Theor Appl Genet (1996) 92: 403–410

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Analysis of somatic hybrids between two sterile dihaploid *Solanum tuberosum L.* breeding lines. Restoration of fertility and complementation of *G. pallida* Pa2 and Pa3 resistance

Received: 21 August 1995 / Accepted: 6 October 1995

Abstract Fourteen somatic hybrids generated by electrofusion of mesophyll protoplasts from a non-flowering dihaploid *S. tuberosum* clone, DHAK-11, and a male-sterile dihaploid clone *S. tuberosum*, DHAK-33, were grown in the greenhouse and subjected to morphological assessments and tests for fertility and resistance to the white potato cyst nematode *Globodera pallida* pathotypes Pa2 and Pa3. The ploidy level of the hybrids ranged from 38 to 63 chromosomes. All hybrids developed flowers with violet petals except for one, hy-56, that possessed red petals. The colour of the tuber skin was purple in all hybrids except in hy-56 where the tuber skin was red. All of the hybrids were female fertile and generated viable seeds. Near-tetraploid hybrids produced the highest number of seeds per fruit and these seeds had a normal size. Hybrids with 58 or more chromosomes produced smaller seeds and less seeds per fruit. The germination frequency of the seeds was not influenced by the chromosome number of the hybrids. Pollen viability was determined and the male fertility of three hybrids was tested. Pollination with these three hybrids gave rise to fruit development, but only one produced viable seeds. The hybrids were tested for resistance to *G. pallida* pathotypes Pa2 and Pa3. A high level of resistance to Pa3, inherited from one parental clone, DHAK-11, and a high level of resistance to Pa2, inherited from the other parental clone, DHAK-33, was combined in four hybrids. These results demonstrate, that protoplast fusion is an efficient method for restoring the fertility of somatic hybrids generated from sterile parent clones, and is a powerful procedure for the complementation of multigenetic disease resistance traits in potato breeding lines.

Key words *Globodera pallida* · *Solanum tuberosum* L. · *Solanum vernei* · Intraspecific somatic hybridization · Fertility

Communicated by G. Wenzel

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Introduction

The heterozygous and tetraploid nature of cultivated potato cultivars (2n=4x=48) complicates the predictability of identifying genotypes with desirable agronomical traits in sexual progeny. Aimed at improvements in breeding efficiency, a number of reports propose dihaploid breeding methods (Wenzel et al. 1979; Ross 1986; Deimling et al. 1988). Dihaploid clones, obtained by either anther cultures (Sopory and Bajaj 1987) or by pollination with *Solanum phureja* (Hermsen and Verdenius 1973), show distinct morphological features and agronomical characteristics due to the reduced ploidy level.

However, one major obstacle in dihaploid potato breeding is that dihaploids extracted from tetraploid cultivars are predominantly male sterile (van Eck et al. 1993; Hutten et al. 1994). Preparatory crosses between *Solanum tuberosum* and *S. phureja* performed in our laboratory confirm a high frequency of sterile, dihaploid plants. Consequently many dihaploid clones with valuable agronomical traits can not be directly employed in sexual breeding programs. However, somatic hybridization by use of protoplast fusion has shown to be an attractive method to circumvent this problem. By protoplast fusion important agronomical traits can be combined into new tetraploid hybrids and concomitantly a high level of heterozygosity is obtained. Intraspecific hybridization between dihaploid *S. tuberosum* clones has been reported by several authors (Austin et al. 1985; Waara et al. 1991; Thach et al. 1993). Interspecific protoplast fusion within different *Solanum* species was originally developed to introduce novel germ plasm from sexually non-compatible *Solanum* species in order to broaden the genetic base of *S. tuberosum* (Pehu et al. 1989). Protoplast fusion is, however, not only a technique to broaden the gene pool of potatoes and to over-

come sexual incompability barriers but is also a rapid procedure for the combination of important multigenetic agronomic traits within the *S. tuberosum* gene pool.

The white potato cyst nematode *Globodera pallida* (Jones et al. 1970; Stone 1972) causes severe damage to potato crops. Three pathotypes of *G. pallida*, Pa1, Pa2 and Pa3, can be distinguished with a set of differentials derived from the wild species *S. multidissectum* and *S. vernei* (Kort et al. 1977). Resistance to pathotypes Pa2 and Pa3 is reported in *S. kurtzianum, S. multidissectum* and *S. vernei* (Ross 1986), *S. circaeifolium* subsp. *circaeifolium cab* (Mattheij et al. 1992), *S. spegazzinii* (Dellaert et al. 1988) and *S. tuberosum* subsp. *andigena* CPC 1673 (Turner 1989). It has been shown that resistance to the pathotypes Pa2 and Pa3 is quantitatively and polygenically inherited, and that resistance to Pa2 and Pa3, derived from the wild species *S. spegazzinii*, is controlled by the *H1* locus on chromosome 5 and by at least two minor loci on chromosomes 4 and 7 (Kreike et al.1994). The genetical complexity of *G. pallida* resistance hampers sexual breeding programs aimed at a complementation of the resistance to the different pathotypes. Until now only a few potato breeding lines, and no commercial potato cultivars, with complete resistance to both Pa2 and Pa3 have been reported.

