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Microprotoplast fusion technique: a new tool for gene transfer between sexually-incongruent plant species

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Summary

Various aspects of a microprotoplast fusion technique and the strategies followed for intergeneric partial genome transfer (one or a few chromosomes) and alien genes from sexually-incongruent donor species to recipient species are described. The essential requirements of the microprotoplast fusion technique are the induction of micronuclei at high frequencies, as well as the isolation and enrichment of sub-diploid microprotoplasts in donor species, efficient fusion of the donor microprotoplasts with normal recipient protoplasts and stable regeneration of plants from fusion products. The results on the production of microprotoplast hybrid plants between the transformed donor lines of *Solanum tuberosum* and *Nicotiana plumbaginifolia* carrying various genetic markers, and a recipient line of *Lycopersicon peruvianum* or *Nicotiana tabacum*, and on the transfer and expression of alien genes (kanamycin resistance, β-glucuronidase) are presented. The data obtained on microprotoplast hybrid plants between *S. tuberosum* and *L. peruvianum* showed that many of the hybrids contained one potato chromosome carrying nptII and GUS, and 24 or 48 *L. peruvianum* chromosomes (monosomic additions), and that they were male- and female-fertile. Various applications of chromosome transfer by this technique, especially for economically-important traits (e.g. disease or stress resistance) from sexually-incompatible wild species, for construction of chromosome-specific DNA libraries through microdissection and microcloning of chromosomes, or by flow-sorting of chromosomes for genome analysis, are discussed.

Introduction

The increasing demand in recent years for the production of cultivars resistant to diseases, insect pests and stress, is insufficiently met by classical hybridization techniques, because of the sexual incongruity between the wild species/relatives (donor sources for resistances) and the cultivated species. Firstly, it is difficult to obtain hybrids in species between which crossing barriers exist. Further, in interspecific or intergeneric crosses, in which only a low percentage of F1 hybrids may be obtained, these are mostly sterile because of the disturbances in various steps of gamete development (reviewed by Sybenga, 1992). In this case several tedious and time-consuming steps have to be carried in order to transfer the desired genes: doubling of the chromosomes of the hybrids to produce amphidiploids, and repeated backcrossing of the amphidiploid with the recipient for several generations, followed by selection to eliminate the undesirable traits of the donor parent and retain the gene of interest. Extensive embryo rescue procedures and growth of plant materials under specific controlled conditions for recovering hybrid plants are the two other requisites for classical hybridization.

Genetic manipulation at the DNA or cellular level makes feasible the transfer of genes across sexual barriers, even from the tertiary gene pool, which constitutes a rich source of germplasm (Gasser & Fraley, 1989; Sybenga, 1989; Potrykus, 1990). Transforma-

tion using recombinant DNA technology requires the availability of the cloned genes for traits of interest with the proper promoter sequences. However, for disease or stress resistance, especially those which are controlled by polygenes, no cloned genes are as yet available.

Somatic hybridization is a suitable method for transfer of genes (polygenic traits, unidentified or uncloned genes) from sexually-incompatible wild species to crop plants, and to generate novel nucleus-cytoplasm combinations (reviewed by Gleba & Sytnik, 1984; Glimelius, 1988; Puite, 1992; Jacobsen et al., 1992; Gilissen et al., 1992; Cardi et al., 1993). To achieve partial genome transfer, generally asymmetric hybridization was carried out using irradiated donor protoplasts and normal recipient protoplasts. However, both symmetric and asymmetric somatic hybridization techniques have so far not given satisfactory results, because the hybrids were mostly unstable, with all or at least several donor chromosomes carrying undesired genes, and were often sterile. Furthermore, several authors reported the damaging effects of irradiation on the genetic composition of fusion products or hybrids, with adverse effects on plant regeneration as well as on growth, phenotype and fertility of the hybrid plants (Famelaer et al., 1990; Wijbrandi et al., 1990; Wolters et al., 1991; Derks et al., 1992; Puite & Schaart, 1993; Schoenmakers et al., 1994).

Another method, metaphase chromosome – mediated gene transfer, has been extensively used in mammalian cell systems for the transfer of specific genes and for the construction of chromosome-specific DNA libraries (see Carrano et al., 1979). The chromosomes were found to integrate into the recipient genome only as small fragments, which could be maintained under selection pressure. In the case of plants, due to the difficulties in cell synchronization, chromosome isolation, flow cytometric sorting and identification of chromosomes, and the absence of clear chromosome banding patterns, this technique has met with limited success only (reviewed by De Laat et al., 1989).

On the other hand, microprotoplast fusion (the fusion of donor microprotoplasts containing one or a few chromosomes with recipient normal protoplasts) can offer a promising tool for the transfer of desirable mono- or polygenic traits, even from a donor species that can not be hybridized sexually with the recipient crop species. Microprotoplast fusion makes feasible the production of monosomic addition lines (one extra donor chromosome and the complete genome of the recipient species) in a single step, thus reducing the number of time-consuming backcrosses necessary for obtaining such lines by generative methods.

In this presentation, various aspects of a microprotoplast fusion technique together with the strategies which enabled the transfer of partial donor genome (one or a few chromosomes) and alien genes will be discussed.

Materials and methods

Genotypes

The transformed cell lines *Solanum tuberosum* (line 413) and *Nicotiana plumbaginifolia* (Doba line) were used as the donor source for the induction of micronuclei and isolation of microprotoplasts. The potato line carries various genetic markers, i.e. kanamycin resistance, β-glucuronidase activity, opine synthesis, hairy root phenotype and hormone autotrophy, while the *N. plumbaginifolia* line carries kanamycin resistance; these markers were introduced by transformation with *Agrobacterium* strains. The details on the origin of these genotypes have been published earlier (Ramulu et al., 1993, 1994).

In-vitro-grown shoot cultures of a hygromycin-resistant transformed line of *Lycopersicon peruvianum* PI 128650 (2n = 2x = 24) (Koornneef et al., 1987) kindly provided by Prof. M. Koornneef, Department of Genetics, Agricultural University, Wageningen, and *Nicotiana tabacum* (2n = 4x = 48) cv. Petit Havana SR1 (Maliga et al., 1975) were used as the recipient lines for protoplast isolation.

