and the tetraploid cultivar Bintje, with regard to their performance in various agronomic traits (Table 4). One of the two hybrid clones with

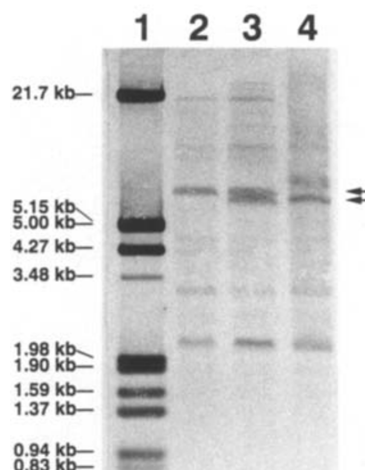

Fig. 1. A representative example of the restriction fragment length polymorphism of the fusion product 35-4 (*lane 3*) and its parents SVP11 (*lane 2*) and SVP 20 (*lane 4*). DNA was restricted with *Eco*RI. The hybridization probe was C5. *Lane 1* shows lambda-DNA restricted with *Hin*dIII and *Eco*RI

Table 2. Summary of data obtained from the fusion experiments

Experiment	Fusion combination	No. of regenerated plants	No. of 4x plants among the regenerants	No. of 4x hybrids	Chromosome number of the 4x hybrids
1	SVP5 (+) SVP1	2	1	1	48
2	SVP11 (+) SVP20	8	4	3	48, 48, 45
3	SVP11 (+) SVP12	3	2	0	
4	SVP20 (+) SVP11	1	0	0	
5	SVP11 (+) SVP10	1	0	0	
6	SVP5 (+) SVP11	3	3	3	48, 48, 48
7	SVP10 (+) SVP11	7	3	0	
8	SVP103 (+) SVP11	1	1	0	
9	SVP103 (+) SY7	25	20	0	
10	SVP104 (+) SY7	25	19	1	48
11	SVP103 (+) SY7	6	1	1	49
		82	54	9	

Table 3. Summarized data on the second series of fusion experiments involving SVP103 (+) SY7

Experiment	Culture volume (µl)	Total no. of fusion products	No. of microcalli
A	200	104	4 (4)[a]
B	200	422	27 (6)
C	5	104	15 (14)
D	5	82	11 (13)
E	5	153	18 (12)

[a] Percentage of microcalli are given in parentheses

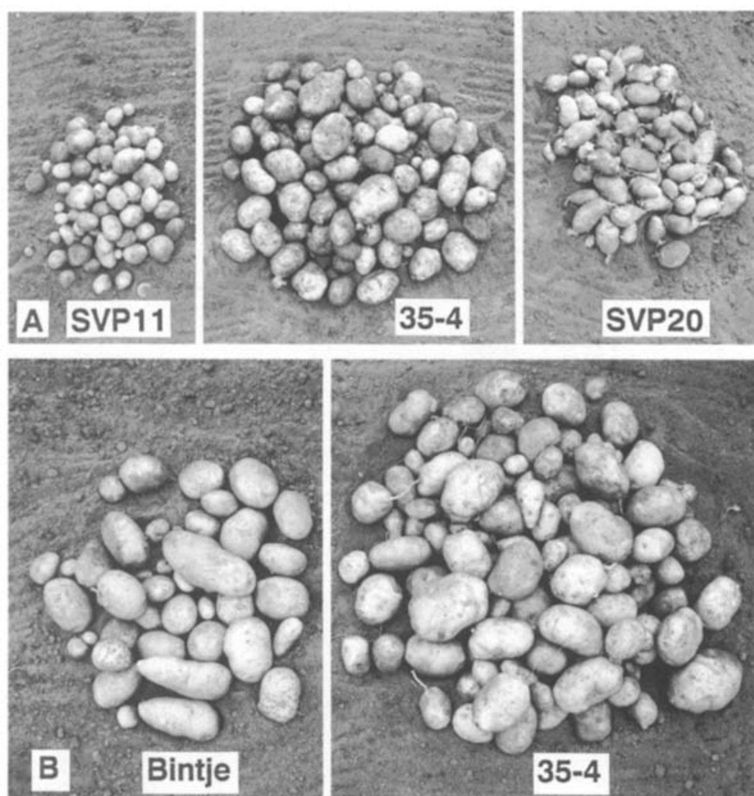

Fig. 2A–B. A comparison of tubers of the hybrid 35-4 with those of the fusion parents SVP11 and SVP20 (**A**) and with the reference cultivar Bintje (**B**)

Table 4. Field data. The sequence of parental clones, somatic hybrids, and cv Bintje (column 1) is given according to the tuber yield (g) i.e., low the high tuber yield (see column 5). Data are the mean value of three field plots, each with eight plants

