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Somatic fusion for combining virus resistances in *Solanum tuberosum* L.

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Summary. Dihaploid genotypes of potato, containing the dominant allele for extreme resistance to PVX and/or PVY, were used in a fusion program in order to analyze the behaviour of the two monogenetic resistances after fusion. Eighteen different fusion combinations were performed and regenerated hybrids were tested by ELISA for their virus resistance. In most of the combinations an addition of the two qualities was found, but a few deviating clones were observed. The possible reasons for the phenotypic disappearance of resistant alleles are discussed.

Key words: Potato – PYX – PVY – Protoplasts – Somatic hybrids

Introduction

The most attractive applied use of cell fusion is the opportunity to combine two genomes asexually – without meiotic segregation – and to propagate such a heterozygous genome construct vegetatively. Thus, cell fusion is a very promising tool for the breeding programs of vegetatively propagated crops such as potato. Potato, however, is tetraploid, making it usually necessary to reduce the ploidy level to dihaploidy before constructing a new tetraploid clone by fusion. Since both fusion techniques – the chemical procedure using poly-ethylene-glycol and the electric technique – are readily reproducible, the analytical synthetic breeding scheme of Wenzel et al. (1979) can be followed and even gene transfer via fusion becomes a realistic goal (Glimelius et al. 1991).

The main emphasis in potato fusion experiments is no longer on the technique, particularly since selection for heterocaryotic hybrids among homocrayotic ones has already been achieved. Presently, investigations concentrate on where to use somatic hybrids in practical breeding programs. In earlier work emphasis was placed on wide fusions between sexually non-compatible *Solanum* species in an attempt to broaden the genetic base of *S. tuberosum*; e.g., the incorporation of resistance from *S. brevidens* to *S. tuberosum* (Austin et al. 1985; Pehu et al. 1990). From fusions between *S. tuberosum* and *S. phureja*, a combination where a sexual cross is also possible, field data on the performance of tetraploid potato hybrids have been published (Mattheij and Puite 1992). For a more direct use in potato breeding, fusions within the tuberosum group would be useful (Deimling et al. 1988; Waara et al. 1989; Chaput et al. 1990; Möllers et al. 1992). We report here on the genetics of the two monogenically inherited dominant alleles *Rx* and *Ry*, inducing extreme resistance to potato virus X (PVX) and PVY, after fusion of dihaploid *S. tuberosum* clones containing these two alleles.

Materials and methods

Plant material and virus strains

In the present study, dihaploid genotypes obtained via wide crosses of *S. phureja* with the cultivars 'Assia', 'Barbara' and 'Heidrun' were screened for extreme virus resistance to PVX and/or PVY. For this purpose, cuttings were grafted on tomatoes, each of which were inoculated with one of the three PVX strains, X 239, X CS, and X Jubel, or the PVY strains

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Y 264, Y 1001, and Y 1466. The potato scions were evaluated after 3 weeks for the presence of top necrosis, indicative of the expression of the *Rx* or *Ry* alleles.

Protoplast fusion and regeneration

Protoplasts were isolated from 3- to 4-week-old shoots propagated in vitro according to Möllers and Wenzel (1992), using 0.1–0.2% macerocyme (Serva) and 0.8% cellulase R-10 (Serva), and purified by flotation. Electrofusion was carried out with the 'CFA 400' (Krüss, Hamburg) cell-fusion apparatus. After fusion, protoplasts were transferred to different liquid culture media: VKM, SKM (Binding et al. 1978) and SKMmod (SKM supplemented with 0.1 M mannitol or 0.2 mg/l zeatin). Three to four weeks later the microcalli were plated on solid CUL medium (Haberlach et al. 1985). After another 1–2 months, macrocalli could be transferred to regeneration medium, rooted and planted in the greenhouse (Möllers and Wenzel 1992).

Hybrid identification

Hybrid identification was preferentially achieved by isozyme analysis, for esterases and peroxidases, which revealed differences between the donor clones used (Deimling et al. 1988). Plant material from in-vitro cultures (100 mg) was ground in 170 µl of 5% sucrose with 0.01% of added β-mercaptoethanol. After centrifugation the clear supernatant was separated on 8–15% DiscPAGE and stained (esterases according to Shaw and Prasad 1970; peroxidases according to Vallejos 1983).