For the wild species *S. vernei* knowledge concerning the genetic background for Pa2 and Pa3 resistance is, scanty. Plaisted et al.(1962) concluded from a series of intercrosses between different *S. vernei* lines that at least two, and probably three, loci are involved in the *G. pallida* resistance. Later it was reported that only by incorporation from various *S. vernei* sources, each with a quantitatively inherited pathotype-specific resistance, could genotypes be obtained with a resistance to a large number of *G. pallida* populations (Turner et al. 1983; Dellaert and Vinke 1987). Ross (1986) summarizing the sources of major resistance genes, reported that there were likely to be four major dominant genes from *S. vernei* inherited in a Mendelian way and that a high level of resistance to Pa2 and Pa3 only seems to occur in potato cultivars that carry several minor genes in addition to the major ones.

In the present paper we describe the use of intraspecific symmetric protoplast fusion between two sterile, dihaploid potato breeding lines in order to achieve restoration of fertility of the hybrids and to achieve a successful complementation of a high level of resistance to *G. pallida* pathotypes Pa2 and Pa3 in a number of hybrids.

Materials and methods

Plant material

Resistance to *G. pallida* pathotypes Pa2 and Pa3 was introduced into different tetraploid *S. tuberosum* breeding lines by the use of sexual crossing with two diploid clones of the wild species *S. vernei*. In wild diploid *Solanum* species approximately 2% of the pollen is non-reduced, so giving rise in crosses with tetraploid plants to a limited amount of tetraploid seeds (Yerk and Peloquin 1988). From these crosses two tetraploid cultivars were selected. *S. tuberosum* N80-APZ-12 (obtained from a cross with *S. vernei* accession no 1642-2) confering partial resistance to Pa2 and a high level of resistance to Pa3, and *S. tuberosum* N81-ARQ-09 (obtained from a cross with *S. vernei* SVP-VT_n-2-62-33-3) confering a high level of resistance to Pa2 and partial resistance to Pa3. Extraction of dihaploids from these two tetraploid lines following pollination with *S. phureja* IVP 101 was carried out. All crosses were made at The Danish Potato Breeding Foundation, Vandel. The results of two representative *S. phureja* crosses are listed in Table 1. From the *S. phureja* crosses two dihaploid clones were selected with respect to resistance to Pa2 and Pa3. These two clones, used throughout this study, were *S. tuberosum*, DHAK-11(code 88-0-02-09), characterized by no flower formation, red tuber skin, partial resistance to Pa2 and resistance to Pa3, and *S. tuberosum*, DHAK-33 (code 89-0-18-01), characterized by white flowers, incomplete anther development, white tuber skin, resistance to Pa2 and partial resistance to Pa3.

Somatic hybrids

Electrofusion of mesophyll protoplasts was performed in order to combine the genomes of the two dihaploid clones. Selection of fusion products and characterization of the somatic hybrids by means of chromosome counting, RAPD (random amplified DNA polymorphism) and isozyme analysis were all accomplished according to Rasmussen and Rasmussen (1995). The hybrids were grown in growth chambers for 16 weeks and then transferred to the greenhouse. Fourteen hybrids were selected, representing a ploidy range of 38–66 chromosomes. Eight hybrids, hy-19, hy-24, hy-30, hy-31, hy-34, hy-49, hy-53 and hy-56, were previously analyzed by chromosome counting, RAPD and isozyme analysis (Rasmussen and Rasmussen 1995). In addition six verified hybrids, hy-61, hy-70, hy-71, hy-72, hy-73 and hy-74, from an additional fusion experiment between DHAK-11 and DHAK-33, were included in the present study.

Transfer to soil

In vitro internodes of regenerated plants were transferred to pith boxes (Vefi, Larvik, Norway), and rooted in the greenhouse in soil ("Mixture-1", Pindstrup, Denmark) supplemented with 0.1 mg/l IAA and 0.3% Previcur-N (Schering A/S, Rødovre, Denmark). The plantlets were hardened under a layer of white plastic for 5 days, then the plastic was gradually removed. After the adaptation period the plants were irrigated with 0.5‰ "Pioner-mixture" supplemented with "Pioner-micronutrient" (P. Brøste A/S, Denmark).

Morphological assessments

Hybrids and parental plants were characterized by petal colour during flowering and by tuber colour after harvest. Assessment of flower and tuber skin colour was according to the Nickerson Color Fan (1957; Published and Distributed by Munsell Color Co., Inc., 2441 N. Calvert Street, Baltimore, Maryland 21218, USA).