Clone	Foliage	Tuber type	General impression of the tubers after harvest	Tuber yield (g)	Underwater weight per 5 kg tubers (g)	No. of tubers	Mean tuber weight (g)
SVP1	senescent	small	3	180 a	387 d	23	8 a
SVP104	senescent	well shaped	6	3,252 b	320 ab	43	77 c
SVP11	senescent	small	5	3,457 bc	341 bc	84	42 b
28-3	green	wild, coarse	5	3,762 bc	375 cd	44	87 cd
83-2	green	wild	5	4,194 bc	312 a	50	84 c
83-1	green	wild, coarse	4	4,518 bc	316 a	57	79 c
Bintje	senescent	well shaped	7	4,722 bc	376 cd	45	105 de
83-3	green	wild, coarse	4	4,777 bc	328 ab	53	90 cd
SY7	senescent	slightly wild	5	4,938 c	434 e	94	45 b
97-3a	senescent	well shaped	6	5,154 bcd	385 d	73	75 c
SVP20	green	wild	6	6,793 cd	339 bc	148	53 b
SVP5	almost senescent	wild	6	7,140 cd	362 cd	168	44 b
35-4	green	coarse	7	14,177 e	357 bcd	123	115 e

The hybrids were derived from the following fusion combinations: hybrid 28-3 from SVP5 (+) SVP1, hybrid 35-4 from SVP11 (+) SVP20, hybrids 83-1, -2, -3 from SVP5 (+) SVP11, and hybrid 97-3a from SVP104 (+) SY7. The scale used for the general impression of the tubers after harvest ranged from 1 (not satisfactory) to 8 (excellent)
Identical letters a, b, c, d, and e in a column indicate data that do not differ at 5% level of significance (Student *t*-test, $P=0.05$)

48 chromosomes, obtained from the SVP11 (+) SVP20 fusions, could not be tested due to insufficient material.

As can be seen in Table 4, the *S. tuberosum* clones (SVP1, 11, 104) exhibited a senescent foliage. The foliage of the *S. phureja* clones (SVP5, 20) was not yet senescent at the day of harvest. However, all hybrids derived from a combination of these *S. tuberosum* and *S. phureja* clones had green foliage. The presence of green foliage at harvest indicates that the tubers may not have reached full maturity.

With regard to tuber type, the *S. tuberosum* tubers were well shaped and often small. On the other hand, the *S. phureja* clones produced tubers with an increased eye depth, irregular shapes, and a large variation in tuber size. The hybrids yielded tubers that were either well shaped or had the *S. phureja* phenotype.

The general impression of the tubers after harvest, a parameter which includes tuber type and yield, had a score between 3 and 7, the highest score being obtained by cv Bintje and hybrid 35-4 derived from the SVP11 (+) SVP20 fusions (Fig. 2). The mean tuber yield from three field plots, each with eight plants, of this hybrid was 14 kg, compared to 4.7 kg for cv Bintje. The high tuber yield resulted from a high number of tubers, while the mean tuber weight of 115 g for hybrid 35-4 was not significantly different from the mean tuber weight of cv Bintje.

Discussion

Selection of the fluorescent fusion products using a micromanipulator and culture in 200 µl of medium has resulted in many putative hybrid microcalli. An increase in plating efficiency from 5 to 14% was obtained when fusion products were cultured in 5 µl droplets of medium (Table 3). The number of regenerated plants in each fusion experiment was rather low, since only 10% of the calli regenerated to shoots. Experiments 9 and 10, involving fusions of bleached protoplasts of SVP103 and SVP104, respectively, with green protoplasts of SY7, which gave a high number of regenerated plants, also resulted in several tetraploid plants. But RFLP analysis revealed only one hybrid, while all others showed the RFLP pattern of SY7. It is unlikely that during the isolation of fusion products using the micropipette mainly homofusions of SY7 protoplasts were selected, although this cannot be completely excluded. The second series of fusions of SVP103 (+) SY7 resulted in the regeneration of three diploid plants, two hexaploid hybrids ($2n = 66-69$), both with two SVP103 and one SY7 genome, as established with Giemsa C-banding of chromosomes (L.P. Pijnacker, personal communication), and one tetraploid hybrid with 49 chromosomes.

Approximately 13% of the regenerated tetraploid plants proved to be hybrids with 48 chromosomes. Although this percentage of tetraploid hybrids is low, it is not uncommon. Previously, in potato, Puite et al. (1986, 1988) and Waara et al. (1989) obtained aneuploid or polyploid hybrids, but no hybrids with 48 chromosomes. Recently, Waara et al. (1990) reported the occurrence of only four stable hybrids ($2n = 4x = 48$) among 51 hybrid regenerants, while Chaput et al. (1990) obtained 18 tetraploid somatic hybrids from 136 selected regenerants.

The analysis on tuber yield of five tetraploid hybrids showed that the yield was similar to that of cv Bintje. Hybrid 35-4 had a significantly higher yield than those of the other hybrids. Also, this hybrid produced three times more yield than that of cv Bintje.

The three hybrids 81-1, -2, -3 from fusions of SVP5 (+) SVP11 did not differ significantly with regard to their performance in tuber yield, underwater weight, and mean tuber weight. Also, Deimling et al. (1988) observed a phenotypic and electrophoretic uniformity between hybrids having the same parents.

