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Asymmetric fusion between wild and cultivated species of potato (*Solanum* spp.) – detection of asymmetric hybrids and genome elimination

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Abstract The objective of this study was to evaluate the suitability of different techniques for a simple and rapid identification of asymmetric hybrids, without the use of selection markers and independent of the fusion partners used. Additionally, the degree of donor DNA elimination was determined. Among 473 viable plants obtained from asymmetric fusion experiments between three di-haploid breeding lines of potato (*Solanum tuberosum*) and diploid wild species (*S. bulbocastanum*, *S. circaeifolium*; X-ray treatment of the wild species) the most promising ones were investigated with three different methods: flow cytometry, RFLP analysis with an oligonucleotide probe $(GATA)_4$, and with single-copy probes. Flow cytometry, which combines a high screening capacity with detailed information about the DNA content and allows a distinction between asymmetric hybrids and chimeras, detected 31 hypo-tetraploid and 42 hypo-hexaploid regenerates among 224 plants. With the oligonucleotide probe $(GATA)_4$ only a few asymmetric hybrids were detected among all regenerates. More than 50% of these asymmetric regenerates were chimeras. Concerning the degree of DNA elimination, the results obtained by RFLP analysis with 17 single-copy probes were correlated with the results obtained by flow cytometry. The maximum DNA elimination of the donor genome was 52%. As a trend, an irradiation dosage of 210 Gy caused a higher DNA elimination in the wild species than a dosage of 70 Gy. No calli were obtained after irradiation of the wild species with 420 Gy.

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Introduction

The transfer of polygenically encoded traits such as various pathogen resistances is of major interest in plant breeding. Conventional breeding by sexual hybridization, however, is often hampered by incompatibility barriers if the genes of interest are localized in wild species. For such cases alternative methods have to be developed. Direct transfer using genetic-engineering technologies is not suitable for polygenically encoded traits, since these techniques are still limited to the transfer of only one or a few genes. The transfer by "bridge crossing" may not be suitable in all cases. Additionally, it has the disadvantage that the breeder has to deal with undesirable traits of the intermediate species. Symmetric fusion between protoplasts of the wild and cultivated species has been conducted successfully by several teams (e.g. Helgeson et al. 1986; Schilde-Rentschler et al. 1993; Menke et al. 1996). The resulting hybrids have the disadvantage that besides the desirable traits all the undesirable characters of the wild species are also combined with the cultivated line. A promising alternative might be asymmetric protoplast fusion. The principle of this method is that a part of the wild species genome is eliminated by irradiation or chemicals before fusion (Dudits et al. 1980). Thus, undesirable traits are transferred to a lesser extent.

Potato is well suited to evaluate the asymmetric protoplast fusion technique. A great number of wild species is known which cannot be crossed sexually with the cultivated species *Solanum tuberosum*. Most of these wild species show excellent resistance properties. Furthermore, a broad knowledge on symmetric protoplast fusion and molecular characterization of the hybrids is available for *Solanum* (Millam et al. 1995).

Concerning asymmetric protoplast fusion, however, only a few reports are available for potatoes, as well as for other crops. For *Solanum*, asymmetric hybrids have so far been obtained only for combinations between *S. tuberosum* and *S. brevidens* (Feher et al. 1992; Puite and Schaart 1993; Xu and Pehu 1993; Xu et al. 1993;) and *S. tuberosum* and *S. pinnatisectum* (Sidorov et al. 1987). The possibility of producing asymmetric hybrids with other wild species has not yet been tested. Also, it is unknown to what degree the wild species genome can be eliminated in order to obtain viable hybrids. Furthermore, knowledge about the effects of partial elimination of the wild species genome on genetic, physiological and morphological properties of the hybrids is scanty. And, most important, highly effective and easy to handle methods which can be used on a wide range of potential wild and cultivated species are still not available for the identification of asymmetric hybrids.

Thus, for an evaluation of the potential benefit of asymmetric protoplast fusion for plant breeding the following questions need to be answered:

(1) How can asymmetric hybrids be reliably identified without limitations in the plant material and with low costs?
(2) Is asymmetric protoplast fusion expected to be applicable to a great number, or even all, combinations between wild species and breeding lines?
(3) How much of the wild-species genome can be eliminated without a significant reduction in the viability of the hybrids and their morphogenetic capacity?

In order to obtain answers to these questions, the asymmetric protoplast fusion technique was applied to several combinations between *Solanum* wild species and *S. tuberosum* breeding lines. The utility of flow cytometry, RFLP analysis with synthetic oligonucleotide probes and with single-copy clones for the identification of asymmetric hybrids was investigated.

Material and methods

Plant material

Symmetric and asymmetric hybrid plants were obtained from several independent protoplast fusion experiments of two diploid wild species with three different di-haploid breeding lines. The wild species were *S. bulbocastanum* (*S. blb.*), BGRC 8006, and *S. circaeifolium* (*S. crc.*), BGRC 27034. Both were obtained from the Dutch-German potato gene bank (FAL Braunschweig, Germany) and were selected because of their good resistance to leaf blight (*Phytophthora infestans*). The diploid breeding lines were obtained from the "Bayerische Landesanstalt für Bodenkultur und Pflanzenbau", Freising, Germany (line H50/52 and line H256/1) and the plant breeding company "Nordkartoffel Zuchtgesellschaft mbH", Ebstorf, Germany (line BP1076/1).

Isolation and irradiation of protoplasts

For protoplast isolation 3–4 week-old in vitro plants were used. Leaves and stem tips were cut and transferred to a solution of 1% macerozyme in 0.5 M sorbitol. The wild species were treated in two parallel ways: one batch was irradiated with X-rays (70 Gy, 210 Gy or 420 Gy), while the other remained non-irradiated as a control.