RAPD

In this study 140 decamer oligonucleotide primers from seven series, AC, AQ, AR, AS, AT, AW and AX (Operon Kit, Alameda, Calif., USA), were tested for the generation of polymorphic RAPD bands. DNA isolation and PCR amplification were performed as described earlier (Rasmussen and Rasmussen 1995).

Pollen morphology and viability

Pollen grains from mature anthers of each hybrid were analyzed three times over a period of 2 weeks. The morphology of the hybrid pollen was compared with that of pollen from a tetraploid standard cultivar which showed 90% uniform pollen size. A population of

Table 1 Interspecific crosses were performed between two tetraploid *S. tuberosum* lines and the diploid *S. phureja* IVP 101. From each cross one plant with a high level of resistance to either *G. pallida* Pa2 or Pa3 was selected and used as a parent plant for somatic hybridization. A phenotypic characterization of the two selected parental clones DHAK-11 and DHAK-33 is listed in Materials and methods

Cross	Offspring					
	Number of fruits	Number of seeds	Dihaploid plants	Female fertile	Male fertile	Selected clone
S. tub. N80-APZ-12 × *S. phureja* IVP 101	8	17	11	1	0	DHAK-11
S. tub. N81-ARQ-09 × *S. phureja* IVP 101	2	11	8	1	0	DHAK-33

pollen showing 90% uniform size was categorized as normal, while pollen with less than 90% uniform size was categorized as varying. Pollen viability was determined by staining with 1% w/v acetocarmine according to Pehu et al.(1989).

Fertility test

Fertility tests of the hybrids were performed by using three tetraploid standard cultivars: *S. tuberosum* cv 89-BHZ-5 (pollen donor B, LKF Vandel), *S. tuberosum* cv DTO-2 (pollen donor D, received from CIP, Lima, Peru), and *S. tuberosum* cv "Gordana" (female G, received from Saatzucht F. Lange, Bad Schwartau, Germany). Prior to opening of the flower buds all recipient flowers in a cluster were emasculated to avoid self-pollination. Each stigma was pollinated three times. Pollen donor B was used to test the female fertility on nine hybrids. In addition, pollen donor D was used for a supplementary test to analyze the female fertility of four of these hybrids. Tests for the male fertility of three hybrids were performed with *S. tuberosum* cv "Gordana" as female partner.

Seed harvest and germination

Mature fruits from each inflorescent cluster were collected in nylon bags. After harvest the fruits were opened and the seeds were transferred to blotting paper to dry. Seed morphology was examined and the seeds were stored at 4°C for at least 8 weeks. Prior to germination seeds were surface sterilized for 20 min in 3% Korsolin (Ferrosan Fine Chemicals A/S, Køge, Denmark) and washed three times in sterile water for 20 min. Seeds were germinated at 24°C in 9-cm Petri dishes on ½ strength MS salt and vitamins (Murashige and Skoog 1962), 1.5% sucrose, 10 mg/l GA_3, 0.8% Bactoagar, pH 5.8. Germination with 15 seeds per Petri dish was performed in the dark for 3 days and then continued in dim light, 5000 lx, for 3–6 weeks. The seeds were transferred to fresh media every 3rd week. When less than 100 seeds were produced from a hybrid all seeds were used for germination. For hybrids that produced more than 100 seeds, 100 seeds were randomly selected and used for germination.

Screening of hybrids for Pa2 and Pa3 resistance

Analysis *of G. pallida* pathotype Pa2 and Pa3 resistance was accomplished by the Dutch Breeding Company, Ropta-ZPC, Metslawier, The Netherlands. Ten tubers from each hybrid were used for the analysis of *G. pallida* resistance. Using the close-container test five tubers from each hybrid were standard inoculated with Pa2 or Pa3 cysts. Three months after inoculation the total number of cysts formed on the roots were scored. The average number of cysts per tuber was calculated, and the level of resistance was categorized into the following groups:

0 cysts per tuber:	complete resistance	CR
$0 < x \leq 8$ cysts per tuber	resistance	RR
$8 < x \leq 12$ cysts per tuber:	partial resistance	R
>12 cysts/tuber	susceptible	S

Results

Morphological assessments

In total 14 somatic hybrids, generated by the fusion of protoplasts from the non-flowering dihaploid clone *S. tuberosum* DHAK-11 and the male-sterile dihaploid clone *S. tuberosum* DHAK-33, were analysed. Selection of putative hybrids, based on vigorous callus growth, and verification of the hybrid nature of the plants by the use of chromosome counting, RAPD and isozyme analysis, was performed as described in Rasmussen and Rasmussen (1995). The somatic hybrids were grown in boxes in a well-defined soil in the greenhouse and only one shoot per plant was allowed to grow. The morphology of the somatic hybrids showed only minor differences in respect of plant height, leaf morphology and leaf pigmentation. Different developmental stages of the greenhouse-grown hybrids, illustrating flowering, fruit setting, seed formation, seed germination and mature tubers, are shown in Fig. 1. Data on the ploidy level, petal colour, tuber colour, female and male fertility, seed germination frequency and resistance to the two *G. pallida* pathotypes Pa2 and Pa3 of the hybrids are shown in Table 2.

