Theor Appl Genet (2000) 101:1250–1258

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Characterization and molecular analysis of transgenic plants obtained by microprotoplast fusion in sunflower

Received: 21 December 1999 / Accepted: 25 March 2000

Abstract Asymmetric somatic hybrid (ASH) plants were obtained by PEG-mediated mass fusion of microprotoplasts from perennial *Helianthus* species and hypocotyl protoplasts of *Helianthus annuus*. The formation of micronuclei in perennial sunflower cell cultures was induced, at early log phase, by addition of the herbicides amiprophos-methyl or oryzalin. Sub-diploid microprotoplasts were isolated by high-speed centrifugation and the smallest enriched by sequential filtration through nylon sieves of decreasing pore size. Fusion products were cultured and the regenerated plants phenotypically, genetically and cytologically characterized. DNA analysis using RAPD markers revealed that 28 out of 53 regenerated plants were asymmetric hybrids. Subsequent nuclear-DNA flow cytometric analysis showed that these plants had a higher DNA content than the receptor *H. annuus*, suggesting that they represented addition lines. Cytological investigation of the metaphase cells of 16 hybrids revealed an addition of 2–8 extra chromosomes in these plants. The phenotype of most ASH plants resembled *H. annuus*. These results indicate that micronuclear induction and asymmetric somatic hybridization represent a potent tool for partial genome transfer aimed at the specific transfer of economically important traits in breeding programs.

Keywords Micronuclei · Microprotoplasts · Chromosome transfer · RAPD · *Helianthus*

Communicated by K. Glimelius

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Introduction

Sunflower (*Helianthus annuus* L.) is an important oil crop and there is a great interest in the quality improvement and stable production of existing sunflower cultivars. This is hampered by their susceptibility to pests and diseases (Fick and Miller 1997; Degener et al. 1999). The transfer of desirable traits in *H. annuus* from related wild species by interspecific crossing is difficult, due to sexual incompatibility and sterility of the hybrids (Atlagic et al. 1995). On the other hand, genetic manipulation and asexual techniques applicable to somatic cells, enable the transfer of genes across sexual borders between related or taxonomically distant species (Sybenga 1992; Rutgers et al. 1997).

Somatic hybridization is potentially useful for the transfer of desirable traits, especially those controlled by polygenes or uncloned genes, from wild species to crop plants, and to generate novel gene combinations, circumventing sexual-crossing barriers (Ramulu et al. 1996; Rutgers et al. 1997). This relatively simple technique, can be applied to most species, but somatic hybrids frequently have a complex genetic constitution and many back-crosses are required for the establishment of a new cultivar. In addition, these hybrids frequently suffer from chromosome instability and a high degree of sterility (Sybenga 1992). In such cases, asymmetric somatic hybridization using microprotoplasts offers the possibility for a more stable and directed gene transfer by introducing only one or a few specific chromosomes from the donor to the recipient genome (Verhoeven et al. 1991; Ramulu et al. 1995; Rutgers et al. 1997).

Initially, micronuclear induction in plants was demonstrated in *Solanum tuberosum*, *Daucus carota*, and *Nicotiana plumbaginifolia* (Morejohn et al. 1987; Verhoeven et al. 1990; Ramulu et al. 1994). Herbicides like amiprophos-methyl (APM) and oryzalin (ORY) are considered as tubulin-binding substances in plant cells, inhibiting microtubule formation and microtubule-dependent processes (Morejohn et al. 1987; Ramulu et al. 1994). Application during mitosis thus leads to de-polymerization of the spindle, and metaphase chromosomes do not

separate into two sister chromatids (Krioutchokova and Onishchenko 1998). Finally the chromosomes become scattered throughout the cell and nuclear envelops are formed, resulting in micronucleated cells after 12 to 24 h of incubation (Verhoeven et al. 1990; Ramulu et al. 1995).

Successful micronuclei induction and isolation of microprotoplasts for asymmetric somatic hybridization has been carried out between species of the genera *Solanum, Lycopersicon* and *Nicotiana* (Ramulu et al. 1995, 1996; Rutgers et al. 1997). For *Helianthus* species, so far, only symmetric somatic hybridization has been reported (Krasnyanski and Menczel 1995; Henn et al. 1998). Here we report the results of asymmetric somatic hybridization between *Helianthus* species and the regeneration of fertile asymmetric somatic hybrid plants. For identification of the transgenic plants at the molecular level, random amplified polymorphic DNA (RAPD) analysis was performed. In addition, the nuclear and chromosome compositions of the partial hybrids were analyzed by flow cytometry and cytological analysis.