After irradiation, the plant tissue was incubated in enzyme solution and gently shaken at 27°C until digestion of the tissue occurred. Then the macerozyme solution was replaced by a 0.25% cellulase RS solution in 0.5 M sorbitol. To enhance the maceration of the tissues, the incubation mix with the tissue or cells was gently sucked up and released several times with a pipette with a large opening. The incubation was terminated when most of the cells were completely spherical. Then the protoplast suspension was filtered through a nylon sieve (mesh width 80 μm; though in case of much contamination additionally through a 40-μm mesh) to remove remaining tissue. Cellulase solution and cell particles were then removed by repeated centrifugation (80–100 g for 5 min) and the pelleted protoplasts were carefully resuspended in 0.5 M sorbitol.

Protoplast fusion and culture

For symmetric fusion, the protoplasts of the wild species and the breeding line were mixed in a ratio of 1:1. For asymmetric fusion, the number of the wild species protoplasts was adjusted 2–3-times higher than the number of the breeding line protoplasts. After mixing, the protoplast suspension was centrifuged (80–100 g for 5 min.). The pellet was resuspended in 0.5 M sorbitol and the concentration was adjusted to 1×10^6 protoplasts per ml. The fusion was carried out using a High Voltage Cell Processor (Bioelectronics Cooperation, Troy, Michigan) with an alternating field of approximately 0.5 Hz, a voltage between 170 V and 220 V/3 mm, and pulse of 30–40 μs and 250–280 V/3 mm.

The protoplast suspension was then mixed with 2.8% alginate solution in 0.5 M sorbitol in a ratio of 1:1 as described by Schilde-Rentschler et al. (1988). This mixture was dropped into a 50 mM $CaCl_2$/0.5 M sorbitol solution. After 2 h (room temperature, darkness) this solution was replaced by a 10 mM $CaCl_2$/0.5 M sorbitol solution. The protoplast-alginate spheres were incubated for 2 days in the dark at 4°C. Then the $CaCl_2$/sorbitol solution was replaced by one of the following media: VKM culture medium (Binding and Nehls 1977), or modified VKM culture medium containing 2% bovine serum albumin or SKM medium (Hunt and Helgeson 1989). Subsequently the protoplasts were incubated at 20°C in the dark. Every week the medium was replaced by fresh medium. As soon as microcalli were visible, the culture medium was replaced by a 20 mM sodium-citrate/0.5 M sorbitol solution which depolymerized the alginate and thus released the calli. The individual calli were placed on MS_{13K} medium (Behnke 1975) and incubated for 2 days at 20°C in the dark. The following cultivation was done at 20°C with a photoperiod of 16 h. Until regeneration, the calli were transferred to fresh medium each month. Only a single shoot was transferred from each regenerating callus to MS medium (Murashige and Skoog 1962) for further cultivation and investigation.

DNA isolation, restriction digestion and transfer

DNA isolation from in vitro plants was carried out according to Saghai-Maroof et al. (1984), as modified by Schweizer (1990). Four different restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII) were tested for polymorphisms between the fusion partners. The best suited restriction enzyme-probe combination was used for the analysis of the fusion products. For hybridization with the oligonucleotide probe $(GATA)_4$ 20 μg of DNA were used; for hybridization with single-copy probes 40 μg of DNA were applied.

After separation of the DNA fragments in a 0.9% agarose gel by electrophoresis, the DNA fragments were transferred to a Hybond N nylon membrane (Amersham). For this transfer either the

vacuum-blot technique (oligonucleotide probe) or the Southern-blot technique (single-copy probes) was used. The DNA was fixed by UV-irradiation for 1 min ($\lambda = 302$ nm).

Labelling and detection

The RFLP analysis was performed using digoxigenin-labelled probes. Two oligonucleotide probes, $(GATA)_4$ and $(GACA)_4$, and 17 single-copy probes (TG probes provided by S.D. Tanksley, Cornell University, Ithaca, USA; and GP probes provided by C. Gebhardt, Max-Planck-Institut für Züchtungsforschung, Köln, Germany) were used. The single-copy probes have been assigned to the following potato chromosomes (Bonierbale et al. 1988; Gebhardt et al. 1989): chromosome 1: TG17, TG24, TG27, TG116; chromosome 2: TG14, GP86; chromosome 3: TG42, TG134; chromosome 4: TG123; chromosome 5: TG69; chromosome 6: TG25; chromosome 7: TG61; chromosome 8: TG16; chromosome 10: TG52, TG63; chromosome 11: TG36; chromosome 12: TG68. As vectors, bluescript M13, pUC 18 and pUC 19 were used. The plasmids were propagated in *Escherichia coli* strains K12, JM 83 and JM 103. The insert of each clone was isolated using "QIAGEN tip 500" according to the instructions of the supplier (Diagen, Düsseldorf, Germany) followed by restriction enzyme digestion, electrophoresis, and isolation of the insert by incubation with gelase according to the instructions of the supplier (Biozym Diagnostik GmbH, Hameln, Germany). The inserts were digoxigenin-labelled according to the instructions of the supplier (Boehringer, Mannheim, Germany). Hybridization of the oligonucleotide probes was carried out according to the instructions of the supplier of digoxigenated $(GATA)_4$ (Fresenius, Oberursel, Germany); hybridization of the single-copy probes was carried out according to Kreike et al. (1990), except that a temperature of 62.5°C was chosen. The immunological detection was done according to Boehringer, Mannheim, Germany. All results were based only on band deletions and band shifts, since band intensity is not specific enough when using a non-radiolabelling detection system.

Flow cytometry

For preparation and staining of plant material a slightly modified method was used as described by Ulrich and Ulrich (1991). A single 2–4-week-old shoot tip of an in vitro plant was cut into small pieces in two drops of a 0.1-M citric acid/0.5% (v/v) Tween 20 solution. This suspension was stirred for 20 min and then filtered through a nylon sieve (mesh width 50 µm). After centrifugation (352 g, 15 min) the pellet was resuspended with 0.5 ml of a 0.1 M citric acid/0.5 % (v/v) Tween 20 solution. Nuclei were stained with 2 ml × 0.025 µM of a DAPI/0.4 M phosphate solution. The samples were analyzed using a PAS II flow cytometer, Partec. As an external standard the breeding line H256/1 was used and adjusted to 25 fluorescence units. The accuracy of the measurements was determined to be ± 2 units.

