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Intergeneric transfer of a partial genome and direct production of monosomic addition plants by microprotoplast fusion

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Abstract Results are reported on the transfer of single, specific chromosomes carrying kanamycin resistance (Kan^R) and β-glucuronidase (GUS) traits from a transformed donor line of potato (*Solanum tuberosum*) to a recipient line of the tomato species *Lycopersicon peruvianum* through microprotoplast fusion. Polyethylene glycol-induced mass fusion between donor potato microprotoplasts containing one or a few chromosomes and normal recipient diploid *L. peruvianum* protoplasts gave several Kan^R calli. A high frequency of plants regenerated from Kan^R calli expressed both Kan^R and GUS, and contained one or two copies of *npt-II* and a single copy of *gus*. Genomic in situ hybridization showed that several microprotoplast hybrid plants had one single potato donor chromosome carrying *npt-II* and *gus* genes and the complete chromosome complement of the recipient *L. peruvianum* (monosomic additions). Several monosomic-addition hybrid plants could be regenerated within the short time of 3 months and they were phenotypically normal, resembling the recipient line. These results suggest that the transfer of single chromosomes is tolerated better than is the transfer of the whole donor genome. The unique advantages of microprotoplast fusion are discussed: these include the direct production of monosomic addition lines for the transfer and introgression of economically important traits in sexually-incongruent species, the construction of chromosome-specific DNA libaries, high-resolution physical mapping and the identification of alien chromosome domains related to gene expression.

Key words Single, specific chromosomes · Transgenes · Microprotoplast fusion · Monosomic additions · Genomic in situ hybridization

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Introduction

In recent years much effort has been devoted to the genome analysis of plants for the identification and isolation of economically important genes. DNA transformation using the isolated, cloned genes makes it feasible to transfer genes across sexual barriers or taxonomic boundaries (Gasser and Fraley 1989; Sybenga 1989; Potrykus 1995). Therefore, detailed molecular genetic maps are being made in various crop species to determine the chromosomal location of specific genes which code for agriculturally important traits, such as disease or stress resistance and quality improvement. Several of these traits are, however, encoded by polygenes, which are clustered within blocks in specific chromosomes or scattered throughout the genome. The identification and isolation of such genes is complicated, and hence these are not yet amenable for DNA transformation. The increasing demand for the production of cultivars resistant to diseases or stress and transfer of apomixis is insufficiently met by conventional breeding methods because of the sexual incongruity between the wild species (donor source for resistances or apomixis) and the cultivated species. In this regard, somatic hybridization can be a useful approach for transferring mono- or polygenically controlled traits and unidentified or uncloned genes from sexually uncrossable species and to generate novel nucleus – cytoplasmic combinations (reviewed by Gleba and Sytnik 1984; Puite 1992; Jacobsen et al. 1994; and Waara Glimelius 1995). To achieve partial genome transfer, asymmetric hybridization has been carried out, using irradiated donor protoplasts and normal recipient protoplasts. However, several studies show that the hybrids obtained were genetically complex, with several donor chromosomes carrying unwanted genes, and were often sterile (Famelaer et al. 1989; Wijbrandi et al. 1990; Puite and Schaart 1993; Wolters et al. 1994).

Additionally, to eliminate the undesired donor chromosomes, symmetric or asymmetric hybrids have to be

repeatedly backcrossed to the recipient parent, followed by selection. It is often difficult, as in the case of sexual hybrids, to eliminate recombined undesirable donor genes, and prevent "linkage-drag" "hitch-hiking" genes (reviewed by Sybenga 1992). On the other hand, the transfer of single chromosomes carrying the target genes between related, but sexually-incongruent species, and the production of monosomic addition lines, can speed up gene introgression through homoeologous chromosome pairing and recombination or other mechanisms of gene transfer (Sybenga 1992; de Jong et al. 1993; Jacobsen et al. 1995).

The present paper reports detailed data on the transfer of single chromosomes from a transformed donor line of potato to a recipient line of the tomato species *L. peruvianum* and the direct production of monosomic addition hybrid plants by microprotoplast fusion.The protocols for the induction of micronuclei and the isolation of sub-diploid microprotoplasts have been reported earlier (Ramulu et al. 1994, 1995). The present paper also provides data on the expression of the alien genes (*npt-II*, *gus*) carried by the potato donor chromosome in microprotoplast hybrid plants. Genomic in situ hybridization has been used to identify the potato addition chromosomes in the microprotoplast hybrids.

Materials and methods

Plant material

A transformed cell line of *Solanum tuberosum* (line 413) (2n = 3x = 36) was used as the donor source for microprotoplasts. The potato line had been transformed with *Agrobacterium tumefaciens* strain LBA 1060KG containing the plasmids pBI121 (with *gus* fused to the CaMV 35 s promotor and nopaline synthase terminator, and with *npt-II* fused to nopaline synthase promotor and terminator) and the *A. rhizogenes* wild-type plasmid pRi1855 (with TL-DNA coding for hairy roots and hormone autotrophy, and TR-DNA coding for auxin and opine synthesis) (Gilissen et al. 1991). Shoot cultures of a highly regenerable hygromycin-resistant transformed diploid recipient line of the wild tomato species *Lycopersicon peruvianum* P.I. 128650 (2n = 2x = 24), kindly provided by Prof. M. Koornneef (Koornneef et al. 1987), were used for protoplast isolation and fusion. Details of the culture conditions and media for cell and shoot cultures were reported earlier (Ramulu et al. 1995).