Material and methods

Plant material, micronuclei induction and recipient protoplast isolation

Protoplasts of the perennial sunflower species *Helianthus giganteus* L. and *Helianthus maximiliani* L., from in vitro culture (Imhoff et al. 1996), were isolated and cultivated as described earlier (Binsfeld et al. 1999a). Three days after the start of cultivation, in early log-phase, the microtubule inhibitors amiprophos-methyl (APM) [O-methyl-O-O-(-4-methyil-6-nitrophenyl)-N-isopropyl-phosphoro thioamidate] and oryzalin (ORY) (3,5-dinitro-N^4,N^4-dipropylsulfanilamide) were added. APM or ORY were applied at 10, 20, 40, 60, 90 and 120 µM for 12, 24, 36, 48, 72 and 96 h. Cytochalasin-B (CB) (20 µM) was applied additionally to the cells, 24 h after treatment with the anti-mitotics. Protoplasts of the cultivar Florom-328 (*H. annuus* L.) were used as receptor cells of the microprotoplasts. The receptor protoplasts were isolated as described by Schmitz and Schnabl (1989).

Isolation and enrichment of the donor microprotoplasts

Forty eight hours after the addition of APM or ORY, the cells were incubated, without shaking, for 4 h at 24°C in a cell wall-digesting enzyme mixture (3% cellulase Onozuka R-10, Serva; 1% macerozyme Onozuka R-10, Serva; 1% bovine serum albumin) in 4 ml of salt solution (336 mM KCl, 16 mM $CaCl_2$ and 3 mM MES, pH 5.6, 600 mosmol kg^{-1}) containing additionally 20 µM of CB and 60 µM of APM. Subsequently the protoplast suspension was purified, as described by Schmitz and Schnabl (1989). Before isolation, the purified micronucleated protoplasts were treated with 10 mM of spermidine, 20 µM of CB and 60 µM of APM and incubated for 4 h at 4°C in 5 ml of CPW5M medium (Fakhrai et al. 1990). Isolation of microprotoplasts was done as described by Verhoeven et al. (1991) and Ramulu et al. (1993). The bands obtained by ultracentrifugation were sequentially filtered through nylon sieves of decreasing pore sizes (30, 20, 15, 10 and 5 µm) (Wilson Sieves, England) and 12- and 3-µm sieves of Millicell (PCF, Millipore, Germany).

Fusion between microprotoplasts and hypocotyl protoplasts, cultivation and plant regeneration

Microprotoplasts and hypocotyl protoplasts were fused using a modified protocol for polyethylene glycol (PEG)-based mass fusion (Ramulu et al. 1995; Henn et al. 1998). Microprotoplasts and protoplasts were mixed 1:1; 2:1 and 3:1, using a density of 1×10^6 protoplasts as a basis (1 ml). The fusion was carried out in Petri dishes (diameter 10 cm) in 1 ml of fusion solution containing 8% (w/v) PEG_{6000}, 90 mM manitol, 60 mM $CaCl_2$, 25 mM glycine and 6% dimethyl sulfoxide (DMSO). After 8 min at RT, the fusion solution was partially removed, 15 ml of buffer solution (240 mM KCl, 100 mM $CaCl_2$, 3 mM morpholinoethane sulfonic acid, pH 7.0) was slowly added and the protoplasts incubated for 20 min at RT. Subsequently, the protoplasts were counted and cultured in KMAR medium (Binsfeld et al. 1999a) as described by Wingender et al. (1996), and plant regeneration obtained as described by Henn et al. (1998).

Nuclear DNA preparation and RAPD analysis

Total DNA was extracted from young fresh leaves of 53 regenerated plants from different and independent calli and their respective parent plants, subjected to RAPD-PCR and analyzed as described by Binsfeld et al. (1999b). All 20 primers used were 10-mer random oligonucleotide sequences, series P1, P2, P3, P5, P7 obtained from Pharmacia (Biotech, FRG) and I04, I10, I11, B1, B5, B12, B18, D1, D3, D13, D20, P160–2, P160–6, P170–4, P170–10 from Roth Random Primer (Roth FRG).