Results

Methods for the identification of asymmetric hybrids

Investigations with the oligonucleotide probe $(GATA)_4$

*Dra*I gave the best results with $(GATA)_4$ (sufficient polymorphisms with clear and marked bands for both parents). With this restriction enzyme-probe combination some regenerates showed all bands of the breeding line, but only few bands of the wild species: (Fig. 1).

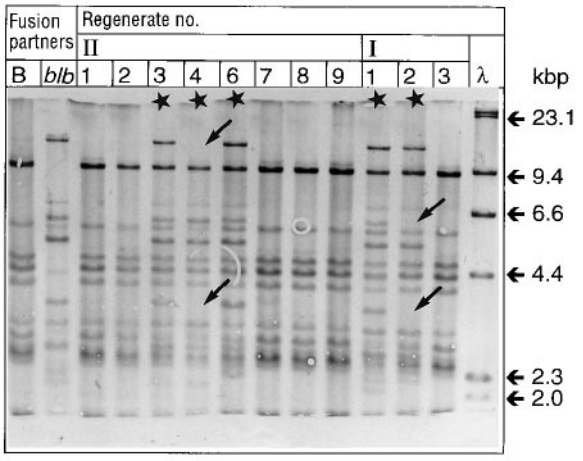

Fig. 1 Restriction patterns of asymmetric hybrids derived from the fusion of H256/1 with *S. blb*. DNA was digested with *Dra*I, vacuum-blotted, and hybridized with the digoxigenin-labelled oligonucleotide probe $(GATA)_4$. ★, hybrid; the *arrow* marks a missing band of the wild species. B, H256/1. *blb*, *S. bulbocastanum*

Most of these asymmetric hybrids lacked only one band of the wild species; in only a few two bands were missing. In a few cases, shifted or additional bands were observed.

Among 473 regenerates which were obtained from the asymmetric fusion experiments, only 21 asymmetric hybrids (= 4%) were detected with *Dra*I in combination with $(GATA)_4$. Among 156 regenerates which were obtained from symmetric fusions, no asymmetric hybrids were found. The use of $(GACA)_4$ instead of $(GATA)_4$ gave nearly identical RFLP patterns. Therefore, no additional information could be obtained by the use of $(GACA)_4$.

Investigation of the regenerates with flow cytometry

The DNA content of the di-haploid *S. tuberosum* (*S. tbr.*) breeding lines (25 fluorescence units) was approximately 12% higher than the DNA content of *S. blb.* (22 fluorescence units). The DNA content of *S. crc.* was between 24 and 25 units, which is approximately 4% less than the DNA content of *S. tbr.* Consequently, the DNA content of a symmetric eutetraploid hybrid was assumed to be 47 units for the combination *S. blb.* + *S. tbr.* and 49–50 units for the combination *S. crc.* + *S. tbr.* Depending on the fusion partners and the composition of the hybrids, the DNA content of euhexaploid hybrids was assumed to be 69–75 units.

Among the 224 regenerates which were classified as hybrids with $(GATA)_4$ and analyzed by flow cytometry, DNA losses of up to 25% were detected. No clear correlation between irradiation dosage and DNA elimination was observed but, as a tendency, the DNA

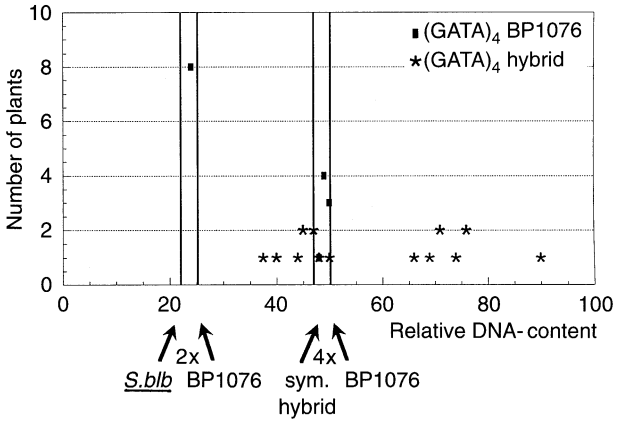

Fig. 2 Number of regenerates, relative DNA content and classification with $(GATA)_4$ of an asymmetric fusion experiment between BP1076 + *S. blb*. Irradiation dosage 210 Gy. A relative DNA content of 47 corresponds to a symmetric hybrid

elimination increased with the irradiation dosage. The maximal DNA loss after irradiation with 70 Gy was 20%, while after irradiation with 210 Gy it was 23 %.

Thirty one regenerates (= 14%) showed a ploidy level between 2x and 4x, and 42 regenerates (19%) showed a ploidy level between 4x and 6x. Figure 2 shows that all regenerates which were classified as breeding line with $(GATA)_4$ had a DNA content corresponding to a di-haploid or tetraploid breeding line. The DNA content of regenerates classified as symmetric hybrids with $(GATA)_4$, however, showed great variability and ranged between approximately 3x and 6x.

Analysis with flow cytometry indicated that some of the hybrids, which were classified as symmetric or asymmetric with $(GATA)_4$, were chimeras. The frequency of chimeras varied between 6% and 21%, depending on the fusion experiment. The highest frequency was detected among the combination H256/1 + *S. crc*. This was the case in the irradiated (21% chimeras) as well as in the non-irradiated (7% chimeras) fusion experiments. Seven of the thirteen regenerates classified as asymmetric hybrids with $(GATA)_4$ were chimeras.

Investigation with single-copy probes

Seventeen single-copy probes (TGs and GPs) in combination with four restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I) were evaluated for polymorphisms between the fusion partners. Most of the single-copy restriction enzyme combinations gave a multiple fragment pattern. Very rarely, just one band per fusion partner was obtained.

The restriction enzyme *Eco*RI was found to be best suited to detect polymorphisms in combination with the single-copy probes employed. For all fusion partners an average of 86% polymorphisms was obtained with this enzyme; less polymorphisms were detected with *Dra*I. *Eco*RV and *Hin*dIII, in some combinations, showed better polymorphisms than *Eco*RI alone, but for the sum of all tested combinations they were not as well suited as *Eco*RI.