The chromosome number of the hybrids ranged from 38 in hy-72 to 66 in hy-61. All hybrids flowered profusely over a period of several weeks. Assessment of petal colour showed that the tetraploid *S. tuberosum* N80-APZ-12 developed red petals. The dihaploid parental clone DHAK-33 developed white petals. All hybrids possessed violet petals except for hy-56 which had red petals.

The tuber skin colour of all hybrids was purple, except for hybrid hy-56 with 46 chromosomes, which possessed a red tuber skin, as did the dihaploid parental clone DHAK-11. The dihaploid parental clone DHAK-33 was characterized by a white tuber skin.

Fertility

The dihaploid parental clone DHAK-11 never generated flowers, while the dihaploid parental clone DHAK-33 developed normal stamens but the anthers never matured. In contrast to the parental clones all tested hybrids produced normal anthers. All hybrids generated plenty of pollen

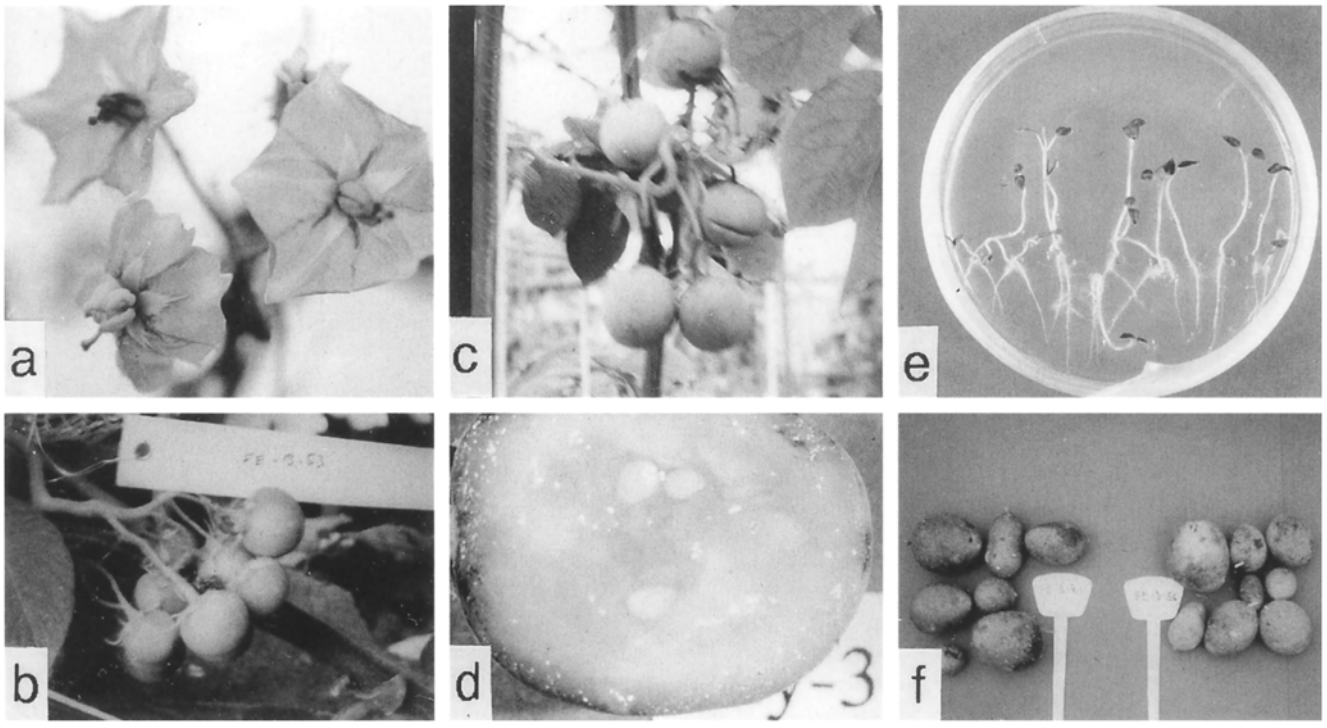

Fig. 1a–f Developmental stages of somatic hybrids generated by the fusion of protoplasts from two dihaploid clones, *S. tuberosum* DHAK-11 and *S. tuberosum* DHAK-33. **a** Flowers from a hybrid, hy-31, with violet petals and mature anthers. **b** Fully developed fruits on hy-53 after pollination with the tetraploid pollen donor B, *S. tuberosum* cv 89-BHZ-5. **c** Fully developed fruits on the standard female partner *S. tuberosum* cv "Gordana" after pollination with hy-31. **d** Cross section of a hy-31 fruit with normal seeds obtained after pollination with *S. tuberosum* cv 89-BHZ-5. **e** Seeds from hy-31 obtained by pollination with the tetraploid pollen donor D, *S. tuberosum* DTO-2, germinated for 3 weeks. **f** Purple tubers from hy-31 (left) and red tubers from hy-56 (right)

grains in the anthers. Some differences in the pollen morphology between the hybrids were observed. Ten hybrids formed pollen with normal size, while three hybrids formed pollen with varying size.