Cytological and flow-cytometric analysis (FCA)

Cells were stained with 10 mM 4,6-diamidino-2-phenylindole (DAPI) for 5 min and micronuclei counted under a UV microscope (Olympus-BH2-PM10-AD). A minimum of 200 cells were counted for each treatment. The relative DNA content of the microprotoplasts, together with the ploidy level and relative DNA content of interphase nuclei of the parents and eight asymmetric somatic hybrid (ASH) plants, was determined by FCA as described (Ramulu et al. 1993; Nagl and Treviranus 1995; Ayele et al. 1996). For isolation of nuclei, 10 µl of freshly isolated protoplasts were incubated for 5 min in 1 ml of chopping solution (sol. A, Partec GmbH kit) and stained with 2 ml of DAPI solution (sol. B, Partec GmbH kit) for 5 min. As a standard, nuclei of *Petunia hybrida* cv F_1 hybrid ''Hit parade blau'' were used (2 C = 2.85 pg, Nagl and Treviranus 1995). The samples were measured on a Partec CA-III flow cytometer (Partec, Münster, FRG), equipped with a HBO-100 mercury high-pressure lamp, with the UG1 excitation filter, TK420, TK560 dicroic mirrors and a CG435 longpass filter. The software DPAC (Data pool application for cytometry, Partec) was used for the calculation of CV-values and evaluation of the diagrams of relative DNA content.

Chromosome preparation

For chromosome analysis, root-tips from 24 greenhouse-grown, asymmetric somatic hybrid (ASH) plants and their parents were collected and pre-treated in an aqueous solution (2 ml) of 2.5 mM 8-hydroxyquinoline for 3 h at 4°C, fixed in (2 ml) 3:1 (v/v) ethanol-acetic acid for 36 h at 4°C, and stored in (2 ml) 70% ethanol at 4°C. Before maceration, the root tips were incubated in 100 µl of enzyme mixture (4% cellulase, Onozuka R-10, Serva, 1% pectolyase Y-23, Seishim Pharmaceutical, 75 mM KCl, pH 4.0; Kakeda et al. 1991) for 30 min at 37°C. They were squashed in 45% acetic acid and stained with DAPI (10 mM) and carmine acetic acid (50%). Photographs were taken using a computer-assisted cooled CCD camera (Photometrics).

Results

Micronucleation

The cultivation of competent and synchronized (G_0/G_1) freshly isolated protoplasts, resulted in a high synchro-