Fifty four fusion products obtained from different experiments with different fusion partners and irradiation dosages were analyzed. In 81 cases a band deletion was found. Both the loss of only one band (Fig. 3), as well as the loss of all specific bands, was observed. Most of the missing bands were specific for the wild species. In 12 cases, however, breeding line-specific bands were missing. A band shift was observed only in one case. This was a breeding line-specific band and not a wild-species-specific band.

Most frequently asymmetric hybrids (at least one missing band) were detected with TG63 (11 regenerates). After hybridization with TG123 and TG36 only one asymmetric hybrid was found. Most bands were missing for the regenerate H256/1 + *S. blb*., no. 2 (I), for which a loss of parental bands could be shown with ten single-copy probes (i.e. 60% of the tested probes).

Comparison of the different methods for detection of asymmetric hybrids

The results of the RFLP analysis with $(GATA)_4$, flow cytometry, and RFLP analysis with single-copy probes for the identification of asymmetric hybrids, were

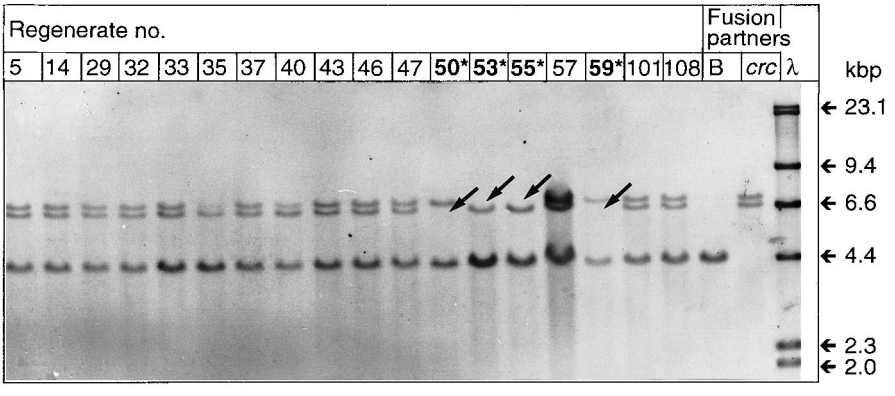

Fig. 3 RFLP pattern of 18 hybrids and their fusion partners. DNA was digested with the restriction enzyme *Hin*dIII, Southern blotted, and hybridized with a digoxigenin-labelled single-copy probe TG63. *, asymmetric hybrid; the *arrow* marks missing band of a fusion partner. B, H256/1., *crc* *S. circaeifolium*

compared for 54 hybrids from different fusion experiments (Table 1). In RFLP analysis with $(GATA)_4$, four (*Dra*I) and three (*Eco*RI) asymmetric hybrids were detected. Combining the results obtained with *Dra*I and *Eco*RI, RFLP analysis with $(GATA)_4$ detected five asymmetric hybrids. Two regenerates were identified to be asymmetric in combination with *Dra*I as well as in combination with *Eco*RI.

By flow cytometry 18 regenerates (33%) with a ploidy level between 2x and 4x and 11 regenerates (20%) with a ploidy level between 4x and 6x were detected. Four regenerates (7%) were identified as chimeras. Only 21 regenerates could not be proven to be asymmetric. However, due to the limited accuracy of flow cytometry, further asymmetric hybrids might be present among the regenerates previously classified as symmetric.

RFLP analysis with single-copy probes revealed that two fusion products did not show any genome part of the breeding line (classified as "W" in Table 1). For two other fusion products no wild species genome parts could be detected (classified as "B" in Table 1). Among the other 50 regenerates, 21 had only a part of the wild species genome. Surprisingly, eight regenerates had partly lost the breeding line genome; three others had lost genetic material of the wild species as well as genetic material of the breeding line. According to the flow cytometry analysis data, as well as according to the RFLP-analysis data with 17 single-copy probes, 18 regenerates were asymmetric.

The results of the RFLP analysis with single-copy probes were transformed to relative genome-content values: for each regenerate the numbers of all detected wild species-specific and breeding line-specific bands,

Table 1 Comparison of results from asymmetric hybrids, characterized by different detection methods

Code of the regenerate	Method of analysis[a]						
	RFLP-analysis with $(GATA)_4$		Analysis with flow cytometry		RFLP-analysis with single-copy probes		
	*Dra*I	*Eco*RI	Rel DNA cont.[b]	Classific.	Wild spec.[c]	Br. line[c]	Classific.
	Irradiation intensity 70 Gy						
H256/1 + *S. crc.* no. 5	sh	sh	50.5	sh (4)	100	100	sh
H256/1 + *S. crc.* no. 14	sh	sh	50	sh (4)	100	100	sh
H256/1 + *S. crc.* no. 29	sh	sh	50	sh (4)	100	100	sh
H256/1 + *S. crc.* no. 32	sh	sh	45.5	ah (1)	88	100	ah (W)
H256/1 + *S. crc.* no. 33	sh	sh	67.5	ah (2)	84	100	ah (W)
H256/1 + *S. crc.* no. 35	sh	sh	72	sh (6)	91	100	ah (W)
H256/1 + *S. crc.* no. 37	sh	sh	47	ah (1)	84	100	ah (W)
H256/1 + *S. crc.* no. 40	sh	sh	42	ah (1)	100	100	sh
H256/1 + *S. crc.* no. 43	sh	sh	47	ah (1)	92	100	ah (W)
H256/1 + *S. crc.* no. 46	sh	sh	47.5	sh (4)	100	97	ah (B)
H256/1 + *S. crc.* no. 47	sh	sh	48	sh (4)	100	100	sh
H256/1 + *S. crc.* no. 50	sh	sh	45	ah (1)	92	100	ah (W)
H256/1 + *S. crc.* no. 53	sh	sh	66.5	ah (2)	68	100	ah (W)
H256/1 + *S.crc.* no. 55	sh	sh	46.5	ah (1)	91	100	ah (W)
H256/1 + *S. crc.* no. 57	sh	sh	70	ah (2)	100	100	sh
H256/1 + *S. crc.* no. 59	sh	sh	47	ah (1)	91	100	ah (W)
H256/1 + *S. crc.* no. 101	sh	sh	46	ah (1)	96	97	ah (WB)
H256/1 + *S. crc.* no. 108	sh	sh	47	ah (1)	100	100	sh
H256/1 + *S. blb.* no. 1	sh	sh	66	ah (2)	100	100	sh
H256/1 + *S. blb.* no. 2	sh	sh	C	C	100	97	ah (B)
H256/1 + *S. blb.* no. 3	sh	sh	67.5	sh (6)	100	100	sh
H256/1 + *S. blb.* no. 5	sh	n.d.	63.5	ah (2)	100	100	sh
H256/1 + *S. blb.* no. 6	sh	sh	66	ah (2)	100	94	ah (B)
H50/52 + *S. blb.* no. 1	sh	sh	45	sh (4)	87	100	ah (W)
H50/52 + *S. blb.* no. 2	sh	sh	64.5	ah (2)	87	100	ah (W)
H50/52 + *S. blb.* no. 4	W	W	44.5	ah (1)	100	0	W
H50/52 + *S. blb.* no. 5	sh	sh	45.5	sh (4)	97	100	ah (W)
H50/52 + *S. blb.* no. 12	ah (W)	ah (W)	44	ah (1)	100	100	sh
H50/52 + *S. blb.* no. 18	ah (W)	sh	47.5	sh (4)	100	100	sh
H50/52 + *S. blb.* no. 21	sh	sh	48	sh (4)	94	100	ah (W)
H50/52 + *S. blb.* no. 25	sh	sh	48	sh (4)	100	100	sh
H50/52 + *S. blb.* no. 27	sh	sh	45	ah (1)	100	100	sh
H50/52 + *S. blb.* no. 35	sh	ah (W)	C	C	100	100	sh
H50/52 + *S. blb.* no. 36	W	W	44.5	ah (1)	100	0	W
H50/52 + *S. blb.* no. 37	sh	sh	49	sh (4)	97	100	ah (W)

