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# Asymmetric protoplast fusions between wild species and breeding lines of potato – effect of recipients and genome stability

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**Abstract** The objective of this study was to investigate if in asymmetric protoplast fusion experiments the ploidy of the recipient line (di-haploid and tetraploid) has an influence on the extent of the asymmetry of the regenerating fusion products. Nineteen different experiments with the wild species *Solanum bulbocastanum* and *Solanum circaeifolium* as donors (irradiated with 210 Gy) and different breeding lines (di-haploid and tetraploid) were carried out. The degree of genome elimination was determined by measuring the relative DNA content using flow cytometry. The data showed that the loss of DNA in hybrid plants was significantly higher for 4*x*, compared to 2*x*, plants as recipients. In addition, the stability of asymmetry in the fusion products was studied. For this purpose differences in asymmetry in individual shoots originating from the same callus were analysed. A large variation in the DNA content of individual shoots was detected. Of the 4*x* to 6*x* shoots 44% had the same DNA content as another shoot originating from the same callus, 19% had a DNA content between 4*x* and 6*x* but different from any other analysed shoot originating from the same callus, 2% were chimeras and 35% had a completely different DNA content (eutetraploid, euhexaploid, eupolyploid or asymmetric with a ploidy level above 6*x*). RFLP-analysis with single-copy probes of 12 regenerates from six calli (two regenerates per callus) confirmed the assumption that the different regenerates of one callus originate from the same single cell. The analysis of selected regenerates cultivated for a period of more than 1 year demonstrated that the genome of asymmetric regenerates might change during cultivation.

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## Introduction

Polygenically inherited traits present in related wild species of crop plants are sometimes difficult to transfer to breeding lines due to crossing barriers. To overcome such barriers somatic hybridisation has shown promising results. Utilising this method pathogen resistances were successfully transferred from wild species to breeding lines of potatoes (e.g. Austin et al. 1993; Darsow et al. 1994; Thieme et al. 1997). However, symmetric fusions transfer not only the desired, but also all unfavourable, genes. Thus a laborious back crossing is inevitable to reduce the number of unfavourable wild-species genes in the offspring of symmetric fusions. To shorten the back-crossing procedure it might be promising to reduce the wild-species genome before protoplast fusion, using the asymmetric somatic-fusion technique. This technique is based on the fragmentation of the wild-species genome by X-rays. After fragmentation it is possible to obtain viable asymmetric fusion products with a reduced content of the wild-species genome. For diploid wild species and di-haploid breeding lines of potato this was shown by Sidorov et al. (1987), Puite and Schaart (1993), Schierbaum et al. (1993), Xu and Pehu (1993), Xu et al. (1993), Valkonen et al. (1994 a), Oberwalder et al. (1997) and Rasmussen et al. (1997). However, it was not possible to reduce the wild-species genome to more than 65% in hybrids with a ploidy content below 4*x* (Xu and Pehu 1993). Results from several investigations [e.g. Dudits et al. (1980), Gleba et al. (1988), Wijbrandi et al. (1990) and Wolters et al. (1991)] indicate that DNA elimination is a complex process, influenced by several factors (Trick et al. 1994). Amongst others these factors could be the X-ray dosage applied for DNA fragmentation, the genetic relationship between the fusion partners,

the ploidy level of the fusion partners and the ploidy level of the regenerates. For example, Puite and Schaart (1993) noticed a polyploidization of the recipient genome in asymmetric hybrids. They assumed that polyploidization is necessary to buffer negative effects of the additional genome from the donor. Their assumption is supported by our experiments with di-haploid breeding lines in which regenerates with a DNA content between 2*x* and 4*x* showed less DNA losses than regenerates with a DNA content between 4*x* and 6*x* (Oberwalder 1996; Oberwalder et al. 1997). Therefore, the aim of the studies reported in this paper was to investigate whether the DNA loss is correlated with the DNA content of the recipient.

In addition we examined whether the loss of portions of the wild-species genome in asymmetric hybrids is a longer-lasting process which leads to a cell mosaic in plants until a stable constellation has been reached, or whether the regeneration process itself involves a selection for balanced asymmetry. It has been shown by other authors (e.g. Rasmussen et al. 1997) that variation in the DNA content of shoots regenerating from one callus does exist. A second aim of our investigations was to learn about the degree of this variation, which is interesting not only for increasing the number of different asymmetric regenerates but also for a better understanding of the stability and rearrangement of asymmetric genomes.