Isolation of donor microprotoplasts and recipient protoplasts

Potato donor microprotoplasts containing one or a few chromosomes were isolated from micronucleated protoplasts according to the procedures described earlier (Verhoeven and Ramulu 1991; Ramulu et al. 1993, 1994). These were used for fusion with shoot-culture protoplasts of the recipient line of *L. peruvianum* (Ramulu et al. 1995). Also protoplasts were isolated from cell suspensions of the donor line of *S. tuberosum* for symmetric fusion, to be used as a control for microprotoplast fusion. Protoplasts were isolated using the same procedure as described for *L. peruvianum*.

Fusions and selection of fusion products

Fusions were carried out using a polyethylene glycol (PEG)-based mass fusion protocol, modified after Menczel et al. (1981) and Derks et al. (1992). The details of the fusion protocol, composition of media and selection of fusion products were reported earlier (Ramulu et al. 1995). When the kanamycin-resistant calli turned green on solid callus growth medium, they were transferred to the regeneration medium without adding kanamycin or hygromycin.

Analysis of kanamycin resistance and GUS activity

Plants regenerated from Kan^R calli were tested for root formation by growing the shoots on MS medium supplemented with 3% sucrose and kanamycin at 50 $mg l^{-1}$. The GUS assay was performed to analyse the pattern of expression in the leaves as described by Jefferson et al. (1987) with some modifications reported earlier (Ramulu et al. 1995).

Dot-blot analysis

Dot-blot analysis was carried out according to Derks et al. (1992), using two species-specific repetitive DNA probes. DNA of the recipient and donor parents in a series of 0–400 ng DNA / dot, and DNA of plants regenerated from Kan^R calli (50–250 ng /dot), were transferred to a Gene Screen Plus membrane (Du Pont) using a Hybrid Dot 96-well manifold from Gibco/BRL. Two identical dot blots were prepared for each analysis. One was probed with pTHG2, a tomato-specific repetitive DNA probe, kindly provided by Dr. P. Zabel, department of Molecular Biology, Wageningen Agricultural University (Zabel et al. 1985). The insert of pTHG2 represents a moderately repetitive DNA fragment that is evenly dispersed on all tomato chromosomes. The other filter was probed with P5L, a potato-specific repetitive DNA probe, kindly provided by Dr. R.G.F. Visser, Department of Plant Breeding, Wageningen Agricultural University (Visser et al. 1988). Hybridization with this fragment occurs predominantly in the telomeric and centromeric regions of the potato chromosomes. The probes were labelled with [P^{32}] dATP. After exposure to X-ray film (Kodak X-Omatic AR), the dots were cut from the membrane and radioactivity was measured with a Tri-carb liquid scintillation analyser.

Southern-blot analysis

Southern-blot hybridization was carried out to determine the presence and copy number of *npt-II* and *gus* genes in plants regenerated from Kan^R calli. Genomic DNA was isolated from leaves according to Bonierbale et al. (1988). The DNA samples were digested with *Hin*dIII, separated on a 0.7% (w/v) agarose gel and blotted onto a Gene Screen Plus membrance. A 1.0-kb *Bam*HI fragment containing the *npt-II* gene, and the *Bam*HI-*Sst*I 1.8-kb fragment containing the 35*S-gus* gene excised from pNG1 and pBI121 respectively and used as DNA probes. After hybridization with random primed-labelled fragments (according to the life Technologies RadPrime Labelling System), the filter was washed according to the Du Pont hybridization protocol and exposed to X-ray film at −80 °C using an intensifying screen.

Flow cytometric analysis of nuclear DNA content

Flow cytometric analysis of nuclear DNA content was carried out for a rapid detection of the ploidy level of plants regenerated from various Kan^R calli derived from microprotoplast fusions. The procedure for the flow cytometric measurement of DNA content in the nuclei of leaves was essentially the same as described earlier (Verhoeven et al. 1990; Verhoeven and Ramulu 1991).

Genomic in situ hybridization

Genomic in situ hybridization (GISH) was performed for determining the chromosome composition of plants regenerated from Kan^R calli.

Young root tips were pre-treated in an aqueous solution of 2 mM 8-hydroxyquinoline for $2\frac{1}{2}$ h at 17 °C and fixed in a solution of 3:1 ethanol:acetic acid for 24 h or more at −20 °C. The fixed root tips were washed in water and incubated in an enzyme mixture containing 0.1% pectolyase Y23, 0.1% cellulase RS and 0.1% cytohelicase in a 10 mM citrate buffer, pH 4.5, for 1 h at 37 °C. The root tips were transferred to a grease-free microscopic slide and the cells were spread according to Pijnacker and Ferwerda (1984). DNA denaturation, in situ hybridization, and detection were all performed according to Schwarzacher and Heslop-Harrison (1993). Total genomic DNA isolated from leaves of the donor potato line was used as a probe and the leaf DNA from the recipient *L. peruvianum* was employed as a blocking DNA. The DNA of the potato donor line and that of *L. peruvianum* were sonicated so as to obtain 0.3–1.0-kb fragments. The potato DNA was labelled with Fluorescein-High Prime kit Fluorescein-12-dUTP (Bhoeringer-Mannheim). The hybridization mix (100 µl per slide) consisted of 50% deionized formamide, 10% sodium dextran sulphate (Sigma). 2 × SSC, 0.25% (w/v) sodium dodecyl sulphate (Sigma), 200 ng of *S. tuberosum* probe DNA and 100 µg of *L. peruvianum* blocking DNA. The hybridization mix was denatured for 10 min at 80 °C and then placed on ice for 5 min. Hybridization was performed overnight (16 h) at 37 °C in a humid chamber. Afterwards, the slides were washed in 2 × SSC buffer for 10 min at 20 °C, in 0.1 × SSC for 3 × 10 min at 42 °C, followed by 2 × SSC again for 15 min at 20 °C. Chromosomes were counterstained with DAPI (4′,6-diamidine-2-phenyl-indole) and propidium iodide (PI). The concentrations of DAPI and PI in the antifade solution Vectashield (Vector Lab; Inc, USA) were 2 µg/ml and 1 µg/ml respectively.