The pollen viability of nine hybrids was in the range of 70–90%, while four hybrids showed a viability lower than 70%. No correlation between the ploidy level of the hybrids and the pollen morphology and viability was detected.

To test the female fertility of the hybrids the tetraploid standard pollen donor B was used. All hybrids generated fruits and developed seeds. The number of fruits per plant varied from 1 in hy-34 to 18 in hy-53. After fruit ripening, the total number of seeds was scored and the seed yield per fruit was calculated; hy-34 produced only one fruit with one seed, while hy-53 produced many fruits (Fig. 1c), of which two were selected containing 191 seeds in total. Other hybrids, like hy-24, gave nine fruits with a total of 39 small seeds.

As a further test of female fertility, hy-30, hy-31, hy-49 and hy-53 were pollinated with the tetraploid standard pollen donor D. A calculation of the number of seeds per fruit showed that the standard pollen donor B gave rise to a 3–4-times higher yield of seeds per fruit compared to the standard pollen donor D. However, the relative number of seeds in the hybrids as a result of pollination with the two pollinators was not changed. The highest number of seeds per fruit for both pollen donors scored was in hy-31.

Near-tetraploid hybrids with 42–48 chromosomes developed seeds with normal size compared to standard seeds (Fig. 1d), while aneuploid hybrids with 58 chromosomes or more developed small seeds. The germination frequency of the seeds was scored after 6 weeks. Seeds with normal size started germinating after 10–12 days, while the small seeds did not start germination until after 3–6 weeks. The germination frequency ranged between 79 and 100%.

To evaluate male fertility three hybrids, hy-31, hy-34 and hy-53, were used as pollen donors for the tetraploid standard cultivar *S. tuberosum* cv "Gordana". Seeds that developed after pollination with hy-31 were harvested and germinated. All seeds had a normal size and 149 out of 162 seeds germinated, giving a germination frequency of 92%. Pollen from hy-34 and hy-53 gave rise to fruit development on *S. tuberosum* cv "Gordana" but these fruits did not develop seeds.

Resistance to *G.pallida*

Analysis of the dihaploid clone DHAK-11 and its tetraploid parent N-80-APZ-12 showed that both clones conferred partial resistance to *G. pallida* Pa2 and resistance to Pa3. DHAK-33 and its tetraploid parent N81-ARQ-09 showed resistance to Pa2 and partial resistance to Pa3. Tubers from all hybrids were analyzed for Pa2 and Pa3 resis-

Table 2 Fourteen somatic hybrids, generated by fusion of *S. tuberosum* DHAK-11 and *S. tuberosum* DHAK-33 protoplasts, were analyzed for chromosome number, petal colour, tuber skin colour, pollen morphology and viability. Nine hybrids were pollinated with the tetraploid standard cultivar B (*S. tuberosum* cv 89-BHZ-5) and four hybrids were pollinated with the tetraploid standard cultivar D (*S. tuberosum* cv DTO-2). Three hybrids hy-31, hy-34 and hy-53 were used as pollen donors for the tetraploid cultivar G (*S. tuberosum* cv "Gordana"). Fertility was listed as F/M: F – female fertile; M – male fertile. Seeds from each cross were harvested and analyzed for the number of seeds per fruit, seed size and germination frequency. Hybrids analyzed for Pa2 and Pa3 resistance were categorized as: RR – resistant ; R – partial resistant; S – susceptible. nt – not tested; *one seed germinated

Clone no.	Chromosome number	Petal colour	Tuber skin colour	Pollen		Fertility	No. seeds per fruit	Seed size	% Germinating seed	*G. pallida* resistance	
				Morphology	%Viability					Pa2	Pa3
N80-APZ-12	48	Red	Red			F/M				R	RR
DHAK-11	24		Red							R	RR
N81-ARQ-09	48	White	White			F/M				RR	R
DHAK-33	24	White	White			F/				RR	R
hy-19	58	Violet	Purple	Normal	50–60	F/nt	B 1,6	Small	85.7	RR	R
hy-24	63	Violet	Purple	Normal	70	F/nt	B 4,3	Small	86.8	RR	R
hy-30	42	Violet	Purple	Normal	80–90	F/nt	B 89 D 24	Normal Normal	99.2 95.7	RR	RR
hy-31	48	Violet	Purple	Normal	70–80	F/M	B 181 D 49 G 90	Normal Normal Normal	94.8 89.2 92.0	RR	S
hy-34	58	Violet	Purple	Normal	50	F/M	B 1 G 0	Small	100*	RR	RR
hy-49	45	Violet	Purple	Normal	90	F/nt	B 96 D 29	Normal Normal	95.3 98.1	RR	S
hy-53	47	Violet	Purple	Varying	70	F/M	B 56 D 23 G 0	Normal Normal	85.0 95.6	RR	RR
hy-56	46	Red	Red	Normal	80	F/nt	B 38	Normal	79.0	RR	R
hy-61	66	Violet	Purple	Varying	5–10	F/nt	B 3,4	Small	80.0	R	S
hy-70	39	Violet	Purple	Normal	80–90	nt	nt			RR	S
hy-71	48	Violet	Purple	nt	nt	nt	nt			RR	RR
hy-72	38	Violet	Purple	Normal	90	nt	nt			R	S
hy-73	43	Violet	Purple	Normal	80–90	nt	nt			RR	R
hy-74	42	Violet	Purple	Varying	60	nt	nt			R	S