Table 1 Continued

Code of the regenerate	Method of analysis[a]						
	RFLP-analysis with $(GATA)_4$		Analysis with flow cytometry		RFLP-analysis with single-copy probes		
	*Dra*I	*Eco*RI	Rel DNA cont.[b]	Classific.	Wild spec.[c]	Br. line[c]	Classific.
	Irradiation intensity 210 Gy						
BP1076 + *S. blb.* no. 3 (I)	sh	sh	37	ah (1)	69	100	ah (W)
BP1076 + *S. blb.* no. 15 (I)	sh	sh	46	sh (4)	100	100	sh
BP1076 + *S. blb.* no. 135 (I)	sh	sh	41.5	ah (1)	76	94	ah (WB)
BP1076 + *S. blb.* no. 143 (I)	sh	sh	43	ah (1)	100	97	ah (B)
BP1076 + *S. blb.* no. 151 (I)	B	B	51	ah (2)	0	100	B
BP1076 + *S. blb.* no. 163 (I)	sh	sh	46.5	sh (4)	100	100	sh
BP1076 + *S. blb.* no. 4 (II)	sh	sh	48.5	sh (4)	100	100	sh
BP1076 + *S. blb.* no. 5 (II)	sh	sh	60.5	ah (2)	100	88	ah (B)
BP1076 + *S. blb.* no. 6 (II)	sh	sh	C	C	100	100	sh
BP1076 + *S. blb.* no. 8 (II)	sh	sh	47	sh (4)	100	100	sh
BP1076 + *S. blb.* no. 10 (II)	sh	sh	43	ah (1)	90	100	ah (W)
BP1076 + *S. blb.* no. 11 (II)	sh	sh	69	sh (6)	100	100	sh
BP1076 + *S. blb.* no. 12 (II)	sh	sh	46.5	sh (4)	100	100	sh
BP1076 + *S. blb.* no. 13 (II)	sh	sh	45.5	sh (4)	100	100	sh
BP1076 + *S. blb.* no. 15 (II)	ah (W)	sh	67	sh (6)	83	100	ah (W)
BP1076 + *S. blb.* no. 16 (II)	sh	sh	C	C	100	100	sh
H256/1 + *S. blb.* no. 2 (I)	ah (W)	ah (W)	65	ah (2)	48	100	ah (W)
H256/1 + *S. blb.* no. 1 (II)	B	B	53.5	ah (2)	0	100	B
H256/1 + *S. blb.* no. 6 (II)	sh	sh	36.5	ah (1)	72	97	ah (WB)

[a] ah (1), asymmetric hybrid, ploidy-level between 2x and 4x
ah (2), asymmetric hybrid, ploidy-level between 4x and 6x
sh (4), symmetric eutetraploid hybrid
sh (6), symmetric euhexaploid hybrid
ah (W), asymmetric hybrid, wild-specific band(s) missing
ah (B), asymmetric hybrid, breeding-line-specific band(s) missing
B, only breeding-line-specific bands
W, only wild-species-specific bands
n.d., no data

[b] relative DNA content (units). For fusions with *S. blb.* the calculated relative DNA content of a 4x hybrid is 47, for fusions with *S. crc.* it is 50

[c] Percentage of the genome of the parental line present in the hybrid

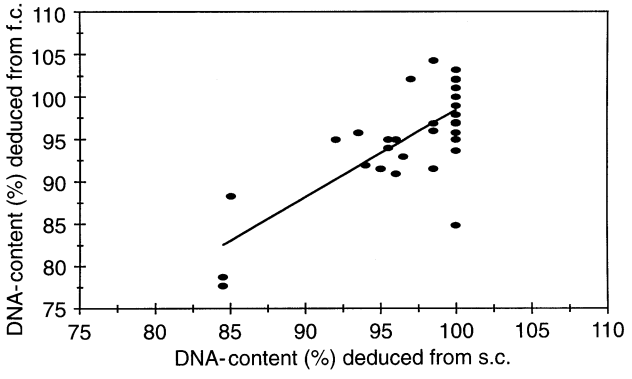

Fig. 4 Correlation between DNA content determined by RFLP analysis with 17 single-copy probes (*s.c.*) and flow cytometry (*f.c.*) ——— line of regression ● individual regenerates

respectively, were summed for all probes applied. This number of specific bands was set in relation to the total number of specific bands for the wild species and the breeding line, respectively. The maximal loss of the wild-species genome was observed to be 52%. No evidence was found for hyper-tetraploid or hyper-hexaploid hybrids with a small content of the wild species genome, even though it cannot be excluded that further asymmetric hybrids were among the regenerates which were classified as breeding line with $(GATA)_4$ and which were not analyzed in detail.