Cell suspension and shoot cultures

Details of the culture conditions and media for *S. tuberosum* and *N. plumbaginifolia* suspension cells were described earlier (Ramulu et al., 1993, 1994). The axenic shoots of *L. peruvianum* were subcultured monthly on MS medium (Murashige & Skoog, 1962) supplemented with 2% sucrose and hygromycin-B (Duchefa) at 25 mg l^{-1}, while those of *N. tabacum* were cultured on MS medium supplemented with 3% sucrose.

Induction of micronuclei in donor cell lines

Early log-phase suspension cells of *N. plumbaginifolia*, one day after subculture, were treated with inhibitors of DNA synthesis, i.e. hydroxy urea at 10 mM or aphidi-

colin at 15 μm for 24 h followed by repeated washing with the culture medium and treatment with the spindle toxins, amiprophosmethyl (APM) at 32 μM or cremart at 3.7 μm for 24 h. For *S. tuberosum*, actively growing early logphase suspension cells at one day after subculture were treated with cremart at 7.5 μm for 48 h. Details on the chemicals (source, structure) and treatment procedures, as well as the optimization of concentrations for inducing the maximum frequencies of micronuclei, have been reported previously (Verhoeven et al., 1990, 1991a, 1991b; Ramulu et al., 1993, 1994).

Isolation of microprotoplasts

After treatment with APM or cremart, suspension cells of *N. plumbaginifolia* or *S. tuberosum* were incubated for 18 h in a cell wall – digesting enzyme mixture, which consisted of cellulase Onozuka-RIO (1%), macerozyme Onozuka-RIO (0.2%) (Yakult Honsha Co, Tokyo, Japan), half-strength V-KM medium (Bokelmann & Roest, 1983) with 0.2 M glucose and 0.2 M mannitol, but no hormones (Ramulu et al., 1993). Cytochalasin – B (20 μM) and cremart (7.5 μM) or APM (32 μM) were added at the time of enzyme incubation to prevent the formation of microfilaments and fusion of micronuclei, respectively, during the protoplast isolation. After enzyme incubation, the samples were filtered through 297 μm and 88 μm nylon meshes and repeatedly washed with half-strength V-KM medium (Bokelmann & Roest, 1983) with macro- and microelements and 0.24 M NaCl (pH 5.6).

The purified dense suspension of mono- and micronucleated protoplasts was loaded onto a continuous iso-osmotic gradient of Percoll and exposed to a high-speed centrifugation at 100.000 g for 2 h (Verhoeven & Ramulu, 1991; Ramulu et al., 1993). The bands obtained after centrifugation contained evacuolated protoplasts, microprotoplasts and cytoplasts, and, were sequentially filtered through nylon sieves of decreasing pore size isolating the smaller sub-diploid microprotoplasts (Ramulu et al., 1993).

Protoplast isolation from shoot cultures and suspension cells

Protoplasts were isolated from leaf pieces of shoot cultures of the recipient lines of *L. peruvianum* and *N. tabacum* after overnight (16 h) incubation in 1% (w/v) cellulase-R10 (Onozuka) and 0.2% (w/v) macerozyme-R10 (Yakult Honsha Co. Ltd., Tokyo, Japan) dissolved in half-strength V-KM medium (Bokelmann & Roest, 1983). This medium contains 0.2 M glucose and 0.2 M mannitol, but no hormones. Protoplast yield ranged between 0.5×10^6 and 4.5×10^6 per gram leaf material of *L. peruvianum*, and between 0.4×10^6 and 1.0×10^6 per gram leaf material of *N. tabacum* in various experiments.

Protoplasts were isolated from cell suspensions of the donor lines of *S. tuberosum* and *N. plumbaginifolia* for symmetric fusions, to be used as controls for microprotoplast fusions. The cell suspensions were incubated for 16 h in 1% (w/v) cellulase-R10 and 0.2% (w/v) macerozyme-R10 dissolved in half-strength V-KM medium (Bokelmann & Roest, 1983). The yield of protoplasts varied from 1.5×10^6 to 3.3×10^6 per 1 ml packed cell volume of *S. tuberosum* suspension cells, and from 1.1×10^6 to 5.0×10^6 per 1 ml packed cell volume of *N. plumbaginifolia* suspension cells in various experiments.

Microprotoplast fusions and symmetric fusions

Table 1 gives details of the characteristics of parental genotypes used for microprotoplast fusions. Fusions were carried out in 2–3 experiments between the donor microprotoplasts and the recipient protoplasts using a polyethylene glycol (PEG)-based mass fusion protocol, modified after Menczel et al. (1981) and Derks et al. (1993), which was briefly reported earlier (Ramulu et al., 1992).

Table 2 shows the details of fusion combinations, and media used for culture, selection and plant regeneration at various periods. Microprotoplasts and protoplasts were mixed in ratios of 1 : 1 or 2 : 1 in 6 cm Falcon Petri dishes in W5 medium and plated at a density of 1×10^6 ml^{-1}. After 20 min, PEG (PEG 4000) was applied at 8–10% (final concentration). After a further 7 min, PEG and W5 medium were carefully removed using a Pasteur pipette, and high pH buffer solution was slowly added. Twenty minutes later, when the microprotoplast-protoplast mix had settled down in the Petri dish, rinsing was done with liquid TM2 or H460M medium, according to the type of fusion. Afterwards, 2 ml of TM2 or H460M medium was added to each Petri dish, the density during culture being 0.2×10^6 ml^{-1}. As a control for microprotoplast fusions (i.e. fusions of microprotoplasts (+) protoplasts), we have also carried out symmetric fusions between the donor cell suspension protoplasts and recipient leaf protoplasts under the same culture conditions and at a similar plating density. The

Table 1. Characteristics of parental genotypes used for microprotoplast fusions

Genotype	Source material (ploidy)	Fusion partner	Selectable markers used	Other markers
Solanum tuberosum, line 413	Suspension cells (2n = 3x = 36)	Donor-microprotoplasts	Kan^R	GUS, OP, HR, HA
Nicotiana plumbaginifolia, line Doba	Suspension cells (2n = 4x = 40)	Donor-microprotoplasts	Kan^R	–
Lycopersicon peruvianum, line PI 128650	Shoot culture (2n = 2x = 24)	Recipient-protoplasts	Hyg^R	–
Nicotiana tabacum, cv. Petit Havana, SR 1	Shoot culture (2n = 4x = 48)	Recipient-protoplasts	–	Streptomycin-resistance

Kan^R : kanamycin resistance; Hyg^R: hygromycin resistance; GUS: β-glucoronidase; OP: opine synthesis; HR: hairy roots; HA: hormone autotrophy.