## Material and methods

### Plant material

For asymmetric somatic hybridisation di-haploid as well as tetraploid breeding lines and varieties, respectively, were used. Two di-haploid breeding lines were provided by the "Bayerische Landesanstalt für Bodenkultur und Pflanzenbau", Freising, Germany (line H256/1), and the plant breeding company "Nordkartoffel Zuchtgesellschaft mbH", Ebstorf, Germany (line BP1076/1). The tetraploid breeding lines and varieties, respectively, and a further di-haploid breeding line were obtained from the "Biologische Bundesanstalt für Züchtungsforschung" Institute for "Züchtung landwirtschaftlicher Kulturpflanzen" at Groß-Lüsewitz, Germany, (variety "Dinia", tetraploid line "GL-Depot no. 6/2/1 VI-88.6164/2NP" abbreviation: GL9 and di-haploid breeding line GL-Depot no. 88) and from the plant breeding company "SaKa-Ragis Pflanzenzucht GbR", Windeby, Germany, (varieties "Pamir" and "Regina"). For some additional fusion experiments we used "Cruza 148", a tetraploid variety from Mexico.

As donor plants the diploid wild species *Solanum bulbocastanum* (Dun.) (S. blb., BGRC 8006) and *Solanum circaeifolium* ssp. *quimense* (Bitt.) (*S. crc*., BGRC 27034) were used. Both wild species were obtained from the Dutch-German potato gene bank (FAL Braunschweig, Germany) and were selected because of their good resistance properties to late blight (*Phytophthora infestans*).

### Protoplast fusion, regeneration and in vitro culture

The fusion of protoplasts, regeneration and in vitro culture was done as described by Oberwalder et al. (1997). Before electrofusion the harvested leaves were irradiated either unfragmented in sorbitol solution or dissected in mazerozyme solution. The irradiation dosage was 210 Gy. For the fusion experiments three groups of fusion partners were set up:

Group I: di-haploid recipient (breeding lines GL-Depot no. 88, H256/1 and BP1076) and the wild species *S. blb*.
Group II: tetraploid recipient (breeding line GL9 and varieties Panda, Pamir and Regina) and the wild species *S. blb*.
Group III: tetraploid recipient (breeding line GL9 and varieties Lyra, Panda and Cruza) and the wild species *S. crc*.

### Flow cytometry

From each regenerating callus at least one shoot was analysed by flow cytometry. For some calli, from which the analysed shoot was asymmetric, up to four further shoots were analysed. Additionally, some plants were analysed several times over a period of more than 1 year.

For flow cytometry a single 2–4-week-old shoot tip of an in vitro plant was employed. The shoot tip was cut into small pieces in a drop of DAPI-solution [70 mM NaCl, 0.2 mM EDTA-acetic acid, 0.1 M Tris, 0.5% (v/v) Tween 20, 4 mg/l Diamidino-2-phenylindol, pH 7.5]. Then, 1.5 ml of DAPI-solution were added and the suspension was filtered (mesh width 50 µm). The sample was put in a refrigerator for at least 10 min and then analysed using a CA II flow cytometer Partec.

The relative DNA content of the di-haploid breeding line H256/1 was taken as a standard and adjusted to 25 fluorescence units. This standard was measured for each of seven samples and the cytometer re-adjusted. Under these conditions the wild species *S. crc*. and *S. blb*. showed a DNA content of 24–25 units and 22 units, respectively. Plants were rated as asymmetric when their DNA content differed by more than three units from the DNA content expected for a symmetric hybrid.

### RFLP-analysis

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. Fifteen different single-copy probes (TG probes kindly provided by S.D. Tanksley, Cornell University, Ithaca, USA) were used for detection. The best-suited restriction enzyme – probe combination was employed for analysis of the fusion products. Further details about the RFLP-analysis are given by Oberwalder et al. (1997). The described method was modified as follows:

(1) nylon membrane from Tropilon-plus (Tropix, Bedford, USA),
(2) chemiluminescence detection ($CDP^{Star}$, Tropix, Bedford, USA),
(3) stripping by two-times washing with solution A (two-fold SSC buffer, 50 mM EDTA, pH 8.0) for 15 min at 62.5°C, two-times washing with solution B [two-fold SSC buffer, 0.1% (w/v) SDS] for 5 min at 62.5°C, and two-times washing with solution C [0.1% (w/v) SDS, 0.2 M NaOH] at 37°C followed by equilibration in two-fold SSC buffer,
(4) up to five re-hybridisations with different probes.