tance. Four hybrids, hy-30, hy-34, hy-53 and hy-71, showed resistance to both Pa2 and Pa3. The highest level of resistance was found in hy-53, with only one Pa2 cyst scored on five tubers. Four hybrids, hy-19, hy-24, hy-56 and hy-73, showed resistance to Pa2 and partial resistance to Pa3. Six hybrids, hy-31, hy-49, hy-61, hy-70, hy-72 and hy-74, showed no detectable Pa3 resistance.

Identification of RAPD marker bands for tuber colour

Tubers from all hybrids possessed a purple skin except for one hybrid, hy-56, with 46 chromosomes, that possessed a red tuber skin (Table 2). DNA polymorphism between the two parental clones, DHAK-11 and DHAK-33, a mixoploid hybrid, hy-61, with 66 chromosomes, a tetra-euploid hybrid, hy-31, with 48 chromosomes and the near-tetraploid hybrid, hy-56, with 46 chromosomes was detected by use of the RAPD technique (Fig. 2). In a detailed RAPD analysis of hy-56, 140 primers were tested (Table 3). A total of 105 polymorphic DNA bands were detected for the two parental clones, and an examination of hy-56 revealed that this hybrid was lacking three unique RAPD marker bands, all identified as DHAK-33 parental bands (Fig. 2). One of the missing DNA bands, a 800-bp band (highlighted by *), was detected by the primer AQ-07 and the other band, a 1500-bp band (highlighted by **) was detected by the primer AW-15. A third missing band, a 394-bp band detected by the primer AC-02, was described earlier (Rasmussen and Rasmussen 1995).

Table 3 RAPD analysis of the two dihaploid parental clones DHAK-11 and DHAK-33 and the hybrid hy-56, performed with 140 decamer oligonucleotide primers

	Score of polymorphic RAPD bands		
	DHAK-11	DHAK-33	Total
Parental clones	54	51	105
hy-56	54	48	102
Abscence of DNA bands in hy-56		3	

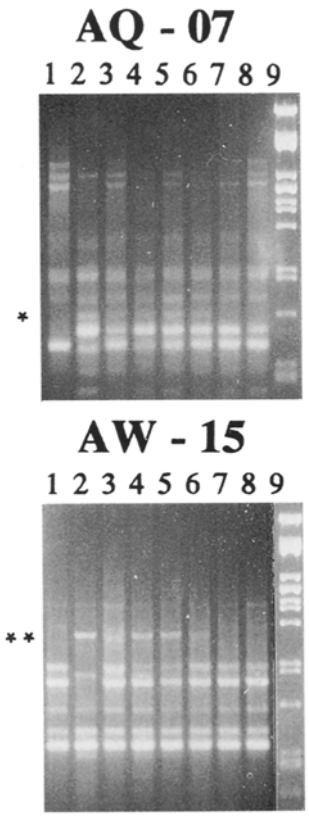

Fig. 2 RAPD profiles of the two parental clones, DHAK-11 and DHAK-33, and four hybrids generated by two decamer oligonucleotide primers AQ-07 and AW-15. Amplified DNA fragments from eight samples were loaded in the following order (the chromosome number of the hybrids is shown in brackets): *lane 1*, DHAK-11(24); *lane 2*, DHAK-33 (24); *lane 3*, a mixture (1:1) of DHAK-11 and DHAK-33; *lane 4*, hy-71 (48); *lane 5*, hy-61 (66); *lane 6*, hy-31 (48); *lanes 7 and 8*, two different extracts of hy-56 (46). *Pst*I-digested λ-DNA was used as DNA size marker (*lane 9*). Unique DHAK-33 DNA bands not amplified in hy-56 extracts are highlighted by * and **