Table 2. Details of fusion combinations, and media used for culture, selection and plant regeneration at various periods

Fusion combinations			Day 5	Day 12 and 19	Day 25	Day 40
Donor	(+)	Recipient	Dilution in CI liquid medium	Dilution and selection in CI liquid medium	Selection on solid CG medium	Regeneration medium
St mpps	(+)	Lp pps	TM2	TM2 + Kan50 + Hyg25	TM3 + Kan100 + Hyg50	TM4
St pps	(+)	Lp pps	TM2	TM2 + Kan50 + Hyg25	TM3 + Kan100 + Hyg50	TM4
St mpps	(+)	Nt pps	H460M	H460M + Kan50	AG + Kan100	MSR1
St pps	(+)	Nt pps	H460M	H460M + Kan50	AG + Kan100	MSR1
Np mpps	(+)	Lp pps	TM2	TM2 + Kan50 + Hyg25	TM3 + Kan100 + Hyg50	TM4
Np pps	(+)	Lp pps	TM2	TM2 + Kan50 + Hyg25	TM3 + Kan100 + Hyg50	TM4

St : *Solanum tuberosum*; Lp: *Lycopersicon peruvianum*; Nt: *Nicotiana tabacum*; Np: *Nicotiana plumbaginifolia*. mpps: Microprotoplasts; pps: Protoplasts: CI: Callus induction medium; CG : Callus growth medium.
Kan 50 : Kanamycin at 50 mg l^{-1}; Kan 100: Kanamycin at 100 mg l^{-1}.
Hyg 25 : Hygromycin at 25 mg l^{-1}; Hyg 50: Hygromycin at 50 mg l^{-1}.

details of the composition of media i.e. TM-2, -3, and -4 used for callus induction and growth and plant regeneration, respectively, have been reported earlier (Shahin, 1985; Derks, 1992). The culture media H460M, AG and MSRI were used with some modifications for callus induction and growth, and plant regeneration, respectively. The medium H460M contained K3 macro elements (Nagy & Maliga, 1976), MS (Murashige & Skoog, 1962) micro elements (5 × diluted), 7.46 g/l Fe EDTA and sugar stock (Kao & Michayluk, 1975). The medium AG consisted of AG micro elements (Caboche, 1980), MS micro elements (10 × diluted), 7.46 gl^{-1} Fe EDTA, vitamins (Morel & Wetmore, 1951), 3% sucrose, 5% mannitol, 0.1 mg l^{-1} naphthalene acetic acid and 1 mg l^{-1} benzyl aminopurine. The MSRI medium contained MS macro- and micro-elements (normal concentration) and 7.46 gl^{-1} Fe EDTA. As shown in Table 2, the hybrid calli were selected using kanamycin and hygromycin selection, or only kanamycin selection, depending on the type of fusion. When the resistant calli turned green on solid callus growth medium, they were transferred to the regeneration medium without adding kanamycin or hygromycin. The regenerated shoots from *S. tuberosum* (+) *L. peruvianum* fusions were rooted on MS medium (Murashige & Skoog, 1962) supplemented with 2% sucrose, and those from *S. tuberosum* (+) *N. tabacum* or *N. plumbaginifolia* (+) *L. peruvianum* were rooted on MS medium supplemented with 2% sucrose and indole butyric acid at 0.025 mg l^{-1}.

Kanamycin resistance and GUS assays

Kanamycin resistance of shoots regenerated from Kan^R calli was determined on the basis of root induction from shoots grown on MS medium supplemented

with 3% sucrose and kanamycin at 50 mg l^{-1}. The GUS assay was performed as described by Jefferson et al. (1987), using a modified extraction buffer containing 50 mM sodium phosphate buffer pH 7.5, 10 mM Na_2-EDTA (ethylene diamine tetra acetic acid), and 0.1% (v/v) Triton X-100. Ironcyanide solution (0.5 mM potassium ferricyanide in water) and X-Gluc solution (1 mM 5-bromo 4-chloro 3-indolyl β-glucuronide in dimethylformamide) were added to the extraction buffer. After an incubation period of 16 h at 37° C, the appearance of a blue colour was indicative of GUS activity. Chlorophyll was removed from stained tissue by ethanol extraction and the presence of GUS staining was observed using a dissection microscope.