Clones SVP5 and SVP20 of *S. phureja* were selected for fusion because of higher production of tubers and, thus, higher tuber yield. All hybrids involving SVP5 or SVP20 in fusion had a significantly higher mean tuber weight than their parents. Thus, these data suggest that the choice of the parental combination for fusion is of great importance for the improvement in agronomic traits of the hybrids.

It can be concluded from the results obtained in the present study that tetraploid somatic hybrids giving higher tuber yield than the diploid parental clones and similar or higher yield than even cv Bintje can be obtained through somatic hybridization. Wider application of this technique in potato breeding through further investigations might demonstrate its importance in the development of new cultivars. This will, however, require well-established diploid breeding programs. It is encouraging that somatic hybridization programs are now included in the commercial potato breeding schemes in The Netherlands.

Acknowledgements. This work was financially supported by the Innovation Fund for Plant Breeding (Inpla), Grant No. 84-02. The help of the following persons is gratefully acknowledged: M. van Deelen (tissue culture), W.R.R. ten Broeke (flow cytometry), P. Dijkhuis (cytology), J.J.H.M. Allefs, J. Jacobs, and J.R.A. de Koning (RFLP analysis), A.E.F. Neele and L. Sijpkes (field experiment). The critical reading of the manuscript by C.M. Colijn-Hooymans, F.A. Krens, and K. Sree Ramulu and L. van Vloten-Doting was highly appreciated.

References

Austin S, Baer M, Ehlenfeld M, Kazmierczak PJ, Helgeson JP (1985) Intraspecific fusions in *Solanum tuberosum*. Theor Appl Genet 71:172–175

Blumenthal AB, Dieden JD, Kapp LN, Sedat JW (1979) Rapid isolation of metaphase chromosomes containing high molecular weight DNA. J Cell Biol 81:255–259

Bokelmann GS, Roest S (1983) Plant regeneration from protoplasts of potato (*Solanum tuberosum* cv "Bintje"). Z Pflanzenphysiol 109:259–265

Chaput M-H, Sihachakr D, Ducreux G, Marie D, Barghi N (1990) Somatic hybrid plants produced by electrofusion between dihaploid potatoes: Bf15 (H1), Aminca (H6) and Cardinal (H3). Plant Cell Rep 9:411–414

Chase SS (1963) Analytic breeding in *Solanum tuberosum* L. – a scheme utilizing parthenotes and other diploid stocks. Can J Genet Cytol 5:359–363

Debnath SC, Wenzel G (1987) Selection of somatic fusion products in potato by hybrid vigour. Potato Res 30:371–380

Deimling S, Zitzlsperger J, Wenzel G (1988) Somatic fusion for breeding of tetraploid potatoes. Plant Breed 101:181–189

Dellaporta SJ, Wood J, Hicks JB (1983) A plant DNA minipreparation II. Plant Mol Biol Rep 1:19–21

Jacobs JJMR, Krens FA, Stiekema WJ, Spanje M van, Wagenvoort M (1990) Restriction fragment length polymorphism in *Solanum* spp for the construction of a genetic map of *Solanum tuberosum* L.: a preliminary study. Potato Res 33:171–180

Masson J, Lancelin D, Bellini C, Lecerf M, Gerche P, Pelletier G (1989) Selection of somatic hybrids between diploid clones of potato (*Solanum tuberosum* L.) transformed by direct gene transfer. Theor Appl Genet 78:153–159

Pijnacker LP, Ferwerda MA, Puite KJ, Schaart JG (1989) Chromosome elimination and mutation in tetraploid somatic hybrids of *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 8:82–85

Puite KJ, Roest S, Pijnacker LP (1986) Somatic hybrid plants after electrofusion of diploid *Solanum tuberosum* and *Solanum phureja*. Plant Cell Rep 5:262–265

Puite KJ, Wikselaar P van, Verhoeven H (1985) Electrofusion, a simple and reproducible technique in somatic hybridization of *Nicotiana plumbaginifolia* mutants. Plant Cell Rep 4:274–276

Puite KJ, Broeke W ten, Schaardt JG (1988) Inhibition of cell wall synthesis improves flow cytometric sorting of potato heterofusions resulting in hybrid plants. Plant Sci Lett 56:61–68

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Waara S, Tegelström H, Wallin A, Eriksson T (1989) Somatic hybridization between anther-derived dihaploid clones of potato (*Solanum tuberosum* L.) and the identification of hybrid plants by isozyme analysis. Theor Appl Genet 77:49–56

Waara S, Wallin A, Eriksson T (1990) Production and analysis of intraspecific somatic hybrids of potato (*Solanum tuberosum* L.). In: Abstr 7th Int Congr Plant Tiss Cell Culture, Amsterdam, June 24–29, 1990, pp 222

Wenzel G, Schieder O, Przewozny T, Sopory SK, Melchers G (1979) Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor Appl Genet 55:49–55