Alternatively, hybrid identification was done at the DNA level (Pehu et al. 1990), using genomic single copy clones GP27, and GP34 of potato kindly provided by Christiane Gebhardt, MPI, Cologne. DNA was extracted according to Saghai-Maroof et al. (1984), reducing the original plant material to 300 mg. For Southern transfer, 5 µg of DNA was restricted with the appropriate restriction endonucleases and separated in 0.8% agarose gels. Membranes (Biodyne B, Pall) were hybridized with radioactively labelled probes.

For all somatic hybrids identified by isozyme and RFLP analysis, ploidy level was determined in root tips to eliminate aneuploid regenerants.

Virus test

In order to test for virus resistance in regenerated hybrids, cuttings were mechanically inoculated with the sap of infected tobacco plants. After a 3-week incubation, virus testing for PVX and PVY was performed with antisera (Boehringer, Mannheim). Each test was repeated three times.

Results

Selection of genotypes for protoplast fusion

From 30 dihaploid clones tested for the presence of the *Rx* and/or *Ry* genes, 14 were selected for the fusion program. Table 1 gives a summary of the genotypes, their origin and resistances.

Protoplast fusion and regeneration of plants

As the aim of the present analysis was to study the combination of two monogenically determined resistances, most of the 18 fusion combinations were performed between clones of the genetic constitution *Rx*, *ry* and *rx*, *Ry*. The most informative combinations are listed in Table 2 (additional data can be obtained from the authors). From a total of 5,140 calli plated, 1,730 plantlets were regenerated, giving a mean of 34% regenerants. The percentage of regenerated plants fluctuated the different experiments, depending on the genotype combination, and fell between 13% (no. 14) and 46% (no. 16). No correlation between the small number of regenerants and the genotype of parents used, e.g., fusion of closely related donor clones or clones obtained from the same cultivar, could be detected.

Table 1. Resistances to PVX (*Rx*) and PVY (*Ry*) in dihaploids derived from potato cultivars

Dihaploid genotype	Cultivar	Resistance genes
H77.401/6		*rx*, *Ry*
H77.421/2		*rx*, *Ry*
H88.1500/12	Assia	*rx*, *Ry*
H88.1512/11	Heidrun	*rx*, *Ry*
H88.1500/4	Assia	*Rx*, *ry*
H88.1512/17	Heidrun	*Rx*, *ry*
H88.1512/4	Heidrun	*Rx*, *ry*
H88.1512/9	Heidrun	*Rx*, *ry*
H88.1512/14	Heidrun	*Rx*, *ry*
H88.1512/28	Heidrun	*Rx*, *ry*
H88.1500/6	Assia	*Rx*, *Ry*
H88.1500/11	Assia	*Rx*, *Ry*
H88.1512/25	Heidrun	*Rx*, *Ry*
H88.1503/1	Barbara	*rx*, *ry*

For some genotypes the lower macerocyme concentration of only 0.1% resulted in a higher production of viable protoplasts. Division of protoplasts varied in the different liquid culture media, depending on the fusion combination. In most cases SKM prevailed over VKM, and sometimes SKMmod was better suited than SKM. Independently of the culture medium, tetraploid protoplasts developed more quickly than the diploids, thus selecting for fused regenerants.

Identification of hybrids

For most combinations hybrid identification could be performed with isozyme analysis of peroxidases (Fig. 1) or esterases. Hybrid identification by RFLP analysis (Fig. 2) was only done when there was no isozyme polymorphism detected.

Single-copy and multi-copy DNA clones derived from the MPI, Cologne, were used for radioactive detection (Fig. 2). Table 3 shows the results of some of the genotype combinations. From a total of 957 regenerants analyzed either by isozyme analysis or RFLP, 31% were identified as hybrids, showing a combination of the typical banding pattern of both

Table 2. Number and percentage of regenerated plants after protoplast fusion

No.	Genotype combination and resistances		Number of calli	Regenerated plants No.	%
	rx, Ry	(+) *Rx, ry*			
1	H77.421/2	(+) H88.1512/4	320	87	27
2	H77.421/2	(+) H88.1512/14	320	75	23
3	H77.421/2	(+) H88.1512/28	640	228	36
4	H88.1500/12	(+) H88.1512/4	500	225	45
6	H88.1500/12	(+) H88.1512/14	300	71	24
7	H88.1500/12	(+) H88.1512/17	439	121	28
	Rx, ry	(+) *Rx, ry*			
12	H88.1512/17	(+) H88.1512/4	440	158	36
13	H88.1512/17	(+) H88.1512/14	285	170	36
	Ry, Rx	(+) *Rx, ry*			
14	H88.1500/6	(+) H88.1512/17	56	7	13
15	H88.1512/25	(+) H88.1512/17	457	190	42
	Rx, Ry	(+) *Ry, rx*			
16	H88.1500/6	(+) H88.1500/12	233	108	46
	Rx, Ry	(+) *rx, ry*			
17	H88.1500/6	(+) H88.1503/1	61	21	34
Total	18 combinations		5140	1730	34