### Preparation of single-copy probes

The RFLP analysis was performed with digoxigenin-labelled probes. Fifteen single-copy probes (TG probes provided by S.D. Tanksley, Cornell University, Ithaca, USA) were used. These single-copy probes have been assigned to the following potato chromosomes (Bonierbale et al. 1988): chromosome 1 – TG17; TG19,

TG116; chromosome 2 – TG14; chromosome 3 – TG42; chromosome 4 – TG123; chromosome 6 – TG115; chromosome 8 – TG16; chromosome 9 – TG3; chromosome 10 – TG52, TG63; chromosome 11 – TG30, TG36, TG44; chromosome 12 – TG68. As vectors bluescript M13, pUC 18 and pUC 19 were employed. The inserts were digoxigenin-labelled according to the instructions of the supplier (Boehringer, Mannheim, Germany).

## Results

### Classification of regenerates

Most of the regenerates showed a DNA level corresponding either to the parental plants, the symmetric hybrids, or else had a DNA content of more than 6*x*. Since these plants were not suited for further investigations, they were not considered in detail in the following tables. The rate of asymmetric regenerates with a ploidy level between 2*x* and 4*x* ranged from 0% to 11% and the rate of asymmetric regenerates with a ploidy level between 4*x* and 6*x* ranged from 0% to 28% for the different fusion experiments (Tables 1–3). The average rate for 4*x* to 6*x* plants for all experiments was approximately 10%. However, a large variation between the fusion experiments was observed.

In addition to these asymmetric plants at least some chimeras were obtained in most fusion experiments. As a trend, the chimeras were more frequent among fusions of group III (10%). Fusions in groups I and II gave approximately the same rate of chimeras (3% and 4%).

The relative DNA contents of the asymmetric hybrids with *S. blb.* as the donor are given in Fig. 1. Regenerates with a wide range of DNA content were obtained. For the fusion experiments with both recipients (2*x* and 4*x*) the regenerates with a 2*x* to 4*x* DNA content lost less of the donor DNA than the 4*x* to 6*x* regenerates.

The individual results of flow cytometry are given in Table 4. Based on the assumption that only part of the genome of the wild species was eliminated, the amount of the wild-species genome contained in the regenerates can be estimated from the flow cytometry results. For the 2*x* recipients this gives a maximum donor-DNA loss of 50% and an average donor-DNA loss of 30%. For the fusion experiments with the same donor (*S. blb.*) and 4*x* recipients a maximum DNA loss of 86% and an average DNA loss of 53% was observed. This is a significantly (*t*-test, $P = 0.05$) higher loss than for the 2*x* recipients. No difference was observed between the donors *S. blb* and *S. crc*. Besides these general trends a strong variation in DNA losses between individual regenerates and the fusion experiments was found.

### Stability of DNA content and RFLP-analysis of individual shoots of selected calli

The examination of every first regenerated shoot of 440 calli obtained from fusions with tetraploid recipients gave the following results (Fig. 2): 9% 4*x* to 6*x* shoots, 7% chimeras and 84% others (eutetraploid,

**Table 1** Analysis of regenerates obtained from fusions between different di-haploid breeding lines and the irradiated diploid wild species *S. bulbocastanum* by flow cytometry. The results are based on the examination of only one shoot per callus

| Fusion partners | Experiment no. | Number of regenerates | | | |
|---|---|---|---|---|---|
| | | Analysed | Hypo-tetraploid | Hypo-hexaploid | Chimeras |
| GL18 + *S. blb* | 1 | 27 | 0 | 0 | 0 |
| H256/1 + *S. blb* | 1 | 5 | 0 | 1 | 0 |
| H256/1 + *S. blb* | 2 | 10 | 1 | 2 | 1 |
| BP1076 + *S. blb* | 1 | 33 | 3 | 1 | 0 |
| BP1076 + *S. blb* | 2 | 18 | 2 | 5 | 2 |
| Five experiments with di-haploid recipients and *S. blb.* | | 93 | 6 (6%) | 9 (10%) | 3 (3%) |