Results

Microprotoplast- and symmetric-fusions, selection and regeneration

Table 1 shows the results on microprotoplast-protoplast fusion (microprotoplast fusion) and protoplast-protoplast fusion (symmetric fusion). After microprotoplast fusion, the frequency of calli obtained on selection medium was 38×10^{-5}, whereas it was 145×10^{-5} after symmetric fusion. The percentage regeneration of Kan^R calli derived from microprotoplast fusion was considerably higher (8%) than that (2.1%) obtained from symmetric fusion-derived calli. Further, the number of shoots regenerated per callus was much higher than that obtained from symmetric fusion (Table 1). Moreover, the shoots derived from microprotoplast fusions generally resembled the recipient parent, whereas those obtained from symmetric fusions showed an abnormal phenotype with poor growth of both shoots and roots.

Kan^R and GUS expression in plants derived from microprotoplast fusion

In all, 98 plants regenerated from nine Kan^R calli were analysed for the expression of Kan^R and GUS (Table 2). The results indicate that the pattern of Kan^R and GUS expression was different in plants regenerated from different Kan^R calli. Some calli (5–2, 5–5, 5–6, 5–7) gave a higher frequency of plants showing the expression of both Kan^R and GUS than did the other calli. Also, some plants showed differential expression of Kan^R and GUS, i.e. some expressed Kan^R but not GUS, and vice versa. Of the 98 plants, 52 expressed both Kan^R and GUS, 11 either Kan^R or GUS, and 36 neither of them. Further, it was observed that the level and type of GUS expression in the leaves were different in fusion plants derived from the same callus as well as in plants from different calli. The potato donor line showed a high level of GUS

Table 2 The pattern of expression of kanamycin resistance (Kan^R) and GUS in plants regenerated from different calli obtained after microprotoplast fusion

Kan^R callus number	No. of plants analysed	No. of plants showing			
		Kan^R+ GUS+	Kan^R+ GUS−	Kan^R- GUS+	Kan^R- GUS−
5–1	15	6	1	1	7
5–2	13	9	3	0	1
5–3	19	6	0	1	12
5–4	5	0	0	0	5
5–5	12	9	0	2	1
5–6	12	10	2	0	0
5–7	14	11	1	0	2
4–1	4	0	0	0	4
4–3	4	1	0	0	3

Table 1 Fusion, selection of calli and plant regeneration from kanamycin-resistant (Kan^R) calli. PPS: protoplasts; MPPS: microprotoplasts

Fusions	No. of donor MPPS or PPS of *S. tuberosum*	No. of recipient PPS of *L. peruvianum*	Calli obtained on selection medium[a]		No. of calli regenerated to shoots (% regeneration)	No. of shoots per callus (mean ± S.E.)
			No. of calli	Frequency ($\times 10^{-5}$ recipient PPS)		
Microprotoplast fusion [donor MPPS (+) recipient PPS][b]	3.6×10^5	1.7×10^5	65	38.2	5.3 (8.1)	13.2 ± 2.3
Symmetric fusion [donor PPS (+) recipient PPS][b]	1.6×10^5	1.6×10^5	232	145	5 (2.1)	3.4 ± 0.4

[a] Selection medium contained kanamycin 100 mg l^{-1} and hygromycin 50 mg l^{-1}
[b] All the data are means (±SE: standard error) from three experiments