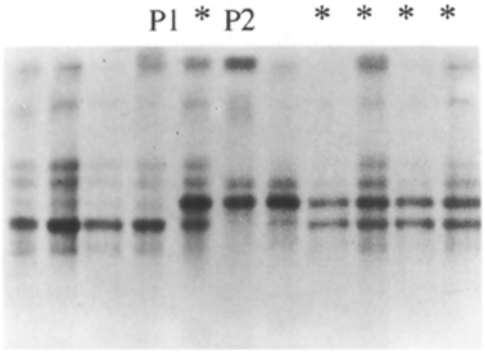

Fig. 1. Somatic hybrid identification with isozymes: esterase stain after electrophoresis. *P1 and P2*, fusion parents. Hybrids are marked with an *asterisk*

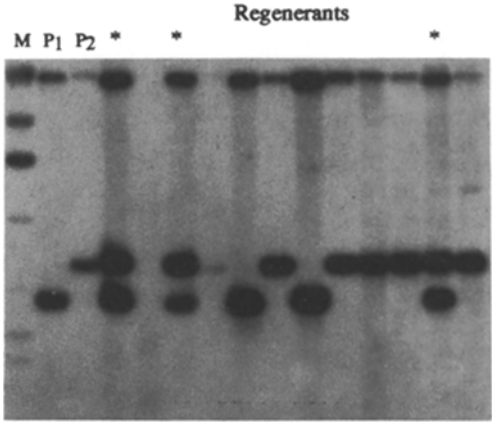

Fig. 2. Somatic hybrid identification with RFLPs. Autoradiography of a Southern blot of genomic DNA (restriction by *Eco*RI; single copy probe GP 27) from fusion parents (*P1 and P2*) and regenerants. Hybrids are marked with an *asterisk*

parental clones. Recovery of hybrids depended strongly on the fused genotypes, as can be seen in the fluctuation of the percentage of detected hybrids: 94% in fusion no. 17 and 2% in fusion no. 12. The ratio of parental types found after fusion is also given. Although the ratio of parental protoplasts was made equal for fusion experiments, there are some parental genotypes which prevail after protoplast fusion, especially in fusions with clone H88.1512/17 (nos. 12 and 13). For all hybrids detected the ploidy level was determined in root tip cells. Between 0% (no. 15) and 46% (no. 17) of the hybrids detected by isozyme analysis or RFLP turned out to be hexaploid or aneuploid. Thus, the yield of tetraploid hybrids is reduced to 8% of the calli originally plated.

Virus test

Table 4 shows the results of the ELISA test for eight of the fusion combinations. In most of these combinations a simple addition of the dominant alleles for resistance was found in the hybrid regenerants. Nevertheless, some of them showed a deviation of up to 40% from the expected addition of traits, e.g., fusion no. 3, where eight from a total of 41 hybrids expressed only the *Rx*, gene four only the *Ry* gene and another four clones showed no resistance at all.

Table 3. Number of hybrids identified by isozyme or RFLP analysis, and the number of homocaryotic fusions of the parents P1 and P2 found

No.	Genotype combination			No. of regenerants analyzed					
	Parent 1 (2x)		Parent 2 (2x)	Total	P1 4x	P2 4x	Hybrids No.	Hybrids %	% Aneuploids
1	H77.421/2	(+)	H88.1512/4	65	30	1	34	52	20
2	H77.421/2	(+)	H88.1512/14	57	9	13	35	61	14
3	H77.421/2	(+)	H88.1512/28	188	115	10	63	33	17
4	H88.1500/12	(+)	H88.1512/4	25	15	2	8	32	
6	H88.1500/12	(+)	H88.1512/14	49	3	1	45	39	30
7	H88.1500/12	(+)	H88.1512/17	66	18	21	27	41	11
12	H88.1512/17	(+)	H88.1512/4	122	120	0	2	2	
13	H88.1512/17	(+)	H88.1512/14	139	133	0	6	4	
14	H88.1500/6	(+)	H88.1512/17	4	0	2	2	50	
15	H88.1512/25	(+)	H88.1512/17	120	84	4	32	27	0
16	H88.1500/6	(+)	H88.1500/12	21	1	16	4	19	
17	H88.1500/6	(+)	H88.1503/1	16	0	1	15	94	46
Total	18 combinations			957			300	31	18