**Table 2** Analysis of regenerates obtained from fusions between tetraploid breeding lines/cultivars and the diploid irradiated wild species *S. bulbocastanum* by flow cytometry. The results are based on the examination of only one shoot per callus

| Fusion partners | Experiment no. | Number of regenerates | | |
|---|---|---|---|---|
| | | Analysed | Hypo-hexaploid | Chimeras |
| Panda + *S. blb.* | 1 | 42 | 8 | 6 |
| Panda + *S. blb.* | 2 | 50 | 1 | 0 |
| GL9 + *S. blb.* | 1 | 26 | 2 | 3 |
| Pamir + *S. blb.* | 1 | 56 | 3 | 0 |
| Regina + *S. blb.* | 1 | 28 | 2 | 0 |
| Five experiments with tetraploid recipients and *S. blb* | | 202 | 18 (9%) | 9 (4%) |

**Table 3** Analysis of regenerates obtained from fusions between tetraploid breeding lines/cultivars and the diploid irradiated wild species *S. circaeifolium* by flow cytometry. The results are based on the examination of only one shoot per callus

| Fusion partners | Experiment no. | Number of regenerates | | |
|---|---|---|---|---|
| | | Analysed | Hypo-hexaploid | Chimeras |
| Lyra + *S. crc.* | 1 | 11 | 2 | 1 |
| Panda + *S. crc.* | 1 | 96 | 6 | 3 |
| Panda + *S. crc.* | 2 | 82 | 9 | 10 |
| GL9 + S. crc. | 1 | 28 | 4 | 1 |
| Cruza + *S. crc.* | 1 | 21 | 2 | 8 |
| Five experiments with tetraploid recipients and *S. crc.* | | 238 | 23 (10%) | 23 (10%) |

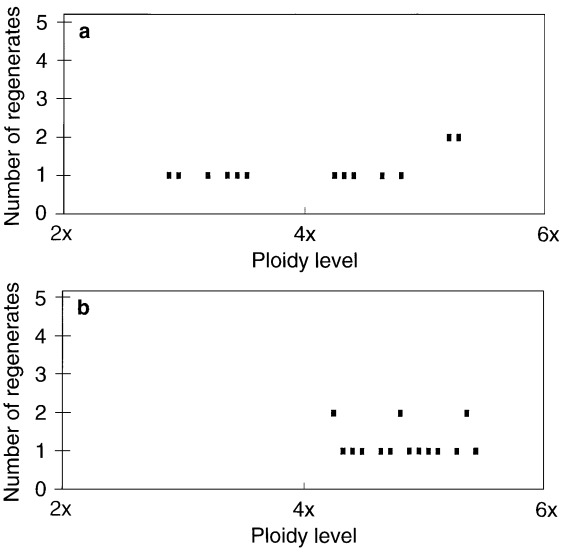


**Fig. 1** Ploidy level of asymmetric regenerates obtained from asymmetric fusion experiments between **a** di-haploid recipients and the diploid wild species *S. bulbocastanum*, **b** tetraploid recipients and the diploid wild species *S. bulbocastanum*

euhexaploid, eupolyploid or asymmetric with a ploidy level above 6*x*). About half of those calli whose first shoot showed a 4*x* to 6*x* DNA content were further examined. Up to four more shoots were isolated and analysed. The results were as follows: 44% 4*x* to 6*x* with the same DNA content as any other shoot of the same callus, 19% 4*x* to 6*x* with a different DNA content than any other shoot of the same callus, 2% chimeras and 35% others (eutetraploid, euhexaploid, eupolyploid or asymmetric with a ploidy level above 6*x*). A detailed analysis of the DNA content of different shoots of some calli is given in Fig. 3. It is evident that for some calli all shoots show the same DNA content, whereas for others pronounced variations in the DNA content between individual shoots can be observed.

For six calli two individual shoots were analysed in detail with 15 different single-copy probes (Table 5). This RFLP-analysis showed a marked similarity between the band pattern of the two shoots of the six calli tested. For example, shoots 1 and 2 of the callus c28 showed an identical band pattern for 14 of the 15 single-copy probes. Among these 14 probes TG17, TG19, TG116, TG52; TG63; TG30 and TG36 showed the same losses of specific wild-species bands. Only the probe TG123 showed a difference between the two shoots.