Karyotype and genomic in situ *hybridization*

Chromosome counts and karyotype analysis were performed on Feulgen-stained root tip metaphase cells of plants regenerated from Kan^R calli as described earlier (Ramulu et al., 1983). As *L. peruvianum* and *S. tuberosum* have small chromosomes and similar karyotypes (Ramulu et al., 1977; Pijnacker & Ferwarda, 1984), their accurate identification through classical cytogenetic methods is difficult. Therefore, genomic *in situ* hybridization (GISH) was carried out for chromosome identification of plants derived from *S. tuberosum* (+) *L. peruvianum* fusions. Actively growing young root tips were pretreated in an aqueous solution of 2 mM 8-hydroxyquiniline for 2.5 h at 17° C and fixed in a solution of 3 : 1 ethanol : acetic acid for 24 h or more. 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 10 mM citrate buffer, pH 4.5, for 1 h at 37° C. The root tips were carefully transferred to a grease-free microscopic slide and the cells were spread according to the technique of Pijnacker & Ferwarda (1984). Various steps of DNA denaturation, *in situ* hybridization and detection/amplification were performed according to Leitch & Heslop-Harrison (1993), Schwarzacher & Heslop-Harrison (1993) and Schwarzacher & Leitch (1993). Total genomic DNA isolated from the leaf material of the donor *S. tuberosum* line 413 was used as probe and the leaf DNA from the recipient *L. peruvianum* plants as blocking-DNA. Labelling of *S. tuberosum* DNA was done either by an indirect method or a direct method. In the indirect method, the DNA was sheared by passage through a syringe until the fragments attained a size of 1–10 kb, while in the direct method, the DNA was sonicated so as to obtain 1.0–2.0 kb fragments. In the indirect method, labelling was done with digoxigenin-11-dUTP (Boehringer-Mannheim) and detected with anti-digoxigenin Fluos (fluorescein isothiocyanate) raised in sheep (Boehringer-Mannheim) and amplified with anti-sheep-FITC raised in rabbit (Boehringer-Mannheim) according to a standard random primer labelling protocol. In the direct method, the labelling was carried out with Fluorescein-high prime kit fluorescein-12-dUTP (Boehringer-Mannheim). The DNA of *L. peruvianum* was sonicated for 10 sec (12 micron amplitude) which resulted in fragments of about 700 bp. The hybridization mix (100 μl) per slide consisted of deionized formamide sodium dextran sulphate (Sigma), 2 × SSC, sodium dodecyl sulphate (Sigma), 200 ng μl^{-1} of *S. tuberosum* probe DNA and 10 μg μl^{-1} of *L. peruvianum* blocking DNA. The hybridization mix was denatured for 10 min at 70° C and then placed on ice for 5 min. Hybridization took place overnight (16 h) at 37° C.

Afterwards, the slides were washed in 2 × SSC buffer for 30 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^{-1} and 1 μg ml^{-1}, respectively.

Results and discussion

Induction of micronuclei in donor cell lines

The treatment of *N. plumbaginifolia* suspension cells with inhibitors of DNA synthesis (10 mM hydroxy urea or 15 μM aphidicolin) for 24 h, followed by treatment with microtubule inhibitors APM (32 μm) or cremart (3.7 μM) for 24 h generally resulted in about 20% micronucleated cells. The treatment of *S. tuberosum* suspension cells with cremart (7.5 μM) gave approximately 15% micronucleated cells. Afterwards, when the treated suspension cells of *N. plumbaginifolia* or *S. tuberosum* were incubated in a mixture of cell wall-digesting enzymes (cellulase, macerozyme) in the presence of cytochalasin-B and APM or cremart for 18 h followed by sieving, the frequency of micronucleated protoplasts increased to a maximum of 40% (Fig. 1A). This was due to a rapid decondensation of metaphase chromosomes, forming micronuclei during enzyme incubation, combined with stable