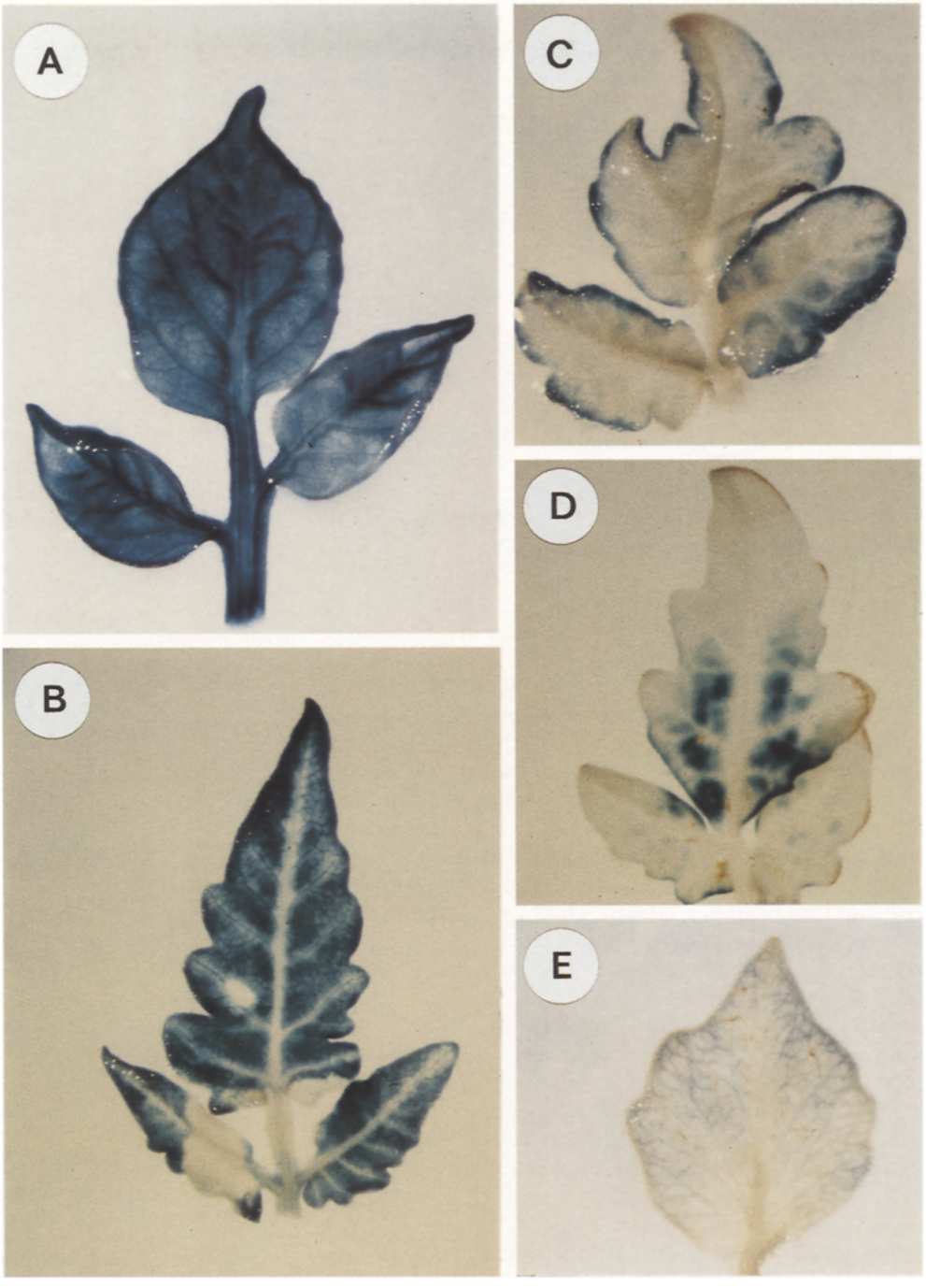

Fig. 1A–E Various patterns of GUS expression in leaves of microprotoplast hybrid plants. **A** GUS expression in the entire leaf, including mesophyll, midribs and veins, in the potato donor line; **B–E** GUS expression in microprotoplast hybrids, i.e. in mesophyll of the leaf, but not in midribs (**B**); predominantly around (periphery) the leaf (**C**); one or more sectors, mainly in the centre (**D**) and the veins (**E**)

expression, i.e. in the entire leaf, including mesophyll, midribs and veins (Fig. 1 A). Of the 52 fusion plants analysed, 15 showed the same pattern as that of the potato donor line, whereas the other plants exhibited different patterns, i.e. GUS expression, including:(1) in the mesophyll of the leaves, but not in the midribs in 11 plants (Fig. 1 B), (2) predominantly around (periphery) the leaf in five plants (Fig. 1C), (3) with one or more sectors mainly in the centre in 14 plants (Fig. 1 D) and (4) in the veins (Fig. 1E), midribs, or both, in six plants.

Dot-blot analysis of microprotoplast fusion plants

To determine the presence of donor potato DNA and to establish the hybrid nature of plants regenerated from different Kan^R calli, some plants from each callus were tested by dot-blot hybridization. The results in Table 3 show that all the plants that were positive for the expression of both Kan^R and GUS, or for either of them, showed hybridization with the potato-specific probe P5L, revealing the presence of potato DNA. In addition, a few plants from calli 5–3, 5–4, 4–1 and 4–3 that were

Table 3 Dot-blot analysis of plants regenerated from microprotoplast fusion-derived Kan^R calli. Plants showing a hybridization signal are reported in parantheses

Kan^R callus number	No. of plants analysed for dot-blot hybridization (i.e. plants showing)			
	Kan^R+ GUS+	Kan^R+ GUS−	Kan^R- GUS+	Kan^R- GUS−
5–1	3(3)	1(1)	–	2(0)
5–2	4(4)	–	–	–
5–3	3(3)	–	–	6(1)
5–4	–	–	–	2(1)
5–5	3(3)	–	1(1)	–
4–1	–	–	–	2(1)
4–3	–	–	–	3(2)

Kan^R-, GUS− also showed the presence of potato DNA, possibly indicating the presence of potato DNA (TR- or TL-DNA) other than Kan^R and GUS DNA.