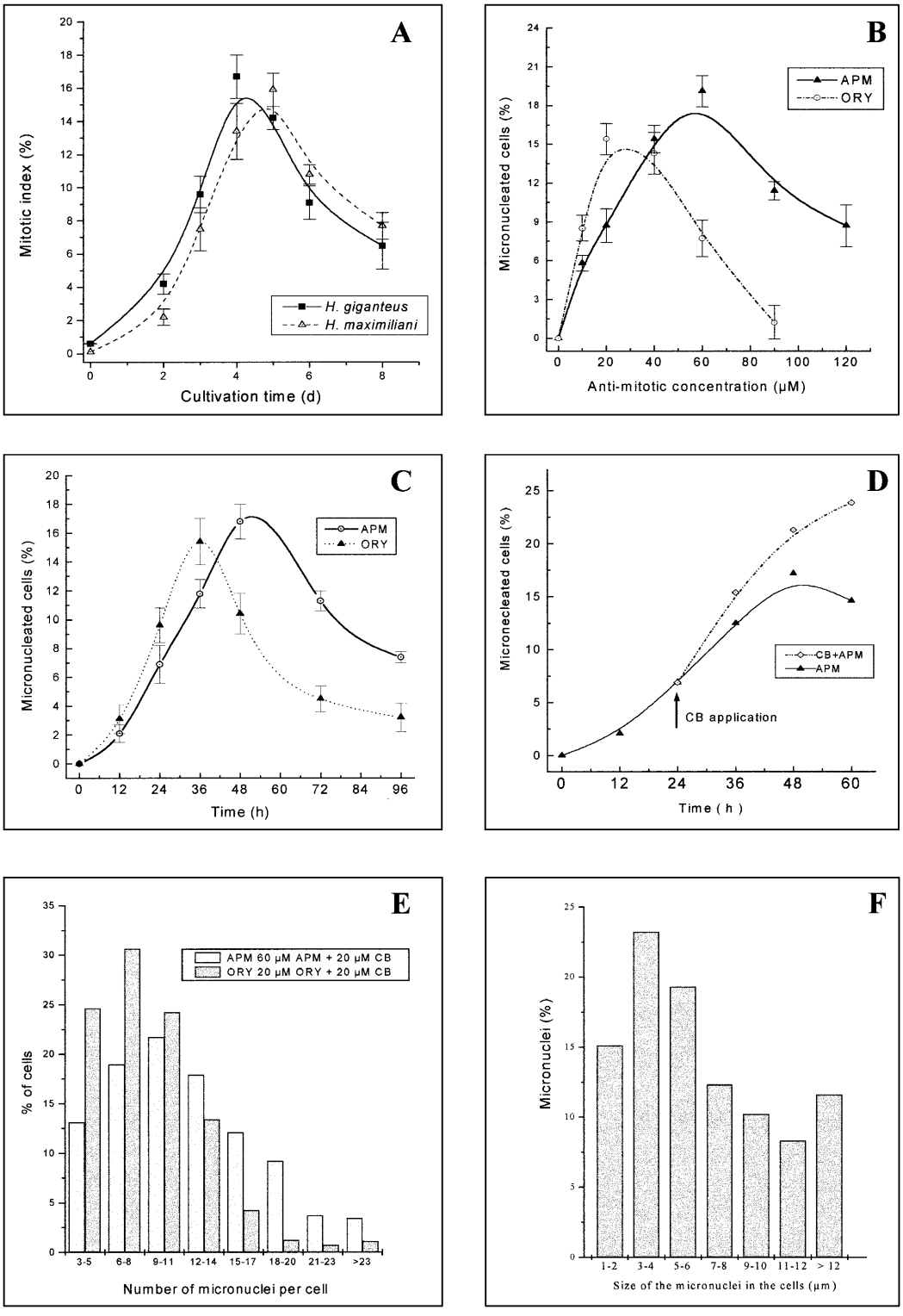

Fig. 1 A–D Process of micronucleation: **A** Percentage of cells in a mitotic state, after initial cultivation; **B** effect of the concentration of the anti-mitotic toxins on the percentage of micronucleated cells of *H. giganteus*; **C** effect of the cultivation time after application of anti-mitotic toxins on the percentage of micronucleated cells; **D** effect of Cytochalasin-B (*CB*) and APM on the percentage of micronucleated cells; **E** percentage distribution of the micronucleated cells in relation to the number of micronuclei per cell; **F** percentage of the micronuclei in relation to their size, before isolation

nized cell growth during the first week in culture (Binsfeld et al. 1999a). As depicted in Fig. 1 A the highest mitotic index (17%) was reached on the 4th day of cultivation. This information was a pre-requisite for testing doses and the timing of supplementation with anti-mitotic toxins (ORY and APM), and provided the first step for an efficient induction of micronuclei in the *Helianthus* suspension-cell culture. On the 4th day of culti-

Table 1 Yield and size of sub-diploid microprotoplasts obtained after ultracentrifugation

Bands formed after ultracentrifugation	Type of micronuclei (%) [a] A ≥ 5 µm	B 6–9 µm	C ≤ 9 µm	Yield of microprotoplasts (× 10^5) (%)	
Large band	10.4	21.7	67.9	6.8	62.4
First small band	19.6	34.4	46.0	2.1	19.3
Second small band	36.5	43.2	20.3	1.3	11.9
Third small band	60.4	33.7	5.9	0.7	6.4
Total	–	–	–	10.9	100.0

[a] n = 630 micronuclei

vation APM or ORY were added at final concentrations of 0–120 µM (Fig. 1B). The most-efficient treatment was obtained with 60 µM of APM yielding 19.2% micronucleated cells, while ORY (at 20, 40 µM) induced significantly ($P < 0.05$) less micronuclei in *Helianthus* cells than APM (Fig. 1B). Treatments with higher concentrations of APM or ORY led to irregular nuclear membrane formation, deformation, a significantly lower number of micronuclei per cell and also to cell death. Lower concentrations were less effective on chromosome scattering and micronuclei formation resulting in larger micronuclei with more chromosomes and a reduced number of micronuclei per cell. Optimal micronucleation was obtained after 36 and 48 h in the presence of ORY and APM respectively (Fig. 1C). CB application (24 h) to micronucleated cells resulted in a significant ($P < 0,01$) increase (23.9%) of micronucleated cells with respect to the control (14.8%) (Fig. 1D). CB alone did not induce micronuclei, but had a stimulatory effect as well as stabilizing and maintaining function, preventing the restitution or fusion of the micronuclei. The efficiency of the method was calculated on the basis of the percent micronucleated cells and on the number of micronuclei per cell. Irrespective of the parental genotype the number of micronuclei per cell varied from 3 to 29 (Fig. 1 E) with an average of 8.4 and 11.6 micronuclei per cell upon ORY or APM treatment. As compared to untreated diploid *Helianthus* cells possessing 34 chromosomes, the mean numbers of chromosomes per micronucleus were 3.7 and 2.8 for the ORY and APM treatments respectively. The size of the micronuclei varied from 1 to 12 µm, with a high percentage (58%) of micronuclei smaller than 6 µm (Fig. 1F).