### Stability of DNA content

The DNA content of 13 selected regenerates was analysed three times over a period of at least 1 year and with intervals of 3–6 months. For 30% of the analysed regenerates a change of the relative DNA content was observed (Table 6) and as a trend, there was a decrease in the DNA content.

## Discussion

DNA loss by asymmetric somatic hybridisation is a complex process, which is influenced by several factors (Trick et al. 1994). The irradiation dosage may be one important factor. Some scientists observed an influence of irradiation dosage on the rate of DNA loss (Melzer and O'Connell 1992), whereas others found no correlation between irradiation dosage and DNA losses (Gleba et al. 1988; Wolters et al. 1991). However, there have to be other factors which also contribute to DNA loss. Furthermore, the large variation observed in individual experiments with the same fusion partners utilising the same procedure needs explaining.

Until now, fusion experiments with *S. tuberosum* as recipient and different wild species as donors and an irradiation dosage between 70 Gy and 500 Gy gave hybrids which contained at least 35% of the wild-species genome (Sidorov et al. 1987; Puite and Schaart 1993; Xu and Pehu 1993; Xu et al. 1993; Valkonen et al. 1994 a, b; Oberwalder et al. 1997). In all these experiments di-haploid recipients were employed. In the experiments of the present study we also used tetraploid recipients. As a result some hypo-hexaploid regenerates were obtained which contained less than 35% of the

**Table 4** DNA content of the asymmetric plants analysed (first determination)

| Donor | Ploidy of the recipient | Regenerate | Relative DNA content[a] | Calculated portion of wild species genome (%)[b] |
|---|---|---|---|---|
| *S. blb* | 2*x* | H256/1 + blb II no. 6 | 36 | 50 |
| *S. blb* | 2*x* | BP1076 + blb I no. 3 | 37 | 55 |
| *S. blb* | 2*x* | BP1076 + blb I no. 135 | 40 | 68 |
| *S. blb* | 2*x* | BP1076 + blb I no. 163 | 44 | 86 |
| *S. blb* | 2*x* | BP1076 + blb II no. 10 | 42 | 77 |
| *S. blb* | 2*x* | BP1076 + blb II no. 13 | 43 | 82 |
| *S. blb* | 4*x* | GL9 + blb no. c12/s1 | 55 | 23 |
| *S. blb* | 4*x* | GL9 + blb no. c25/s1 | 68 | 82 |
| *S. blb* | 4*x* | Pamir + blb. no. c38/s1 | 56 | 27 |
| *S. blb* | 4*x* | Pamir + blb. no. c22/s1 | 58 | 36 |
| *S. blb* | 4*x* | Pamir + blb. no. c41/s1 | 66 | 73 |
| *S. blb* | 4*x* | Panda + blb I no. c21/s1 | 54 | 18 |
| *S. blb* | 4*x* | Panda + blb I no. c23s1 | 59 | 41 |
| *S. blb* | 4*x* | Panda + blb I no. c8/s1 | 60 | 45 |
| *S. blb* | 4*x* | Panda + blb I no. c28/s1 | 61 | 50 |
| *S. blb* | 4*x* | Panda + blb I no. c6s1 | 62 | 55 |
| *S. blb* | 4*x* | Panda + blb I no. c4/s1 | 63 | 58 |
| *S. blb* | 4*x* | Panda + blb I no. c2/s1 | 64 | 64 |
| *S. blb* | 4*x* | Panda + blb I no. c34/s1 | 67 | 77 |
| *S. blb* | 4*x* | Panda + blb II no. c46/s1 | 60 | 45 |
| *S. blb* | 4*x* | Regina + blb. no. c21/s1 | 53 | 14 |
| *S. blb* | 4*x* | Regina + blb. no. c3/s1 | 53 | 14 |
| *S. blb* | 4*x* | Regina + blb. no. c9/s1 | 67 | 77 |
| *S. crc* | 4*x* | Cruza + crc c19/s1 | 61 | 44 |
| *S. crc* | 4*x* | Cruza + crc c13/s1 | 64 | 56 |
| *S. crc* | 4*x* | GL9 + crc no c15/s1 | 53 | 12 |
| *S. crc* | 4*x* | GL9 + crc no c31/s1 | 59 | 36 |
| *S. crc* | 4*x* | GL9 + crc no c20/s1 | 64 | 56 |
| *S. crc* | 4*x* | GL9 + crc no c11/s1 | 69 | 76 |
| *S. crc* | 4*x* | Lyra + crc. no c10/s1 | 53 | 12 |
| *S. crc* | 4*x* | Lyra + crc. no c12/s1 | 65 | 60 |
| *S. crc* | 4*x* | Panda + crc I no c6/s1 | 60 | 40 |
| *S. crc* | 4*x* | Panda + crc I no c61/s1 | 60 | 40 |
| *S. crc* | 4*x* | Panda + crc I no c7/s1 | 61 | 44 |
| *S. crc* | 4*x* | Panda + crc I no c64/s1 | 64 | 56 |
| *S. crc* | 4*x* | Panda + crc I no c28/s1 | 68 | 72 |
| *S. crc* | 4*x* | Panda + crc I no c45/s1 | 70 | 80 |
| *S. crc* | 4*x* | Panda + crc II no c7/s1 | 53 | 12 |
| *S. crc* | 4*x* | Panda + crc II no c61/s1 | 60 | 40 |
| *S. crc* | 4*x* | Panda + crc II no c75/s1 | 60 | 40 |
| *S. crc* | 4*x* | Panda + crc II no c13/s1 | 61 | 44 |
| *S. crc* | 4*x* | Panda + crc II no c8/s1 | 61 | 44 |
| *S. crc* | 4*x* | Panda + crc II no c15/s1 | 62 | 48 |
| *S. crc* | 4*x* | Panda + crc II no c64/s1 | 64 | 56 |
| *S. crc* | 4*x* | Panda + crc II no c51/s1 | 66 | 64 |
| *S. crc* | 4*x* | Panda + crc II no c55/s1 | 66 | 64 |