Southern-blot hybridization of microprotoplast fusion plants using *npt-II* and *gus* probes

Figure 2 shows the patterns of hybridization bands in the potato donor parent and in some representative examples of fusion plants and Table 4 gives a summary of the results. The potato donor parent contained three *npt-II* bands of 3.7, 6.1 and 8.4 kb. Some fusion plants showed only one *npt-II* copy (6.1 or 7.3 kb) while others showed two *npt-II* copies (3.7 and 8.4 kb). The occurrence of the 7.3 kb band is probably due to a change in the fragment size from 6.1 kb (a manuscript on the *npt-II* and *gus* inserts is in preparation). Regarding *gus*, the donor potato line contained three bands (4.0, 7.0 and 8.4 kb), and two of these (7.0 and 8.4 kb) were observed in fusion plants. The data in Table 4 show that all plants, except one from callus 5–3), that were Kan^R+ and GUS+ contained one or two *npt-II* copies and a *gus* copy. Also one plant from callus 5–1 was Kan^R+, GUS− but contained no *npt-II* copies which appear to have been lost during vegetative propagation of the shoots. Further, some plants (from calli 5–1, 5–2, 5–5, 4–3) contained *npt-II* and *gus* copies but did not express either Kan^R or GUS, suggesting gene silencing.

Ploidy level, chromosome composition, phenotype and fertility of microprotoplast fusion plants

Flow cytometric analysis of nuclear DNA content was carried out for a rapid determination of the ploidy level of plants regenerated from various Kan^R calli derived from microprotoplast fusion. It can be seen from Table 5 that most of the plants from early regenerating calli showed a diploid (G1 = 2C) DNA content, except for some plants from callus 5–1 which showed a tetraploid (G1 = 4C) DNA level. On the other hand, the plants from late regenerating calli showed ploidy levels ranging from tetraploid to hexaploid (G1 = 4C – 6C).

Genomic in situ hybridization (chromosome painting) was carried out on a total of 43 plants regenerated from various Kan^R calli to determine their chromosome composition (Table 5). From the early regenerating calli, 18 plants showed 24 *L. peruvianum* chromosomes and one *S. tuberosum* chromosome (2n = 24 Lp + 1 St = monosomic addition at the diploid level) (Figs. 3 B, C). The monosomic addition hybrid plants at the di-

Fig. 2A–C Southern-blot hybridization using *npt-II* (*N*) and *gus* (*G*) probes in the potato donor line (**A**) and in microprotoplast fusion plants (**B**: hybridization with *npt-II* probe; **C** hybridization with the *gus* probe). L. recipient line of *L. peruvianum* containing no *npt-II* or *gus* copies. 1–3 Examples of fusion plants with one *npt-II* copy (7.3 kb) and one *gus* copy (7.0 kb). 4–8 Examples of fusion plants with two *npt-II* copies (3.7 and 8.4 kb) and one *gus* copy (8.4 kb)

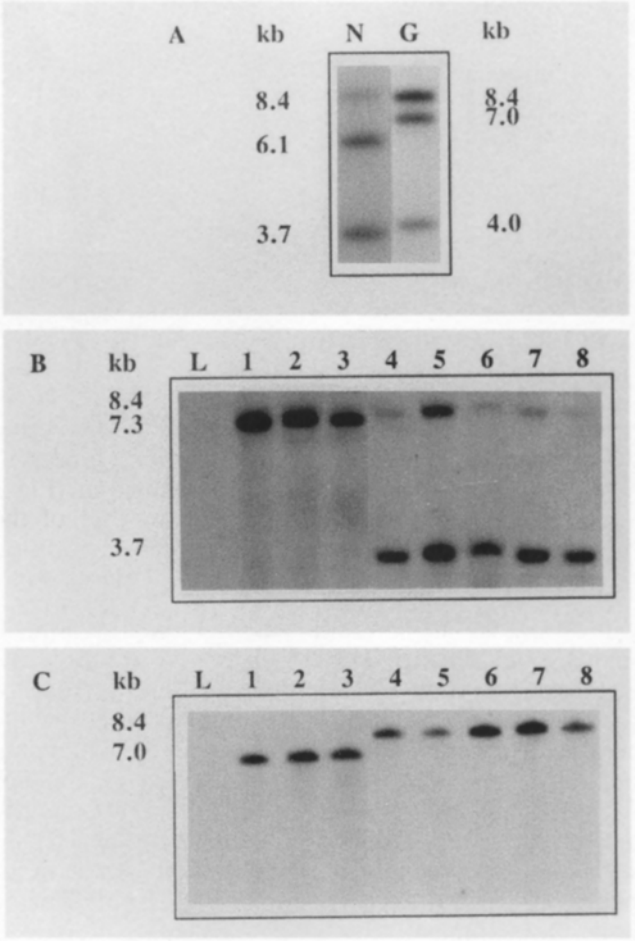

Table 4 Southern–blot hybridization of plants regenerated from Kan^R calli, using *npt-II* and *gus* probes. Plants showing *npt-II* and *gus* inserts are reported in parantheses

Kan^R callus number	No. of plants analysed for Southern hybridization (i.e. plants showing				No. of *npt-II* copies (size in kb)	No. of *gus* copies (size in kb)
	Kan^R+ GUS+	Kan^R+ GUS−	Kan^R- GUS+	Kan^R- GUS−		
5–1	6(6)	1(0)	1(1)	–	2 (3.7, 8.4)	1(8.4)
5–2	–	2(2)	–	–	1(6.1)	1(7.0)
5–3	6(5)	–	–	2(0)	1(7.3)	1(7.0)
5–4	–	–	–	1(0)	–	–
5–5	9(9)	–	2(2)	1(0)	2(3.7, 8.4)	1(8.4)
4–1	–	–	–	1(0)	–	–
4–3	–	–	–	2(1)	2(3.7, 8.4)	1(8.4)