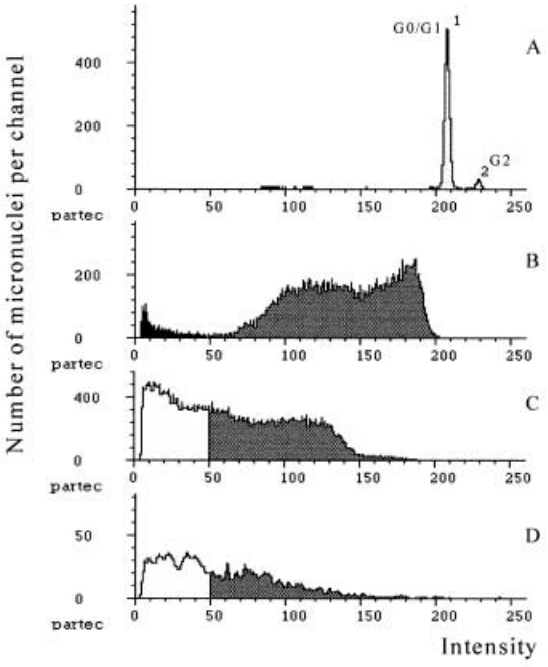

Fig. 2A–D Relative DNA content of interphase nuclei of freshly isolated protoplasts and micronuclei of *H. giganteus*: **A** normal diploid (2n = 34 chromosomes) protoplasts; **B** microprotoplasts after sequential filtration through sieves of 30-, 20-, 15- and 12-µm; **C** microprotoplasts after filtration through sieves with a 10- and 5-µm pore size and **D** microprotoplasts obtained after filtration through sieves of 3-µm pore size

Isolation and enrichment of microprotoplasts

In order to achieve optimal yields and stabilization of the microprotoplasts, the addition of APM (60 µM) and CB (20 µM) during protoplasting, together with cold treatment and spermidine supplementation, were found to be crucial. Ultracentrifugation of undigested cells resulted in 90% less microprotoplasts while omission of APM and CB reduced the average number of micronuclei per cell to more than 25% and the yield of microprotoplasts to about 40%. After ultracentrifugation two large and three small bands were formed from top to bottom in the Percoll gradient at various distances (usually from 15 to 30 mm). The bands were separately analyzed and the microprotoplasts classified (Table 1). Type A was found predominantly (60.4%) in the third small band, type B (43.2%) in the second small band, and type C (46.0 and 67.9%) in the first small and the large band respectively. The yield of non-enriched (selected) microprotoplasts from about 1.5×10^6 packed cells used per experiment, ranged from 1.0 to 2.0×10^6. After enrichment it was possible to recover small sub-diploid microprotoplasts on a mass scale ($2.0–10.0\times10^5$) with more than 70% being DAPI-positive. Flow-cytometric analysis of control cells (Fig. 2A), with 2n = 34 chromosomes, and sequentially filtered microprotoplasts (Fig. 2B, C, D), revealed a positive correlation ($r = 0.91$) between microprotoplast size and DNA amount confirming an efficient enrichment procedure.