For regenerates with a DNA content up to 4x, the DNA content calculated with the method described above was correlated with the DNA content determined by flow cytometry (Fig. 4). The coefficient of correlation (r^2) was 0.55, and the slope of the regression line was 1.02.

Discussion

Comparison of the techniques to identify asymmetric hybrids

Three different techniques (RFLP analysis with oligonucleotide probes, with single-copy probes, and flow cytometry) were used to detect asymmetric hybrids among regenerates from ten asymmetric fusion

experiments. With $(GATA)_4$ in combination with two restriction enzymes, *Dra*I and *Eco*RI, five asymmetric hybrids could be found among 54 regenerates. Comparing this analysis with the results of the two other methods showed, however, that only a few asymmetric hybrids were detected with $(GATA)_4$. Even regenerates, which had lost up to 32% of the wild species genome according to the RFLP analysis with single-copy probes, could not be proven to be asymmetric with the oligonucleotide probe. From 12 hybrids with at least 10% loss of the wild species genome (according to single-copy probes) only two were identified as asymmetric with $(GATA)_4$. Therefore, the suitability of the fingerprint technique with oligonucleotide probes specific for simple repeats, for the identification of addition lines or for chromosomal aneuploidy (Beyermann et al. 1992) and somaclonal variation (Weising et al. 1991), could not be extended to the identification of asymmetric potato hybrids. Even though Campos et al. (1993) found somaclonal variations with this method, it is more likely that many changes were not detected. This assumption was supported by the results of Vosman et al. (1992). Despite morphological differences they did not find any variations with $(GATA)_4$ in tomato. Similar results were obtained by Rus-Kortekaas et al. (1993) with a GACA-containing microsatellite probe. An explanation for these results was given by Arens et al. (1995). They proved for tomato that GATA-containing microsatellites were not dispersed over the total genome.

Flow cytometry is well suited for a rapid determination of the relative DNA content of cells. However, asymmetric hybrids can only be detected if the DNA loss or the DNA addition is greater than the accuracy of the measurements. In our study this meant that at least 8% of the wild species genome had to be added or lost for classification as an asymmetric hybrid. A differentiation between homologous and heterologous symmetric hybrids is only possible if the difference between the DNA content of the diploid fusion partners is greater than the accuracy of the measurements. In our study, hints for symmetric heterologous and symmetric homologous protoplast fusion products could only be obtained for the combination BP1076 + *S. blb*., but not for the other combinations. All regenerates obtained from the fusion of BP1076 with *S. blb*. and classified as homologous fusion products with $(GATA)_4$ were grouped into the same category using flow cytometry. However, among the regenerates classified as homologous fusion products with flow cytometry, heterologous fusion products were detected using $(GATA)_4$ and single-copy probes. This clearly demonstrated that flow cytometry, although giving information on the asymmetry of fusion products and the ploidy level (different from RFLP analysis), was not suited to give information on the origin of the DNA.

A great advantage of flow cytometry compared to the RFLP analysis was in the detection of chimeras. Among the 54 regenerates analyzed with $(GATA)_4$, single-copy probes, and flow cytometry, one asymmetric- and three symmetric-hybrids were chimeras. The high percentage of chimeras among plants regenerated after an asymmetric fusion might be due to genetic instability during the regeneration process. Genetic instability was observed during the organogenesis of tobacco by Bates (1990). A high genetic instability can be deduced for the regenerates which were identified as asymmetric hybrids with $(GATA)_4$ because of the high number (>50 %) of chimeras among them.

The analysis with single-copy probes is a laborious and time-consuming process for the classification of regenerates. However, besides the comparison of DNA sequences, this is the most-sensitive method for the detection of genomic differences (Gebhardt et al. 1989). Single-copy probes have already been used several times for the characterization of asymmetric potato hybrids (Feher et al. 1992; Puite and Schaart 1993; Xu and Pehu 1993). Puite and Schaart (1993) also used flow cytometry, but did not compare both methods for their suitability to detect asymmetric hybrids. In our study we were able to show that both flow cytometry and RFLP with single-copy probes gave similar results concerning the amount of eliminated DNA. Among the 54 analyzed regenerates, 36 were identically classified with both methods. For seven regenerates, flow cytometry showed DNA losses which were not detected with the 17 single-copy probes. In these cases probably none of the single-copy probes used were specific for the eliminated DNA sequences. This demonstrates that the information obtained with single-copy probes is correlated directly with the number of probes used. In another seven regenerates, RFLP with single-copy probes revealed DNA changes which were not detected with flow cytometry. Since the total DNA content was unchanged, and RFLP gave a different banding pattern, it has to be supposed that these changes were point mutations, insertions, inversions or minor deletions which gave DNA losses below the detection limit of flow cytometry.

In summary it has to be stressed that each of the tested methods has its own specific advantages and disadvantages and gives a reliable classification only for a fraction of the regenerates analyzed. Therefore, we propose the following procedure for the identification of asymmetric hybrids: for an initial screening, flow cytometry seems to be best due to its low cost and work requirement. Only regenerates identified as asymmetric by this screening method should then be analyzed with single-copy probes for the identification of the eliminated DNA sequences.

Amount of DNA elimination

Even if there was no clear correlation between irradiation dosage and DNA elimination, irradiation with

210 Gy showed a tendency for a higher DNA elimination than irradiation with 70 Gy. This indicates that DNA elimination is influenced not only by the irradiation dosage, but also by other factors. A contribution of several factors besides the irradiation dosage would well explain why Gleba et al. (1988) and Wolters et al. (1991) observed no correlation between irradiation dosage and DNA elimination, whereas Melzer and O'Connell (1992) found such a correlation.