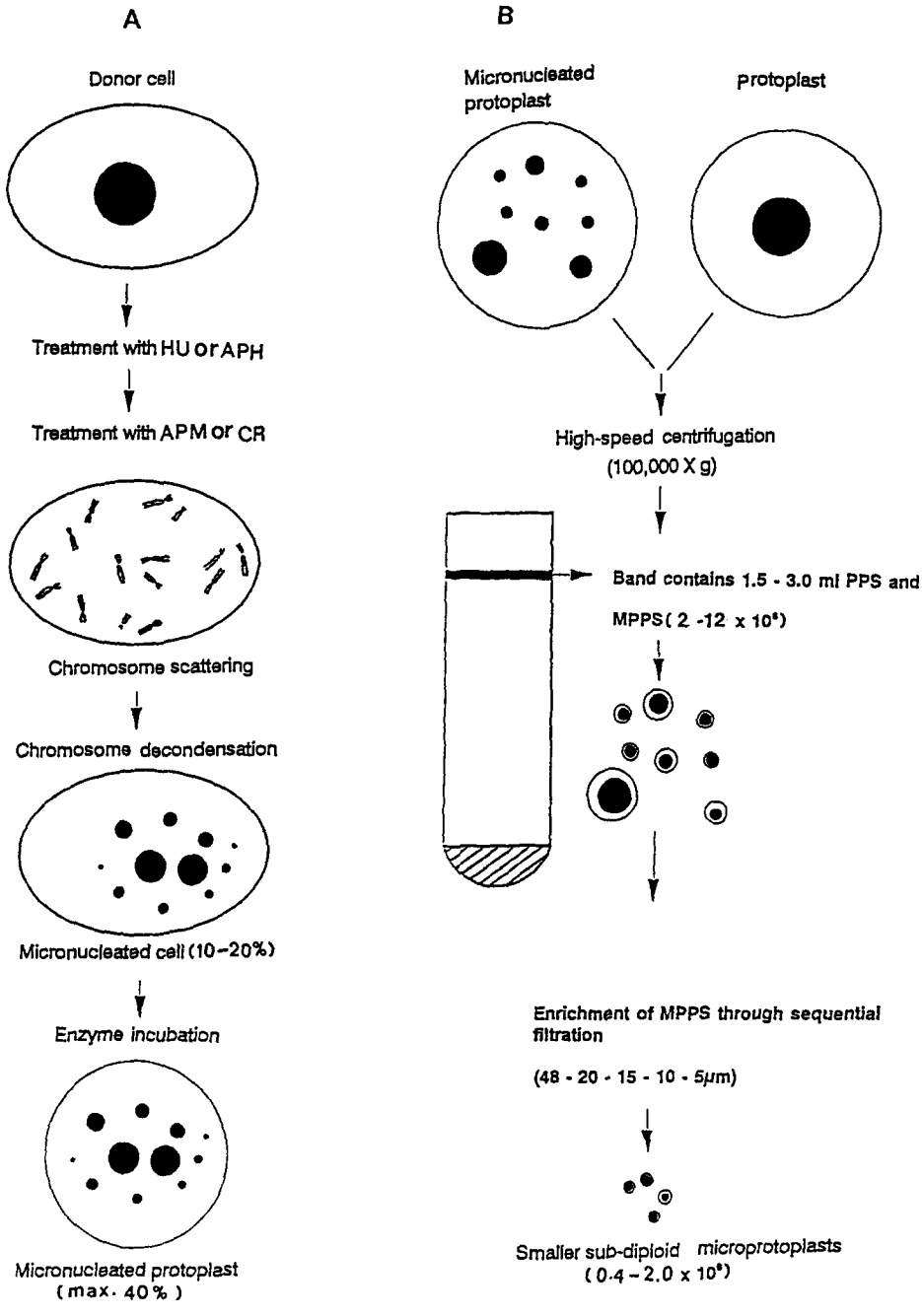

Fig. 1A. Induction of micronuclei in donor suspension cells after treatment with hydroxy urea (HU 10 mM, 24 h) or aphidicolin (APH 15 μM, 24 h) followed by treatment with amiprophos-methyl (APM 32 μM, 48 h) or cremart (CR 3.7 μM or 7.5 μM, 48 h). *Fig. 1B.* High-speed centrifugation of micronucleated and mononucleate protoplasts for the isolation of individual protoplasts (PPS) and microprotoplasts (MPPS) and enrichment of MPPS by sequential filtration through nylon sieves of decreasing pore size.

maintenance of micronuclei already formed prior to the enzyme incubation without fusion and restitution.

Isolation and enrichment of smaller sub-diploid microprotoplasts

After enzyme treatment and purification, the dense suspension of the mixture of micronucleated protoplasts

Table 3. Plant regeneration from kanamycin-resistant calli (Kan^R) derived from fusions between the donor microprotoplasts (mpps) or protoplasts (pps) and recipient mesophyll protoplasts (pps) of various species

Fusion combinations		No. of Kan^R calli obtained	No. of Kan^R calli regenerated to plants	No. of regenerated plants obtained	Duration of plant regeneration
Donor	(+) Recipient				
S. tuberosum mpps	(+) *L. peruvianum* pps	184	16	210	3
S. tuberosum pps	(+) *L. peruvianum* pps	465	10	34	7
S. tuberosum mpps	(+) *N. tabacum* pps	7	5	15	4
S. tuberosum pps	(+) *N. tabacum* pps	2[a]	–	–	–
N. plumbaginifolia mpps	(+) *L. peruvianum* pps	21	12	24	8
N. plumbaginifolia pps	(+) *L. peruvianum* pps	3[a]	–	–	–

[a] : These calli did not develop more than 2–3 mm size and eventually turned brown and perished.

was collected from the surface of the sucrose solution, loaded onto a continuous isoosmotic gradient of Percoll and exposed to a high-speed centrifugation at 100.000 g for 2 h (Fig. 1B). After centrifugation, several bands formed containing evacuolated protoplasts, microprotoplasts and cytoplasts. About 12 ml of packed cell volume of suspension cells used per experiment gave rise to the bands containing a mixture of 1.5 to 3.0 ml of protoplasts and microprotoplasts of different sizes, the yield of which ranged from 2–12 $\times$ 10^6 in various experiments. By using sequential filtration of the mixture of protoplasts and microprotoplasts through nylon sieves of decreasing pore size (48–20–15–10–5 μm), it was possible to recover smaller subdiploid microprotoplasts on a mass scale (0.4–2.0 $\times$ 10^6). These microprotoplasts contained a small rim of cytoplasm and plasmamembrane around them, and were FDA-positive. These were used for fusions with the recipient leaf protoplasts.

Fusion, selection of hybrid calli and plant regeneration

After fusions using PEG-based mass fusion protocol, heterokaryons and hybrid calli were selected on medium containing kanamycin and hygromycin for fusions of *S. tuberosum* (+) *L. peruvianum* and *N. plumbaginifolia* (+) *L. peruvianum*, and on medium containing kanamycin alone for *S. tuberosum* (+) *N. tabacum*. Table 3 gives details on various fusion combinations, Kan^R calli and plant regeneration. The fusions of donor microprotoplasts with recipient protoplasts gave regeneration of several plants from Kan^R calli in all the combinations (Fig. 2A–C). On the other hand, in the case of symmetric fusions of *S. tuberosum* (+) *L. peruvianum*, plants were regenerated from Kan^R calli at a low frequency only (Table 3). In other symmetric fusions, i.e. *S. tuberosum* (+) *N. tabacum* and *N. plumbaginifolia* (+) *L. peruvianum*, no fusion products could be obtained.

Characterization of plants regenerated from Kan^R calli

Table 4 gives data on plant phenotype, chromosome composition and Kan^R and GUS assays of plants derived from various fusion combinations.

S. tuberosum (+) L. peruvianum. Of the total of 111 plants analysed for plant phenotype, 67 resembled the recipient parent *L. peruvianum*, though 19 of the 67 plants showed bigger leaves and stems and more vigorous growth. Three of the 19 plants contained 48 *L. peruvianum* chromosomes and one *S. tuberosum* chromosome (as verified by genomic *in situ* hybridization), and expressed both Kan^R and GUS from *S. tuberosum*.

Fifteen plants resembled the recipient parent *L. peruvianum* in general appearance, but distinctly differed in leaf morphology and colour (Fig. 2D). Eleven of these plants contained 24 *L. peruvianum* chromosomes and one *S. tuberosum* chromosome (Fig. 2E), and expressed Kan^R and/or GUS.

The other 29 plants were intermediate in phenotype, i.e. between that of *L. peruvianum* and *S. tuberosum* (Table 4). One of these showed 71 *L. peruvianum* chromosomes, 5 *S. tuberosum* chromosomes and 2 chromosomes with interchanged or reciprocally-translocated parts of *L. peruvianum* and *S. tuberosum* chromosomes, and expressed Kan^R and GUS. The tests for Kan^R and