[a] Relative DNA content of *S. crc.* = 24, of *S. blb.* = 22, of di-haploid breeding lines = 25, and of tetraploid breeding lines = 48–50

[b] Percentage of the genome of the wild species present in the hybrid

wild-species genome. These observed DNA losses are in accordance with the results of Rasmussen et al. (1997) who also used tetraploid recipients and two different diploid wild species. Based on RAPD-analysis they calculated a 74–95% loss of the wild-species genome. In our studies a minimum 12% of the wild-species genome was present. In average, DNA losses were higher for fusion experiments with 4*x* than with 2*x* recipients. Thus, it can be assumed that for potato the ploidy level of the fusion partners may have an influence on the rate of DNA elimination. As a consequence, tetraploid recipients are better suited for the production of asymmetric hybrids with a low content of the wild-species genome. However, differing from Rassmussen et al. (1997), we observed no influence of the donor on DNA elimination.

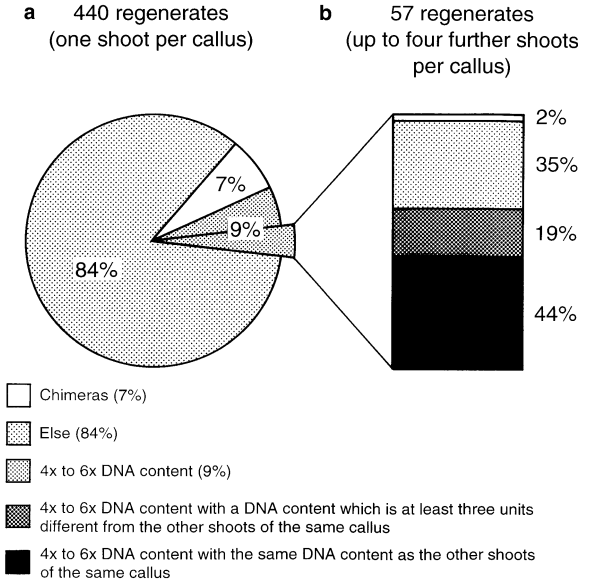


**Fig. 2a, b** Classification of regenerates obtained from asymmetric fusions between tetraploid recipients and the diploid wild species *S. bulbocastanum* and *S. circaeifolium.* **a** all regenerates, **b** about half of those calli (4% from 9%), whose first shoot showed a 4*x* to 6*x* DNA content (up to four further shoots were further examined)