Table 4. Virus resistances in somatic hybrids

No.	Genotype combination and resistances			Number of plants	Resistances in regenerants				% *RX, RY*
					RX, RY	*RX, ry*	*rx, RY*	*rx, ry*	
	rx, Ry		*Rx, ry*						
1	H77.421/2	(+)	H88.1512/4	20	20	–	–	–	100
2	H77.421/2	(+)	H88.1512/14	28	26	–	1	1	93
3	H77.421/2	(+)	H88.1512/28	41	25	8	4	4	61
6	H88.1500/12	(+)	H88.1512/14	30	30	–	–	–	100
7	H88.1500/12	(+)	H88.1512/17	14	14	–	–	–	100
	Rx, Ry		*Rx, ry*						
14	H88.1512/25	(+)	H88.1512/17	19	17	2	–	–	89
	Rx, Ry		*rx, ry*						
17	H88.1500/6	(+)	H88.1503/1	7	7	–	–	–	100

Surprisingly also in combination no. 14, where the two dominant alleles were present in one parent, the *Ry* allele was no longer expressed after in-vitro handling in about 10% of the hybrids. The number of hybrids tested with the combination *Rx, Ry*(+) *rx, ry* (no. 17) is too small to allow a definitive conclusion.

Discussion

Potato breeding at the diploid level followed by somatic hybridization of selected clones to obtain a new and superior tetraploid cultivar is only of value if an addition of the traits of both fusion parents can be obtained. The aim of the present study was to analyze in different fusion combinations the expression of the two monogenic dominant alleles, *Rx* and *Ry*, causing extreme resistance to PVX and PVY.

The methodology of protoplast fusion and regeneration developed by Möllers and Wenzel (1992), slightly modified in our fusion program, can be routinely applied for all genotypes of spp *tuberosum*. The rate of regenerated plants, about 10% over all experiments, is equivalent to the results obtained with the slightly different techniques employed by other research groups (e.g., Waara et al. 1992). In the procedure used, the low number of isolation steps and the number of media transfers necessary during regeneration are advantageous compared to the protocols of Haberlach et al. (1985) or Schilde-Rentschler and Ninnemann (1988).

Hybrid identification was performed at the molecular level, since interspecific fusions show only few morphological differences, not allowing for an early identification of fusion hybrids. Furthermore, isozyme and RFLP markers can be applied during the in-vitro phase as soon as shoots are present. With both procedures a rather large number of genotypes can be screened.

Although the number of plants tested for virus resistance is at present limited, deviations from the simple addition of traits were found. Especially in fusions no. 2 and 3, 10–40% of regenerants show fewer resistances than expected (Table 4). Different causes for these results are possible: (1) numerical or structural chromosome mutations during the regeneration phase, (2) a gene dose effect, and (3) the influence of the parental genotype and interactions with the cytoplasm.

Genome and chromosome mutations during the callus phase are well-known phenomena (Gleba and Sytnik 1984). In our analysis of ploidy level based on chromosome counts in potato root tip cells, exact chromosome number could not always be determined. In their fusion experiments with *S. brevidens* and *S. tuberosum* to integrate resistance to potato leaf roll virus, Austin et al. (1985) suggested somaclonal variation as a possible interpretation of the deviating results. Apart from genome and chromosome mutations, base substitution can also occur. For most of the deviations found in our fusion program, regenerants did not show resistance to PVY, suggesting that the gene *Ry* preferentially mutates during the in-vitro phase.

To check whether, in contrast to sexual hybridization, the extreme resistances to PVX and PVY is somatic fusion are dependent on a gene dose effect or not, two *Rx* or *Ry* alleles were combined in hybrids nos. 14, 15 and 16 respectively. From the presently available data no dosage effect can be deduced.

It is also of interest to determine whether the different genotypes or cytoplasms employed exert any effect on the expression of the resistance genes. In fusions nos. 1–3 one parent is H77.421/2 and the other fusion parents are diploids obtained from the same cultivar and probably of nearly identical cytoplasm. In fusion no. 1 a clear addition of traits is found, whereas the loss of resistance to PVX and PVY in fusions nos. 2 and 3 can neither be explained by the genotype of fusion parent H77.421/2 nor traced back to the combination of genome and cytoplasm.

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