RFLP analyzis with single-copy probes indicated that DNA of the non-irradiated breeding line was eliminated in two hybrids. Previously Wijbrandi et al. (1990b), using asymmetric hybrids between *Lycopersicum esculentum* and *L. peruvianum*, showed that some alleles were missing in most hybrids and that even whole chromosomes of the recipient were lost in some hybrids. A possible explanation for this phenomenon might be that, shortly after the fusion event, chromosome or chromosome-fragment exchanges with subsequent elimination of the exchanged DNA fragments might occur. In contrast to these results, Xu and Pehu (1993) detected no elimination of the recipient DNA in five asymmetric hybrids between *S. tuberosum* and *S. brevidens* using 21 single-copy probes. Instead they observed several non-parental bands. In contrast, we observed a non-parental band for only one regenerate. Non-parental bands might be caused by extensive DNA rearrangements (Xu and Pehu 1993) or by minor DNA changes such as point mutations. An explanation for the deviating results of Xu and Pehu (1993) might be plant specific differences of the fusion partners, a different irradiation technique, or different culture conditions.

The degree of donor DNA elimination observed in our studies correlated well with the results obtained for the asymmetric breeding line wild species fusion experiments in potato reported by other authors (Sidorov et al. 1987; Feher et al. 1992; Puite and Schaart 1993; Xu and Pehu 1993; Xu et al. 1993). These authors found only minor losses of the wild species genome. The highest loss of the wild species genome (65%) was observed after irradiation with 300 Gy–500 Gy (Xu and Pehu 1993). These, as well as our, results indicate that DNA elimination was limited to hypo-tetraploid and hypo-hexaploid hybrids. This is in agreement with the results of Imamura et al. (1987), Müller-Gensert and Schieder (1987), Famelaer et al. (1989), Yamashita et al. (1989), Wijbrandi et al. (1990a) and Wolters et al. (1991), but stands in contrast to the results of Dudits et al. (1980), Gupta et al. (1984), Bates et al. (1987), Dudits et al. (1987), Gleba et al. (1988), Agoudgil et al. (1990) and Hinnisdaels et al. (1991) who found only small amounts of donor DNA in the hybrids. These different results indicate that DNA elimination is a complex process, influenced by several factors. The differences in genome elimination might be due to different physical, genetic and cellular criteria (Trick et al. 1994). Not only the irradiation rate, but also the degree of relationship between the fusion partners, the ploidy level of the fusion partners and the cultivation conditions, might all contribute to different DNA elimination rates. Finally, the method of hybrid selection and the calculation of DNA elimination is of decisive influence on the reported DNA losses. The significance of these individual factors should be investigated in further studies. Additionally, there is need to investigate, whether the technique of asymmetric protoplast fusion is of advantage in plant breeding, e.g. for the transfer of desired polygenically encoded traits. Important premises for these investigations, such as the development of a simple and rapid identification procedure for asymmetric hybrids and an adequate number of asymmetric hybrids, are now available.

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References

Agoudgil S, Hinnisdaels S, Mouras A, Negrutiu I, Jacobs M (1990) Metabolic complementation for a single gene function associated with partial and total loss of donor DNA in interspecific somatic hybrids. Theor Appl Genet 80:337–342

Arens P, Odinot P, van Heusden AW, Lindhout P, Vosman B (1995) GATA- and GACA-repeats are not evenly distributed throughout the tomato genome. Genome 38:84–90

Bates GW (1990) Asymmetric hybridization between *Nicotiana tabacum* and *N. repanda* by donor-recipient protoplast fusion: transfer of TMV resistance. Theor Appl Genet 80:481–487

Bates GW, Hasenkampf CA, Contolini CL, Piastuch WC (1987) Asymmetric hybridization in *Nicotiana* by fusion of irradiated protoplasts. Theor Appl Genet 74:718–726

Behnke M (1975) Regeneration in Gewebekulturen einiger dihaploider *Solanum tuberosum* Klone. Z Pflanzenzüchtg 75:262–265

Beyermann B, Nürnberg P, Weihe A, Meixner M, Epplen JT, Börner T (1992) Fingerprinting plant genomes with oligonucleotide probes specific for simple repetitive DNA sequences. Theor Appl Genet 83:691–694

Binding H, Nehls R (1977) Regeneration of isolated protoplasts to plants in *Solanum dulcamara* L.. Z Pflanzenphysiol 85:279–280

Bonierbale MW, Plaisted RL, Tanksley SD (1988) RFLP maps based on a common set of clones reveal modes of chromosomal evolution in potato and tomato. Genetics 120:1095–1109

Campos V, Schilde L, Ninnemann H (1993) Verification of genetic stability using RFLPs and different DNA-probes. EAPR 12th Conference Trienniale, Paris, pp 449–450

Dudits D, Fejer O, Hadlaczky G, Koncz C, Lazar GB, Horvath G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179:283–288

Dudits D, Maroy E, Praznovsky T, Olah Z, Gyorgyey J, Cella R (1987) Transfer of resistance traits from carrot into tobacco by asymmetric somatic fusion: regeneration of fertile plants. Proc Natl Acad Sci USA 84:8434–8438

Famelaer I, Gleba YY, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherep NN, Negrutiu I, Jacobs M (1989) Intrageneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Nicotiana sylvestris* obtained by gamma-fusion. Plant Sci 61:105–117

Feher A, Preiszner J, Litkey Z, Csanadi GY, Dudits D (1992) Characterization of chromosome instability in interspecific somatic hybrids obtained by X-ray fusion between potato (*Solanum tuberosum* L.) and *S. brevidens* Phil. Theor Appl Genet 84:880–890

Gebhardt C, Ritter E, Debener T, Schachtschabel U (1989) RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor Appl Genet 78:65–75

Gleba YY, Hinnisdaels S, Sidorov VA, Kaleda VA, Parokonny AS, Boryshuk NV, Cherep NN, Negrutiu I, Jacobs M (1988) Intergeneric asymmetric hybrids between *Nicotiana plumbaginifolia* and *Atropa belladonna* obtained by "gamma fusion". Theor Appl Genet 76:760–766