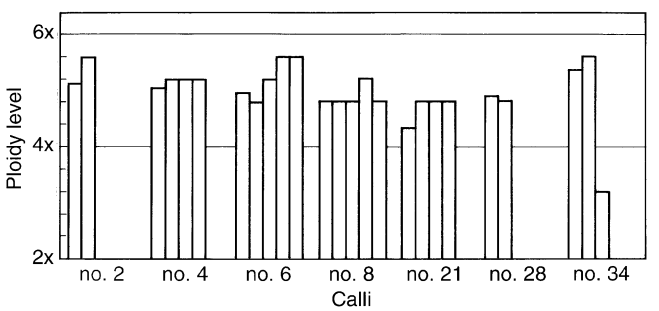


**Fig. 3** Ploidy level of individual shoots of selected calli obtained by asymmetric fusion between the tetraploid cultivar "Panda" and the wild species *S. bulbocastanum.* Experiment no. 1

We also obtained highly asymmetric 4*x* to 6*x* regenerates with the 2*x* recipient. It has however, to be considered that these regenerates might contain several partial genomes of the donor. Therefore, it might also be possible that these regenerates contained more donor DNA than is assumed and they were not considered for further analysis.

## Stability of asymmetry

In several calli up to five individual shoots per callus were analysed for their DNA content. Surprisingly, a strong variation between the individual shoots was observed. Different shoots of the same callus showed different ploidy levels, and even chimeras could be found among non-chimeric shoots.

**Table 5** Chromosome-specific analysis of 12 regenerates from six calli of the asymmetric fusion between Panda + *S. blb.* sh: symmetric hybrid; ah (w): asymmetric hybrid, wild-specific band(s) missing; b: only breeding line-specific bands; w: only wild species-specific bands; *: chromosome no.; v: shift of a band; C: callus; s: shoot

| Regenerate no. | TG 17 (I)* *Eco*RV | TG19 (I)* *Eco*RV | TG 116 (I)* *Hin*dIII | TG14 (II)* *Hin*dIII | TG 42 (III)* *Hin*dIII | TG 123 (IV)* *Eco*RI | TG 115 (VI)* *Hin*dIII | TG 16 (VIII)* *Hin*dIII | TG3 (IX) *Hin*dIII | TG 52 (X)* *Eco*RV | TG 63 (X)* *Hin*dIII | TG 30 (XI) *Hin*dIII | TG 36 (XI)* *Eco*RI | TG44 (XI) *Eco*RI | TG 68 (XII)* *Hin*dIII |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| C4/s1 | ah (w) | b | sh | ah (w) | sh | sh | b | sh | sh | sh | ah (w) | sh | sh | sh | sh |
| C4/s2 | b | b | ah (w) | ah (w) | sh | sh | b | b | sh | sh | ah (w) | sh | sh | sh | sh |
| C6/s2 | sh | b | sh | ah (w) | sh | b | sh | sh | b | ah (w) | sh | sh | sh | sh | sh |
| C6/s3 | ah (w) | sh | sh | ah (w) | sh | sh | sh | sh | b | ah (w) | sh | sh | sh | sh | sh |
| C8/s1 | ah (w) | b | sh | sh | sh | sh | b | | sh | sh | ah (w) | sh | sh | sh | sh |
| C8/s4 | ah (w) | sh | sh (v) | sh | sh | sh | b | | sh | sh | ah (w) | sh | sh | sh | sh |
| C21/s1 | b | b | | sh | sh | b | ah (w) | sh | sh | b | | b | sh | b | b |
| C21/s2 | b | b | | sh | sh | b | sh | sh | sh | b | | b | b | b | b |
| C28/s1 | b | b | ah (w) | sh | sh | sh | sh | sh | sh | ah (w) | b | b | b | sh | sh |
| C28/s2 | b | b | ah (w) | sh | sh | b | sh | sh | sh | ah (w) | b | b | b | sh | sh |
| C46/s1 | ah (w) | sh | | ah (w) | sh | | sh | | sh | b (v) | | | b | ah (w) | sh |
| C46/s2 | ah (w) | sh | | ah (w) | sh | | sh | | sh | b (v) | | | b | ah (w) | sh |