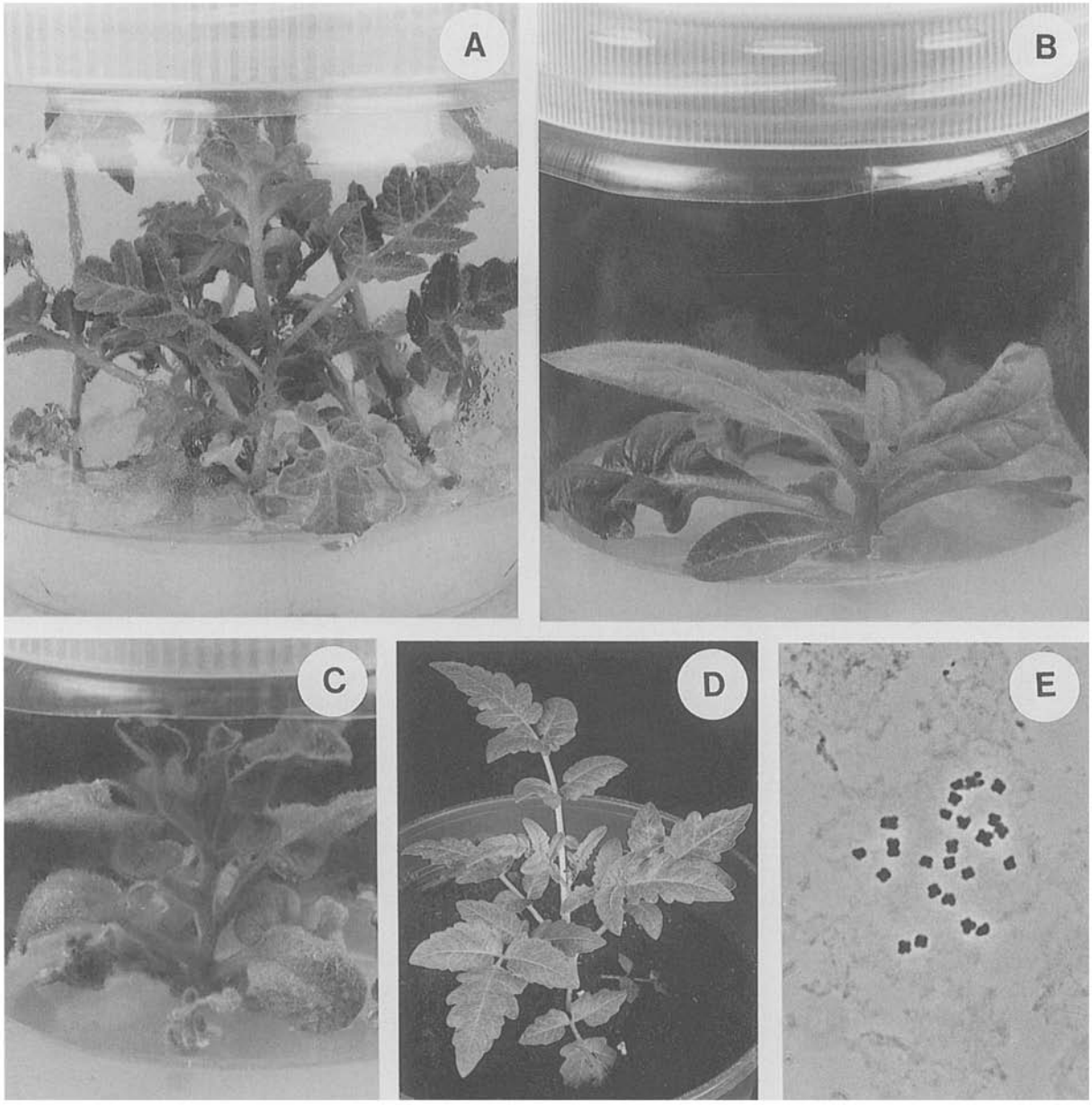

Fig. 2. Shoots regenerated from various fusion combinations. A: Shoot from *S. tuberosum* MPPS (donor) (+) *L. peruvianum* PPS (recipient); B: Shoot from *S. tuberosum* MPPS (donor) (+) *N. tabacum* PPS (recipient); C: Shoot from *N. plumbaginifolia* MPPS (donor) (+) *L. peruvianum* PPS (recipient). *Fig. 2D, E.* Microprotoplast hybrid plant (D) derived from *S. tuberosum* (+) *L. peruvianum* fusions which expressed the donor traits Kan^R and GUS, and contained one *S. tuberosum* chromosome and 24 *L. peruvianum* chromosomes (E) (also verified by genomic *in situ* hybridization.

GUS assay showed that, out of a total of 87 plants, 58 expressed Kan^R and/or GUS.

S. tuberosum *(+)* N. tabacum. The phenotype of all the 14 plants resembled that of the recipient line of *N. tabacum* and expressed the donor traits, i.e. Kan^R and GUS (Table 4). Seven of the plants also showed another donor *S. tuberosum* trait, i.e. anthocyanin pigmentation on stems and leaf midribs. The analysis of chromosome composition is in progress.

Table 4. Characterization of microprotoplast hybrid plants derived from various fusion combinations

Fusion Combinations			Plant phenotype		Chromosome Composition		Kan^R and GUS assays	
Donor	(+)	Recipient	No. of plants analysed	Phenotype	No. of plants analysed	Chromosome number	No. of plants analysed	No. of plants expressing Kan^R and/or GUS
St mpps	(+)	Lp pps	67	Recipient	3	48 Lp + 1 St	87	58
			15	Recipient, but distinct	11	24 Lp + 1 St		
			29	Intermediate	1	71 Lp + 5 St + 2 Lp.St*		
St mpps	(+)	Nt pps	14	Recipient	nd		14	14
Np mpps	(+)	Lp pps	19	Intermediate	nd		nd	

St : *S. tuberosum*; Lp: *L. peruvianum*; Nt: *N. tabacum*; Np: *N. plumbaginifolia*.
nd : not determined; *: 2 chromosomes with interchanged or reciprocally-translocated parts of *L. peruvianum* and *S. tuberosum* chromosomes.

N. plumbaginifolia (+) L. peruvianum. All of the 19 plants were intermediate in phenotype and leaf morphology between that of the two parents (Table 4).

The key factors for partial genome transfer (transfer of one or a few donor chromosomes) through the microprotoplast fusion technique, as achieved for example in *S. tuberosum* (+) *L. peruvianum* fusions, are the efficient induction, isolation and enrichment of microprotoplasts in the donor line and efficient plant regeneration from the recipient line. The fact that after microprotoplast fusion, plant regeneration occurred at a high frequency and within 3 months, and that the microprotoplast hybrid plants generally resembled the recipient line, suggests that the transferred partial genome can be better tolerated than is the whole donor genome. Partial genome transfer through microprotoplast fusion might overcome complex genetic interactions which can occur between donor-recipient nuclear and cytoplasmic genomes after symmetric fusions, leading to unstable plant regeneration or even no regeneration in some species combinations (Wolters et al., 1994).