Discussion

Conventional breeding of tetraploid heterozygous potato lines has been shown to be inefficient because of the complex segregation of valuable agronomic characters such as yield and disease resistance. A widely advocated alternative is breeding at the diploid, instead of the tetraploid, level and restoration of the tetraploid level by use of protoplast fusion (Wenzel et al. 1979; Ross 1986; Deimling et al. 1988). Dihaploid breeding lines can be generated by the pollination of tetraploids with a *S. phureja* dihaploid inducer line. Pollination with *S. phureja* may stimulate unfertilized ovules in the tetrapolid parent to develop parthenogenetically (Hermsen and Verdenius 1973) or may add some genetic information to the dihaploid progeny (Waugh et al. 1992). However, a major bottle-neck in dihaploid potato breeding is the high number of non-tuberizing and of non-flowering dihaploids in the progeny. From a study where tetraploid varieties of *S. tuberosum* were crossed with *S. phureja*, Hutten et al. (1994) reported that 68% of the vigorous dihaploids formed flowers but only 4% had a pollen stainability higher than 60%. Our results with crosses between *S. phureja* and two tetraploid *S. tuberosum* breeding lines also reveal a high frequency of stertile dihaploids (Table 1). In two crosses with *S. phureja* we obtained no male-fertile dihaploid plants and 6% and 9% female-fertile plants, respectively. In spite of the low fertility rate many dihaploids convey valuable genetic characters. The dihaploid plants in the present study were tested for resistance to *G. pallida* pathotypes Pa2 and Pa3 and the two clones that possessed the highest level of resistance to Pa2 or to Pa3 were selected for protoplast fusion.

The fusion products gave rise to somatic hybrids with a broad ploidy range from 38 to 63 chromosomes. Only two euploid hybrids with 48 chromosomes were scored. It is likely that variation in the chromosome number of somatic hybrids after protoplast fusion depends both on the genotypes used as parental plants and on the experimental conditions during the fusion and the regeneration procedure. In a study with intraspecific protoplast fusion between two *S. tuberosum* cultivars, Waara et al. (1992) obtained mainly hybrids which either lost or gained only one chromosome, while Chaput et al. (1990) demonstrated a wide distribution range from the diploid to the octoploid level in intraspecific *S. tuberosum* hybrids.

All somatic hybrids in the present study generated tubers and flowers (Table 2). The flowers from all hybrids were violet, except for one hybrid, hy-56, that possessed red flowers. The tetraploid *S. tuberosum* N80-APZ-12, the mother plant of the non-flowering dihaploid DHAK-11 clone, also had red petals. The flowering dihaploid clone DHAK-33 developed white petals.

The dihaploid clones DHAK-11 and DHAK-33 had tubers with red and white skins, respectively, while all hybrids produced tubers with purple skin, except hy-56 which produced a red tuber skin. No obvious differences in the form and size of the tubers from the different hybrids were observed. Tuber skin colour is dependent on anthocyanin pigmentation in the phelloderm layer of the tuber. The genes controlling the production and distribution of anthocyanin pigmentation have been shown to be excellent marker genes (De Jong 1991). From a study of anthocyanin pigmentation in potato van Eck et al. (1993) proposed a genetic model with five main loci as the genetic elements responsible for the colour of flowers and tuber skin. The *D* and *P* loci are assumed to be the basic factors involved in the biosynthesis of red and blue anthocyanin, respectively. Locus *R* is tissue specific for the pigmentation of tubers while locus *F* is tissue specific for the pigmentation of the flowers. Loci *D/d*, *P/p* and *F/f* were mapped on chromosomes 2, 11 and 10, respectively. Dihaploids with the genetic combination *D-ff* develop white petals, while *D-F*-combinations develop red petals. Expression of the anthocyanin genes in the tubers are regulated by the *R/r* loci. The *rr* constitution results in white tubers, while *R*-results in pigmented tubers determined by *D* and/or *P*.

The dihaploid parental clone DHAK-11 developed red tubers. According to the genetic model of van Eck et al.

(1993) it can be predicted that the genetic composition of DHAK-11 is *D-pp-F-R-*. The dihaploid parental clone DHAK-33 possessed faint purple stems (Rasmussen and Rasmussen 1995) but developed white petals and white tuber skin. From these phenotypic characters the genetic composition of DHAK-33 is *ffrr* in combination with *P* and either *D* or *d*. Purple tuber skin was a phenotypical character for all hybrids in this study, except for hy-56, indicating that the genetic composition of these hybrids was *D-(D)-P-ppF-ffR-rr* which allows expression of both blue and red anthocyanin in the tuber skin cells. In a study on intraspecific somatic hybridization between different *S. tuberosum* clones, Möllers et al. (1994) reported that fusion combinations in which one of the parent clones had a red tuber skin showed dominance over light yellow although the red tuber skin colour of the hybrids was not so intense as that of the parent clones. Our results show that in somatic hybrids between two dihaploid *S. tuberosum* clones with red and white tuber skins, respectively, a dominance of purple tuber skin was detected in the hybrids. We conclude that the dominance of purple tuber skin colour may be due to at least one *D-* and one *P*-allele in combination with at least one modifying *R*-allele conferring tissue-specific expression of anthocyanin in the tubers.