**Table 6** Long-term DNA stability. + : no DNA change; − : DNA change; C: callus; s: shoot

| Regenerate no. | Date of regeneration | Relative DNA content at different dates of analysis[a] | | | |
|---|---|---|---|---|---|
| | | Nov. 96 | May 97 | Nov. 97 | Stability |
| Panda + blb I no. C2/s1 | Apr. 96 | 64 | 66 | nd | + |
| Panda + blb I no. C4/s1 | Apr. 96 | 63 | 64 | 58 | − |
| Panda + blb I no. C6/s1 | Apr. 96 | 62 | 62 | nd | + |
| Panda + blb I no. C8/s1 | Apr. 96 | 60 | 56 | 56 | − |
| Panda + blb I no. C8/s4 | May 96 | nd | 60 | 59 | + |
| Panda + blb I no. C21/s1 | Jun. 96 | 54 | 56 | 57 | + |
| Panda + blb I no. C23s1 | Jun. 96 | 59 | 56 | 58 | + |
| Panda + blb I no. C28/s1 | Jun. 96 | 61 | 55 | 57 | − |
| Panda + blb I no. C28/s2 | Jun. 96 | nd | 54 | 54 | + |
| Panda + blb I no. C34/s1 | Jun. 96 | 67 | 68 | 66 | + |
| | | Jun. 96 | May 97 | Nov. 97 | |
| Pamir + blb. no. C22/s1 | Mar. 96 | 58 | 60 | 61 | + |
| Pamir + blb. no. C38/s1 | Mar. 96 | 56 | nd | 51 | − |
| Pamir + blb. no. C41/s1 | Mar. 96 | 66 | 68 | 68 | + |

[a] Relative DNA content of *S. blb.* = 22, of di-haploid breeding lines = 25, and of tetraploid breeding lines = 48–50

It is well known that calli, and plants regenerated from calli, may show genetic variation (somaclonal variation). Chimeras were previously observed among regenerates of protoplast-fusion experiments by several other authors (Fahleson et al. 1988; Ramulu et al. 1989; Ward et al. 1994; Valkonen et al. 1994a; Menke et al. 1996; Rasmussen et al. 1997). For our experiments the variation between the individual shoots of the same callus, as well as the occurrence of chimeras, can be explained by two possibilities:

(1) The callus resulted from an aggregation of two or more micro-calli. However, this is not likely. As demonstrated by RFLP analysis, the band patterns of individual shoots of the same callus corresponded well with each other, which would not be expected following an aggregation of different cell lines.
(2) The genome of a callus obtained by asymmetric fusion was not stable. Genome instability after fusion has already been reported by other groups. For example, Gilissen et al. (1992) noted that out of 24 highly asymmetric calli only eight remained stable over a period of more than 1 year. One explanation, given by Feher et al. (1992) for asymmetric fusion products, is the occurrence of chromosomal deficiencies of the donor genome due to irradiation with X-rays. Another explanation is that the regeneration of a plant from a callus is a very complex process and may be possible only if a special balance of genome and genome products is present.

Due to the genome differences between individual shoots of the same callus the following consequences arise for the work with asymmetric fusion products:

(1) The genome analysis of a single shoot of a callus is not representative for the whole callus. The result is only valid for the shoot which has been analysed and only for the time of analysis.
(2) The strong variation among shoots of calli which produce at least one asymmetric shoot might be used for the detection of shoots with further DNA-losses and a different asymmetry. Our results show that it is more likely to find such shoots among those calli with at least one asymmetric shoot than among the total of all regenerated calli.

In addition to the DNA change during regeneration, which resulted in shoots with different DNA contents, a change in DNA content was also observed during the vegetative propagation of the regenerates. Genetic instability was also reported by Feher et al. (1992) who noted the loss of kanamycin resistance during the vegetative cultivation of asymmetric potato plants. Thus, it can be concluded that the loss of portions of the wild-species genome in asymmetric hybrids is a long-lasting process which leads to a plant cell mosaic until a stable constellation has been reached and which is not completed with the regeneration process.

The experiments described above give an impression of the problem of the long-lasting genetic instability of asymmetric hybrid plants. In addition, although the yield in highly asymmetric plants is higher when tetraploid recipients were used, it is still low. Both aspects limit the value of the technique for transferring specific traits from wild species to breeding lines. In our experience it may be more efficient to produce symmetric

hybrids and reduce the portion of the wild-species genome by backcrossing with varieties or advanced breeding lines (Oberwalder et al., in preparation).

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