Further, the chromosome/genome composition in the microprotoplast hybrid plants depends on 1) the segment of the donor genome (one or a few chromosomes) transferred to the recipient protoplasts at the time of fusion, and 2) genetic stability after fusion. The first stage is prone to endoreduplication leading to polyploidization. During the callus phase, aneuploidy and chromosome structural changes can occur (Ramulu et al., 1989; Pijnacker & Ramulu, 1991). For variation in the segment of the donor genome present in the fusion products, different processes might occur. As outlined in Fig. 3, when the donor microprotoplast carrying a potato chromosome with two chromatids

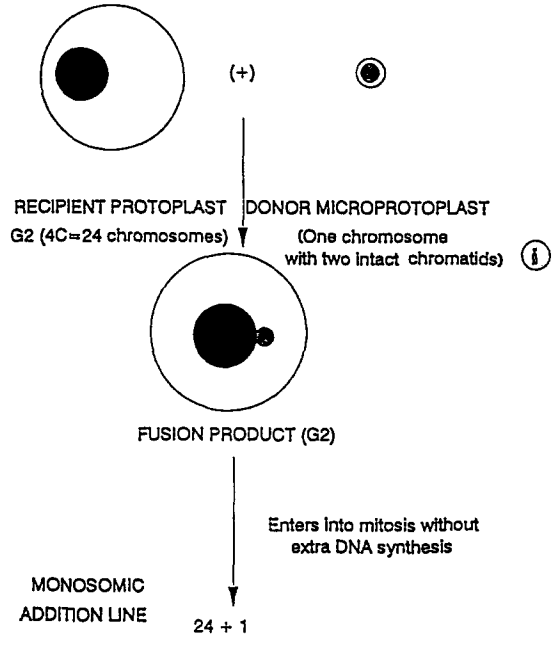

Fig. 3. Production of a monosomic addition line after fusion of a recipient protoplast with a donor microprotoplast.

together, due to the action of spindle toxins APM or cremart used for inducing micronuclei (Ramulu et al., 1988, 1994) in resting phase (= G2) fuses with a recipient G2 *L. peruvianum* protoplast, the fusion product may directly progress to mitosis (without an extra DNA synthesis), giving rise eventually to a microprotoplast hybrid with only one potato chromosome and a complete genome of *L. peruvianum* (*monosomic addition line*: 2n = 24 + 1). On the other hand, after fusion of the donor microprotoplast containing a chromosome

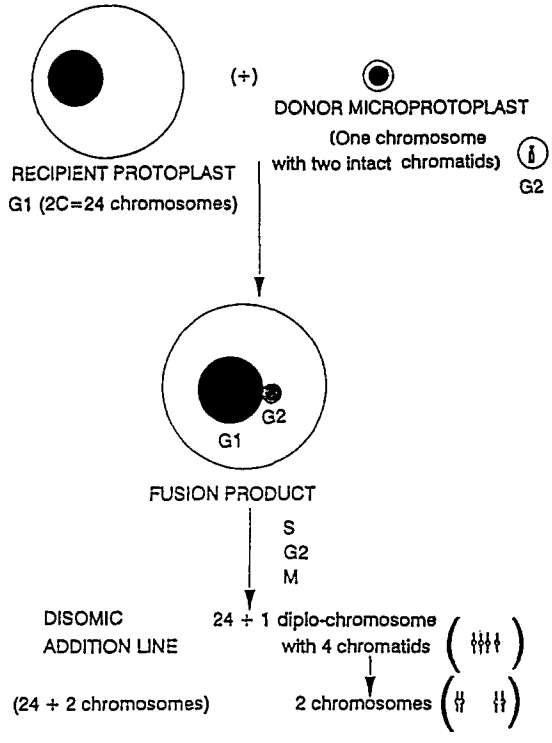

Fig. 4. Production of a disomic addition line after fusion of a recipient protoplast with a donor microprotoplast.

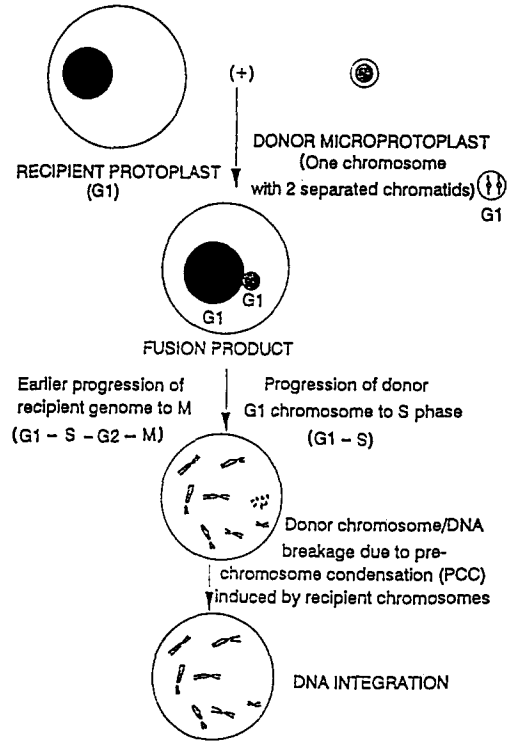

Fig. 5. Donor DNA integration in recipient chromosomes after fusion of a recipient protoplast with a donor microprotoplast.