Gupta PP, Schieder O, Gupta M (1984) Intergeneric nuclear gene transfer between somatically and sexually incompatible plants through asymmetric protoplast fusion. Mol Gen Genet 197:30–35

Helgeson JP, Hunt GJ, Haberlach GT, Austin S (1986) Somatic hybrids between *Solanum brevidens* und *Solanum tuberosum*: expression of a late blight resistance gene and potato leaf roll resistance. Plant Cell Reports 3:212–214

Hinnisdaels S, Bariller L, Mouras A, Sidorov V, Del-Favero J, Veuskens J, Negrutiu I, Jacobs M (1991) Highly asymmetric intergeneric nuclear hybrids between *Nicotiana* and *Petunia*: evidence for recombinogenic and translocation events in somatic hybrid plants after "gamma" fusion. Theor Appl Genet 82:609–614

Hunt GJ, Helgeson JP (1989) A medium and simplified procedure for growing single cells from the *Solanum* species. Plant Science 60:251–258

Imamura J, Saul MW, Potrykus I (1987) X-ray irradiated-promoted asymmetric somatic hybridisation and molecular analysis of the products. Theor Appl Genet 74:445–450

Kreike CM, de Koning JRA, Krens FA (1990) Non-radioactive detection of single-copy DNA-DNA hybrids. Plant Mol Biol Rep 8:172–179

Melzer JM, O' Connell MA (1992) Effect of radiation dose on the production of, and the extent of, asymmetry in tomato asymmetric somatic hybrids. Theor Appl Genet 83:337–344

Menke U, Schilde-Rentschler L, Rouss B, Zanke C, Hemleben V, Ninnemann H (1996) Somatic hybrids between the cultivated potato *Solanum tuberosum* L. and the 1EBN wild species *Solanum pinnatisectum* Dun.: morphological and molecular characterization. Theor Appl Genet 92:617–926

Millam S, Payne LA, Mackay GR (1995) The integration of protoplast fusion-derived material into a potato breeding programme – a review of progress and problems. Euphytica 85:451–455

Müller-Gensert E, Schieder O (1987) Interspecific T-DNA transfer through plant protoplast fusion. Mol Gen Genet 208:235–241

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Puite J, Schaart JG (1993) Nuclear genomic composition of asymmetric fusion products between irradiated transgenic *Solanum brevidens* and *S. tuberosum*: limited elimination of donor chromosomes and polyploidization of the recipient genome. Theor Appl Genet 86:237–244

Rus-Kortekaas W, Smulders MJM, Arens P, Vosman B (1993) Direct comparison of levels of genetic variation in tomato detected by a GACA-containing microsatellite probe and by random amplified polymorphic DNA. Genome 37:375–381

Saghai-Maroof M, Soliman K, Jorgensen R, Allard W (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci, USA 81:8014–8018

Schilde-Rentschler L, Boos G, Ninnemann H (1988) In: Puite KJ, Dons JJM, Huizine HJ, Kool AJ, Korneef M, Krens FA (eds.) Progress in plant protoplast research. Kluver Academic Publishers, Dordrecht Boston London, pp 195–196

Schilde-Rentschler L, Ruoss B, Schierbaum B, Ninnemann H (1993) Availability of new genetic resources from wild species for potato breeding. EAPR 12th Conference Trienniale, Paris, pp 99–100

Schweizer G (1990) Charakterisierung und RFLP-Analyse spezifischer Genomkomponenten bei *Solanum* und ihr Einsatz zur Identifikation somatischer Hybride von *S. tuberosum*-Zuchtlinien. Dissertation, Tübingen, Germany

Sidorov VA, Zubko MK, Kuchko AA, Komarnitsky IK, Gleba YY (1987) Somatic hybridization in potato: use of gamma-irradiated protoplasts of *Solanum pinnatisectum* in genetic reconstruction. Theor Appl Genet 74:364–368

Trick H, Zelcer A, Bates GW (1994) Chromosome elimination in asymmetric somatic hybrids: effect of gamma dose and time in culture. Theor Appl Genet 88:956–972

Ulrich I, Ulrich W (1991) High-resolution flow cytometry of nuclear DNA in higher plants. Protoplasma 165:212–215

Vosman B, Arens P, Rus-Kortekaas W, Smulders MJM (1992) Identification of highly polymorphic DNA regions in tomato. Theor Appl Genet 85:239–244

Weising K, Ramser J, Kraemmer D, Kahl G, Epplen JT (1991) Oligonucleotide fingerprinting in plants and fungi. Birkhäuser 294:312–329

Wijbrandi J, Wolters AMA, Koornneef M (1990 a) Asymmetric somatic hybrids between *Lycopersicon esculentum* and irradiated *Lycopersicon peruvianum*. 2. Analysis with marker genes. Theor Appl Genet 80:665–672

Wijbrandi J, Zabel P, Koornneef M (1990 b) Restriction fragment length polymorphism analysis of somatic hybrids between *Lycopersicon esculentum* and irradiated *L. peruvianum*: evidence for limited donor genome elimination and extensive chromosome rearrangements. Mol Gen Genet 222:270–277

Wolters AMA, Schoenmakers HCH, van der Meulen-Muisers JJM, van der Knaap E, Derks FHM, Koornneef M, Zelcer A (1991) Limited DNA elimination from the irradiated potato parent in fusion products of albino *Lycopersicon esculentum* and *Solanum tuberosum*. Theor Appl Genet 83:225–232

Xu YS, Pehu E (1993) RFLP analysis of asymmetric somatic hybrids between *Solanum tuberosum* and irradiated *S. brevidens*. Theor Appl Genet 86:754–760

Xu YS, Murto M, Dunckley R, Jones MGK, Pehu E (1993) Production of asymmetric hybrids between *Solanum tuberosum* and irradiated *S. brevidens*. Theor Appl Genet 85:729–734

Yamashita Y, Terada R, Nishibayashi S, Shimamoto K (1989) Asymmetric somatic hybrids of *Brassica*: partial transfer of the *B. campestris* genome into *B. oleracea* by cell fusion. Theor Appl Genet 77:189–194