Table 5 Flow cytometric measurement of nuclear DNA content, genomic in situ hybridization (for determining the chromosome composition), phenotype and fertility of microprotoplast fusion plants. Lp: *L. peruvianum* chromosomes; St: *S. tubersoum* chromosomes; Lp. St: recombinant chromosomes of *L. perurianum* and *S. tuberosum*; nd: not determined

Kan^R callus number	No. of plants analysed	Nuclear DNA content (G1)	Chromosome composition	Phenotype	Pollen fertility %
Early regenerating calli[a]					
5–1	7	4C	48 Lp + 1 St	Robust	8
	7	4C	48 Lp	Robust	9
	1[c]	2C	24 Lp	Recipient	74
5–3	5	2C	24 Lp + 1 St	Distinct[d]	80
	1(5–3–7)	2C	24 Lp + 1 St	Distinct[d]	86
	1[c]	2C	24 Lp	Recipient	88
5–5	11	2C	24 Lp + 1 St	Distinct[e]	nd
	1(5–5–20)	2C	24 Lp + 1 St	Distinct[f]	nd
4–1, 4–3, 5–4	4[c]	2C	24 Lp	Recipient	70
Late regenerating calli[b]					
5–2	1	4C	48 Lp + 1 St	Robust	nd
	1	6C	71 Lp + 5 St + 2 Lp.St	Intermediate	nd
5–6	2	4C	46 Lp + 4 St + 4 Lp.St	Intermediate	nd
5–7	1	5C	59 Lp + 5 St + 2 Lp.St	Intermediate	nd

[a] Shoots regenerated in 3 months
[b] shoots regenerated during, 4–10 months
[c] Plants with 24 *L. peruvianum* chromosomes and potato donor DNA or traits (Kan^R, GUS);
[d–f] Resembled the recipient parent, but were distinct [d] Dark green, rigid leaves; [e] Slender, light green leaves, less vigorous; [f] Curly and dark green leaves

ploid level generally resembled the recipient diploid parent *L. peruvianum*, but were distinct in leaf morphology and color as compared to the recipient parent (Fig. 3 A). Pollen fertility was similar (80%) to that of the diploid regenerants or the recipient parent. In addition to the diploid monosomic addition hybrid plants, some tetraploid monosomic addition hybrid plants were obtained. As can be seen from Table 5, callus 5-1 gave seven plants containing 48 *L. peruvianum* chromosomes and one *S. tuberosum* chromosome (2n = 48 Lp + 1 st = monosomic addition at the tetraploid level) (Fig. 3 D) and seven plants with 48 *L. peruvianum* chromosomes. One plant showed 24 *L. peruvianum* chromosomes only. This plant was Kan^R +, GUS − when tested after regeneration. But, eventually Southern analysis revealed no *npt-II* or *gus* inserts in this plant, which were apparently lost during vegetative propagation of the shoots. The phenotype of the monosomic addition hybrid plants at the tetraploid level was indistinguishable from the other tetraploid regenerants, being robust and vigorous as compared to the recipient diploid parent, thus suggesting the buffering capacity of the recipient tetraploid genome.

All the monosomic addition hybrid plants showed the presence of *npt-II* and *gus* copies, and some diploid plants showed 24 *L. peruvianum* chromosomes and potato donor DNA or traits. However, two monosomic addition plants (5-3-7 and 5-5-20) showed no *npt-II* or *gus* copies, which seem to have been lost during the callus phase or else during shoot propagation after regeneration.

Further, to determine the type of plants obtained from the late regenerating calli, the chromosome composition and phenotype of some plants were analysed (Table 5). The results show that the late regenerating calli gave rise to polyploid (4x–6x) chromosome numbers for *L. peruvianum* with one or a few *S. tuberosum*