(2-chromatid chromosome) with a recipient G1 protoplast, and following S-G2-M in the immediate cell cycle, the hybrid cell can contain a 4-chromatid (diplo) chromosome, the centromere of which can separate, giving rise to two copies of a given chromosome (*disomic addition line*: 2n = 24 + 2) (Fig. 4). Further, due to delayed DNA replication of the donor chromosomes in the immediate cell cycle after fusion, and when the recipient genome progresses to metaphase, the latter can induce pre-chromosome condensation (PCC) of the donor S-phase chromosomes, leading to DNA or chromosome breakage (Fig. 5). This process can also occur in the later cell cycles of the fusion product, if the donor chromosomes are delayed in undergoing anaphase segregation, forming micronuclei. When the micronuclei enter into the S-phase while the recipient genome is already in metaphase, the S-phase micronuclei can undergo PCC, and consequently DNA or chromosome breakage (Sperling, 1982). The released donor DNA may integrate into the recipient genome through transformation events, or repair processes. When genetic instability occurs during the callus phase, which is multicellular and heterogeneous in cell cycle stages, microprotoplast hybrids containing intact or modified donor chromosomes, and/or with integrated donor DNA or chromosome segments, might be recovered. The possible mechanisms for DNA integration in mitotic and meiotic cell cycle occurring in various plant species have been extensively discussed by Sybenga (1989, 1992). In generatively – produced addition lines, the integration of donor DNA/chromosome segments apparently occur by similar mechanisms: double strand breaks of the donor chromosome during its disintegration at some stage in meiosis/mitosis, followed by repair-induced invasion and integration of donor DNA into the host chromosomes (reviewed by Sybenga, 1992). In many instances of segment transfer in wheat after ionizing irradiation, interstitial inserts are observed, often of considerable size, which are probably the result of similar mechanisms.

The results also show that several plants expressed both Kan^R and GUS. Some plants regenerated from the same Kan^R callus (e.g. *S. tuberosum* (+) *L. peruvianum* fusions) did not express either Kan^R or GUS, probably due to the deletion of the donor chromo-

some/chromosome segment, or inactivation of the gene. Several microprotoplast hybrid plants derived from *S. tuberosum* (+) *L. peruvianum* fusions, which expressed Kan^R of GUS, contained the donor *S. tuberosum* chromosome as well as the npt II and GUS DNA sequences (as determined by Southern-blot hybridization), suggesting that the expression of the genes is linked to the presence of the donor chromosome. Many of the microprotoplast hybrid plants containing 24 *L. peruvianum* chromosomes and 1 potato chromosome (monosomic additions at the diploid level), or 48 *L. peruvianum* chromosomes and 1 potato chromosome (monosomic additions at the tetraploid level) appeared to be sufficiently male- and female-fertile, as adjudged by the production of several berries and of seed progeny obtained after backcrossing with different self-incompatible genotypes of *L. peruvianum* (results in progress). The progeny tests, which are being currently carried out for analysis of the transmission, showed that some plants were GUS-positive, indicating sexual transmission of the alien genes to the seed progeny.

Conclusions

The results obtained show that through microprotoplast fusion technique, it is possible to transfer single, chromosomes and alien genes between sexually-incongruent species. This technique offers some unique advantages for alien gene transfer, genome analysis and gene cloning, as outlined below.

1. Transfer of desirable traits, e.g. from wild to the cultivated species. Important traits like disease- or stress-resistance, encoded by polygenes, might be clustered within blocks or scattered throughout the genome. In this regard, microprotoplast fusion can be a useful method for the transfer of clustered resistance genes. Also, the transfer of individual chromosomes from non-hybridizing wild to cultivated species by this technique provides perspectives for the transfer, localization and inheritance of non-host resistance genes.
2. Production of monosomic or disomic addition lines in a single step, thus avoiding time-consuming back-cross or self-pollination generations necessary to obtain such lines by generative methods. From monosomic addition lines, it is possible to obtain substitution or recombinant lines, including those with recombinant chromosomes (intergenomic translocations) after backcrossing or selfing, depending upon the degree of homoeologous pairing between the donor and recipient chromosomes (Sybenga, 1992; Parokonny et al., 1992; Jacobsen et al., 1994). Thus, the transfer of chromosomes from non-hybridizing, but related wild species to the cultivated species makes it feasible to obtain a greater insight into genome organization/evolution of the species, genome/chromosome homoeology and somatic genome compatibility, which are important for introgressive breeding (Sybenga, 1992; Sybenga et al., 1994; de Jong et al., 1993; Jacobsen et al., 1994; Wolters et al., 1994).
3. Construction of chromosome-specific DNA libraries. Genome analysis and molecular plant breeding concepts rest on dense linkage maps based on RFLPs. A high marker saturation, i.e., 1cM or less, is not readily attainable with customary techniques involving random selection of recombinant DNA for RFLPs from shot-gun libraries. It can be substantially facilitated by directly cloning DNA from individual chromosomes or chromosome segments, either by microdissection *via* micromanipulation using glass needles or laser optical trapping, or by flow cytometric sorting of metaphase chromosomes and amplification by PCR (Fukui et al., 1992; Jung et al., 1992; Wang et al., 1992; Schubert et al., 1993; Lucretti et al., 1993; Schondelmaier et al., 1993; Arumuganathan et al., 1994), using monosomic, disomic, telosomic or ditelosomic additions. Chromosome additions of various kinds can be produced, through microprotoplast fusion, in new genetic backgrounds with marked differences in the karyotype/DNA content from the recipient chromosomes.
4. Studies of the three-dimensional structure of chromosomes and spatial arrangement of the donor chromosomes and their relationship with stability of gene expression and transmission (Gleba et al., 1987; Appels, 1989; Heslop-Harrison & Bennett, 1990; Nanninga et al., 1992; Oud & Nanninga, 1992; Montijn et al., 1994). Interspecific or intergeneric microprotoplast hybrid plants with one or a few chromosomes carrying alien genes facilitate an efficient analysis of the fate of introduced genes (deletion, inactivation or co-suppression, structural alteration) in somatic and generative cycles, because the transferred chromosomes can be identified using molecular cytogenetic methods (genomic *in situ* hybridization) and

the chromosome observations can be linked to the presence or absence of the genes.

5. Gene localization on the chromosome. Genes can be localized if a sufficient population of addition lines is produced by microprotoplast fusion. Monosomic addition and recombinant lines can be useful as mapping tools to assign RFLP markers to specific regions on chromosome arms (Islam & Shepherd, 1991; Rogowsky et al., 1991).

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