We discovered only one hybrid, hy-56, that gave rise to tubers with red skin, Fig. 1f. This particular hybrid was subjected to further study and it was shown by RAPD analysis that, of 140 decamer oligonucleotid primers tested, a total of 105 polymorphic DNA bands from the two dihaploid parental clones DHAK-11 and DHAK-33 could be detected (Table 3). Analysis of hy-56 showed that in this particular aneuploid hybrid three DNA bands were lacking. These bands were all shown to be unique for the dihaploid parental clone DHAK-33. Therefore, we conclude that of the two chromosomes lacking in hy-56 at least one was a DHAK-33 chromosome, and more likely both were. The red tuber skin of hy-56 may be caused by the abscence of the dominant *P* allele responsible for the synthesis of blue anthocyanin. This indicates that one of the missing chromosomes in hy-56 is chromosome 11.

The female fertility of the hybrids was tested by crosses with two different tetraploid *S. tuberosum* pollen donors and expressed as the number of seeds per fruit. A 3–4-fold higher seed-production capability of the pollen donor B compared to pollen donor D was observed (Table 2), while the relative order of the seed yield per fruit in the hybrids was the same, irrespective of the pollinator. Seed production was highly efficient in hybrids within a chromosome range of 42–48, while the aneuploid hybrids with 58–63 chromosomes showed a lower seed yield per fruit. As a further analysis of the hybrid female fertility a germination test of seeds from all crosses was performed. A correlation between the size of the seeds and the time for initiation of germination was observed. The small seeds produced by hy-19, hy-24, hy-34 and hy-61 germinated after 3–6 weeks, while normal seeds germinated within 2–3 weeks. The germination delay of the small seeds may be due to either the high ploidy level or the reduced endosperm in these seeds.

The male fertility of three somatic hybrids was tested with the standard cultivar *S. tuberosum* cv "Gordana" as female partner. Fruits were developed from the three crosses, but only pollen from hy-31 gave rise to seed formation. The germination frequency of the seeds was 92%, which is of the same magnitude as the germination frequency of seeds developed from crosses where hy-31 was used as pollen donor.

Ten randomly selected tubers from each of the hybrids were used to test the resistance against *G. pallida* pathotypes Pa2 and Pa3. For the classification of the resistance levels we have employed the system developed by the ZPC company, Metslawier, The Netherlands, with the only modification that a subgroup named partial resistance, R, scoring $8<x<12$ cysts per tuber was included (see Materials and methods).

The tetraploid cultivar N-80-APZ-12 and its dihaploid progeny DHAK-11 carried partial resistance, R, to Pa2 and resistance, RR, to Pa3. The tetraploid N-81-ARQ-09 and its dihaploid progeny DHAK-33 carried resistance, RR, to Pa2 and partial resistance, R, to Pa3. Analysis of 14 somatic hybrids revealed that in four (26%) a complementation of a high resistance to both Pa2 and Pa3 was obtained. Five hybrids (33%) conferred a similar resistance as the dihaploid parentals, while six hybrids have lost the Pa3 resistance, but still conferred partial Pa2 resistance.We have thus demonstrated for the first time that the quantitative resistance traits of the pathotypes Pa2 and Pa3 from either of two parental clones can be combined in somatic hybrids produced by the use of protoplast fusion. Furthermore, all the hybrids were shown to be fertile, which opens up new possibilities for protoplast fusion in future breeding programs.

The transfer of Pa2 and Pa3 resistance from the diploid wild species *S. circaifolium* subsp. *circaifolium* to a diploid *S. tuberosum* subsp. *tuberosum* by use of both somatic hybridization and sexual crossing, where the wild-type was used as female partner, has been reported (Louwes et al. 1992; Mattheij et al. 1992). In the somatic as well as the crossed hybrids these authors found a high transmission rate of Pa2 and Pa3 to the F_1 generation and a slightly lower transmission rate of resistance to *Phytophtora infestans*. A number of other valuable agronomic traits have been transferred by protoplast fusion. In a study with two dihaploid genotypes of potato, containing the monogenic dominant alleles for resistance to PVX (virus X) and virus PVY (virusY), 18 different fusion combinations were performed (Thach et al. 1993). In most of these combinations an addition of the two qualities was found, although a few deviating clones were observed. Additionally, the production of frost-tolerant somatic hybrids and their adaptability to cold were achieved by the fusion of mesophyll protoplasts from a frost-tolerant diploid species *Solanum commersonii* and a dihaploid *S. tuberosum* (Cardi et al. 1993). These authors found that except for one these hybrids were female fertile but male sterile.

Taken together, these reports demonstrate the potential of protoplast fusion in practical plant breeding. Inclusion of several of the recently discovered chromosomal mar-

kers (Ritter et al. 1991; Leonards-Schippers et al. 1992; Gebhardt et al. 1993; Kreike et al. 1993, 1994) in the studies on multigenetic disease-resistant traits may contribute to the localization and regulation of these genes *in planta*.

Acknowledgements The authors thank Kirsten Sørensen for skillful technical assistance with the RAPD analysis. This work was supported by The Danish Agricultural Ministry, Grant A.U. 92–2.

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