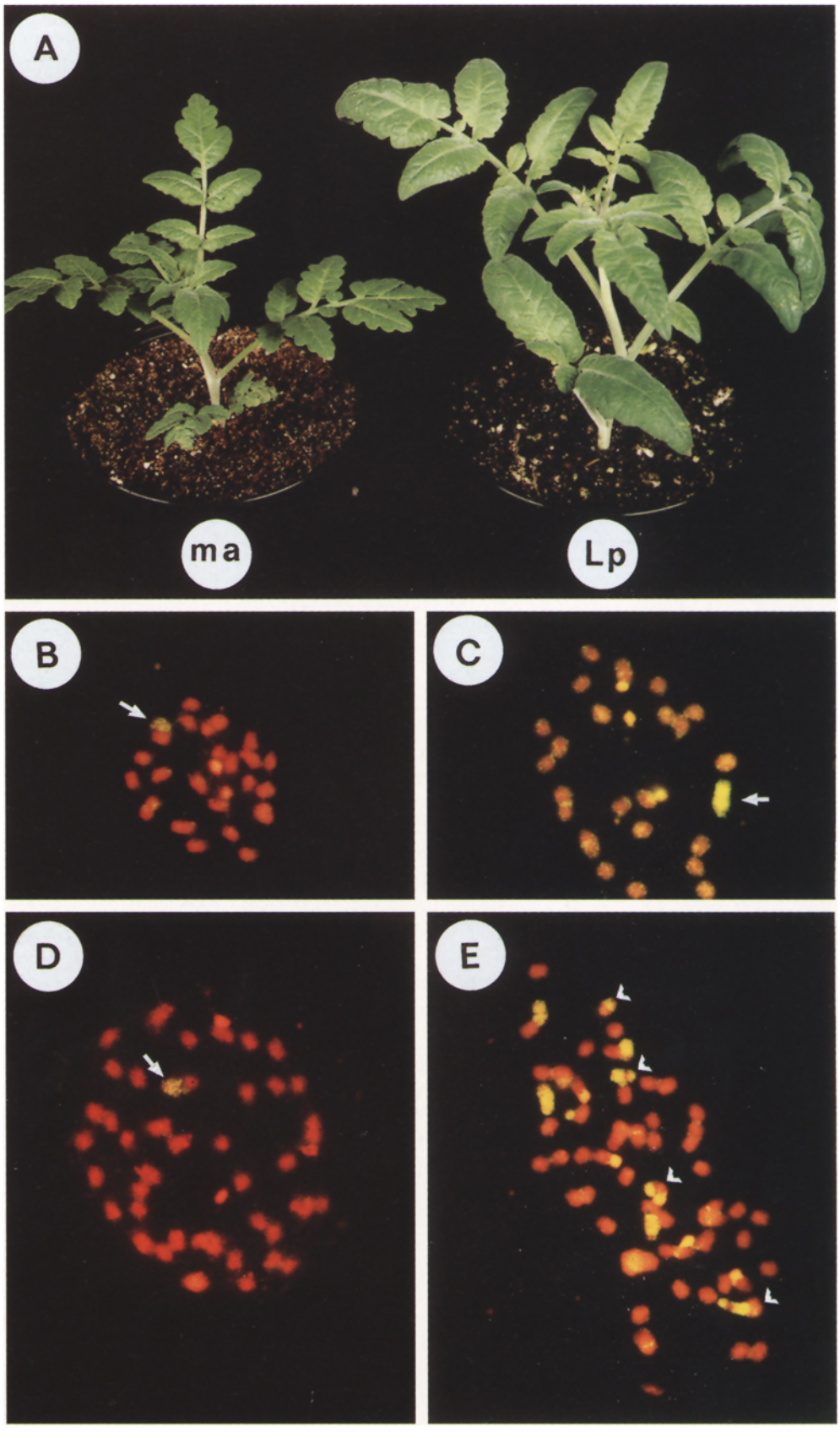

Fig. 3A–E Plant phenotype and chromosome composition of monosomic addition hybrid plants. **A** Plant phenotype and leaf morphology of a monosomic addition plant (*ma*) in comparison with the recipient *L. peruvianum* line (*Lp*); **B–E** Genomic in situ hybridization to mitotic-cell spreads of microprotoplast hybrids. Yellow-green fluorescence of FITC-labelled total genomic potato DNA shows hybridization to the potato DNA and enables the detection of potato (yellow-green) chromosomes (marked with arrows). Unlabelled chromosomes of *L. peruvianum* fluoresce orange-red with propidium iodide. Metaphase chromosome complements of diploid monosomic addition plants from callus 5-3 (**B**) and 5-5 (**C**) showing 24 *L. peruvianum* chromosomes and one *S. tuberosum* chromosome (*arrow*). Metaphase chromosomes of a tetraploid monosomic addition plant from callus 5-1 (D) showing 48 *L. peruvianum* chromosomes and one *S. tuberosum chromosome* (*arrow*) . Chromosome complement of a microprotoplast hybrid plant derived from a late regenerating callus (5-6) (E) showing 46 *L. peruvianum* chromosomes, four *S. tuberosum* chromosomes, and four recombinant chromosomes of *L. peruvianum* and *S. tuberosum* (*arrowheads*). The satellites of the NOR chromosomes of *L. peruvianum* appear light-orange or yellow-orange in color in some of the metaphase plates

chromosomes. Some plants showed recombinant chromosomes, i.e. with exchanged segments of *L. peruvianum* and *S. tuberosum* chromosomes (Fig. 3E). These plants were robust or intermediate in phenotype between *L. peruvianum* and *S. tuberosum*.

Discussion

The results obtained in the present study show that microprotoplast fusion makes feasible the transfer of single, specific potato donor chromosomes carrying alien genes (*npt-II*, *gus*), and the production of monosomic addition hybrid plants. To our knowledge, this is the first time that monosomic addition hybrid plants have been produced between sexually incompatible species through a single-chromosome transfer technique. Thus, these data demonstrate that microprotoplast fusion can bypass sexual barriers, resulting in the direct production of monosomic addition plants, without the intensive or tedious backcrosses which are necessary for producing such lines by sexual methods or after symmetric somatic hybridization (reviewed in Sybenga 1992; Jacobsen et al. 1994, 1995). The key factors for the transfer of single chromosomes by microprotoplast fusion are the efficient induction, isolation, and enrichment of sub-diploid microprotoplasts in the donor line, fusion between the donor microprotoplasts and recipient protoplasts, and high plant regenerability from protoplasts of the recipient line. The selection applied during callus induction and growth after microprotoplast fusion on double selection medium containing kanamycin and hygromycin resulted in an efficient recovery of the microprotoplast hybrid plants, inhibiting the growth of recipient protoplasts and homokaryons. The donor microprotoplasts containing one or a few chromosomes did not divide, and therefore this was an additional advantage in fusions by avoiding contamination of the donor partner, while selecting the fusion products by Kan^R.

Further, the results show that the Kan^R calli gave regeneration to several plants expressing Kan^R and GUS. The regeneration capacity of the recipient line was thus unaffected by the microprotoplasts carrying one or a few chromosomes and a small amount of cytoplasm. The facts that many microprotoplast hybrid plants were obtained within the short period of 3–4 months after fusion, and that they were phenotypically normal, resembling the recipient line, suggest that the transferred partial genome can be tolerated better than the whole donor genome. Partial genome transfer through microprotoplast fusion might be a method to overcome complex genetic interactions which can occur between donor-recipient nuclear and cytoplasmic genomes after symmetric fusion, leading either to unstable, or else to failure of, plant regeneration and subsequent deviant genotypes in some species combinations (Gleba et al. 1987; Puite and Schaart 1993; Wolters et al. 1994; Waara and Glimelius 1995).

It is evident that genomic is situ hybridization (GISH) is a potentially useful tool for the identification of the recipient *L. peruvianum* chromosomes and the donor *S. tuberosum* chromosomes, which have small and morphologically identical chromosomes (Ramulu et al. 1977; Pijnacker and Ferwarda 1984). The present results on GISH reveal that the early regenerating calli derived from microprotoplast fusions gave monosomic addition hybrid plants at diploid or tetraploid level. The latter can originate from the fusion between a donor microprotoplast with a single chromosome and a recipient endoreduplicated protoplast with 24 diplochromosomes, undergoing mitosis directly (Pijnacker and Ramulu 1991). On the other hand, the late regenerating calli gave rise to microprotoplast hybrid plants which showed late polyploidization of the recipient *L. peruvianum* genome after fusion, and thus the doubling of the potato donor chromosome. As is well known, a callus is in an unorganized state and prone to various kinds of genetic instabilities, such as polyploidization, aneuploidy, and structural chromosome changes, which accumulate at high frequency with increasing age of the culture (Pijnacker and Ramulu 1991). The diploid monosomic addition hybrid plants generally resembled the recipient parent in phenotype, but they could be distinguished by their different leaf color and morphology and growth, suggesting an effect of the potato addition chromosome, as has been shown in generatively produced monosomic additions (reviewed in Sybenga 1992).

Some microprotoplast hybrid plants showed recombinant chromosomes in somatic cells of potato and *L. peruvianum*, implying the occurrence of translocations (interchange of segments) due to mitotic instability. The occurrence of recombinant chromosomes has previously been reported in both symmetric and asymmetric somatic hybrids (Parokonny et al. 1992; de Jong et al. 1995; Jacobsen et al. 1995). The present results, which demonstrate that some fusion plants showed 24 *L. peruvianum* chromosomes and potato donor DNA or traits (Kan^R, GUS) without the potato chromosome, clearly indicate the loss of the potato addition chromosome either during the regeneration step (callus phase), or in the vegetative propagation of plants (in vitro or in vivo). Delayed replication of the addition chromosome and mitotic failure can occur, subsequently leading to chromosome breakage and integration of donor DNA into the recipient genome. The possible mechanisms for DNA integration have been discussed previously (Sybenga 1989, 1992; Ramulu et al. 1995).

Several microprotoplast hybrid plants expressed both of the donor (transgene) traits, Kan^R and GUS, showing the presence of the relevant gene copies as well as of the potato donor chromosome. Southern analysis revealed that the potato donor line contained three T-DNA inserts of *npt-II* and *gus*, which were integrated into three different chromosomes. This was evident from the occurrence of two of these in different monosomic-addition hybrid plants. The other T-DNA insert carry-

ing the *gus* copy of 4.0 kb without the *npt-II* gene was not recovered among the regenerated plants, because the fusion products were selected on the basis of kanamycin resistance (a manuscript on the *gus* and *npt-II* inserts and on chromosome identification by RFLP analysis is in preparation). Differential *gus* expression in the leaves, and the silencing of *npt-II* or *gus* copies in some of the microprotoplast hybrids, might be due to phenomena such as the effect of the chromosomal integration site of transgenes, copy number, or DNA methylation (reviewed by Flavell 1994).

The direct production of monosomic-addition plants, through microprotoplast-mediated transfer of single chromosomes carrying the desirable alien genes, can speed up introgression in sexually incongruent, but related species, i.e. by meiotic chromosome pairing and recombination (de Jong et al. 1993, Jacobsen et al. 1994; Sybenga et al. 1994). By using a combination of approaches, for example the transposon system which makes feasible the tagging of the whole haploid set of chromosomes with selectable markers (Pereira et al. 1992), or one-to-one fusion which needs no selectable markers (Spangenberg et al. 1991; Verhoeven et al. 1991), microprotoplast fusion can facilitate the construction of a complete set of monosomic addition lines. Thus, microprotoplast fusion offers some unique advantages including: (1) the transfer of economically important traits (disease or stress resistance) controlled by polygenes which are clustered within blocks in specific chromosomes, or unidentified genes, e.g. those controlling apomixis or non-host resistance, (2) the construction of chromosome-specific DNA libraries by DOP-PCR microcloning of addition chromosome fragments (Schondelmaier et al. 1993), (3) high-resolution physical mapping, using fluorescence in situ hybridization on extended DNA fibres of the addition chromosomes (regional mapping) and (4) identification of the spatial arrangement of the alien addition chromosomes related to gene expression and transmission (Gleba et al. 1987; Heslop-Harrison and Bennett 1990).

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