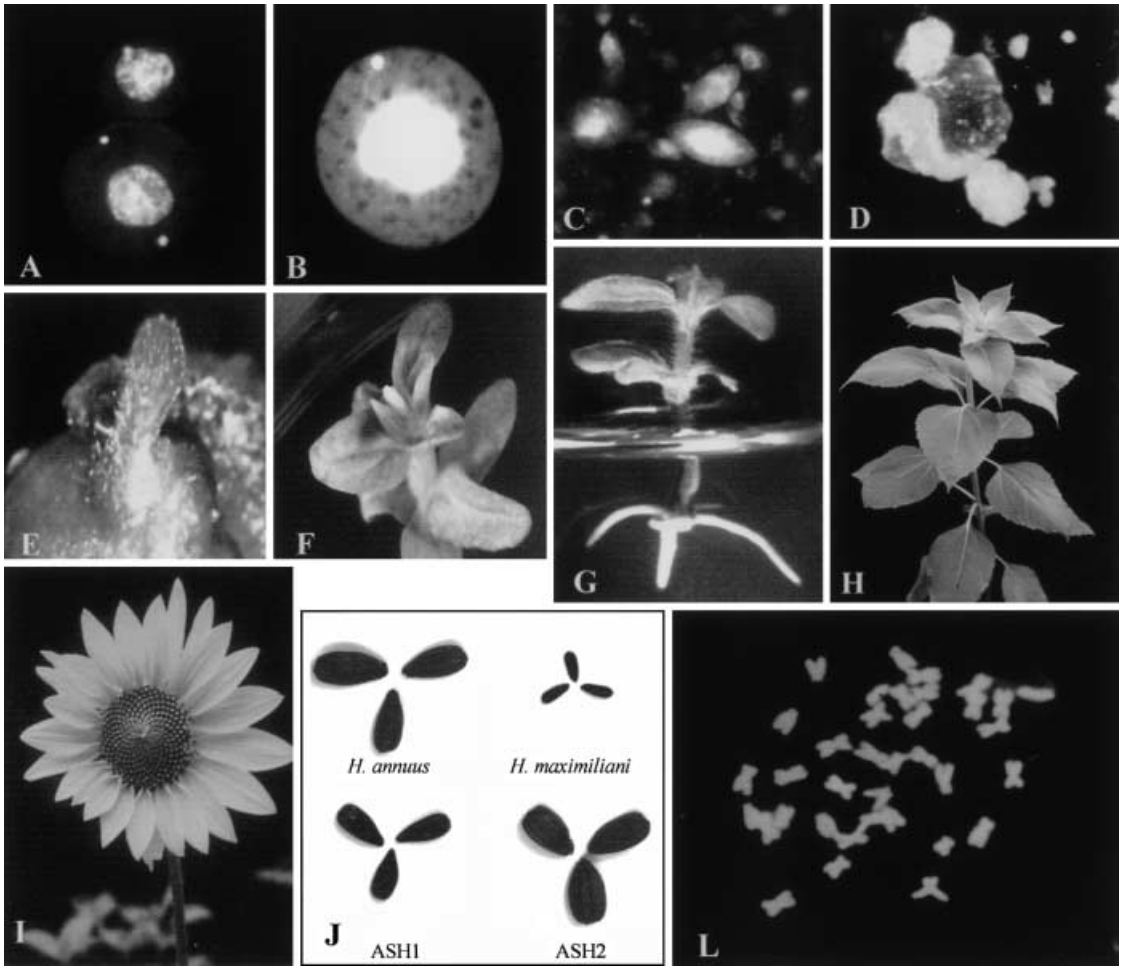

Fig. 3A–L Plant regeneration of asymmetric somatic hybrid (*ASH*) plants after fusion between donor microprotoplasts of *H. giganteus* or *H. maximiliani* with hypocotyl protoplasts of *H. annuus*. **A** Asymmetric fusion product with two small micronuclei incorporated in the receptor cell, stained with the DNA-fluorochrome DAPI, **B** ideal asymmetric fusion with one micronucleus in the receptor protoplast stained with the DNA-fluorochrome DAPI, **C** different types of microcallus development in agarose droplets after 4 weeks in culture, **D** green calli showing the initial differentiation process, **E** globular calli showing shoot bud formation, **F** shoot elongation, **G** root development of regenerated shoots, **H** acclimatized regenerated ASH plants in the greenhouse, **I** flowering ASH plants, with a varied form of the ray flowers, **J** seed obtained from parent and ASH plants **L** metaphase chromosomes of a ASH plant, stained with the DNA-fluorochrome DAPI, showing the presence of two chromosomes (34+2) in addition

Fusion, cultivation and plant regeneration

The percentage of fusion products in different microprotoplast : protoplast ratios (1:1; 2:1 and 3:1) varied from 12.7 to 17.9%, and were not significantly different from each other. In general a 2:1 ratio was used because increased hypocotyl protoplast fusions were obtained with less microprotoplasts and multiple fusions with more. The fusion of several microprotoplasts with one protoplast, could also be avoided by reducing the fusion time from 15 min to 8 min. The identification of the partial heterokaryons was only possible if the fusion products were stained with DAPI (Fig. 3A, B).

Subsequently, about 18–20% of the fusion products were recovered and embedded in agarose droplets at an initial density of 2.6×10^4 protoplasts ml^{-1}. Generally, cell division started after 4–5 days and in 10 days a division frequency of 50–55% with respect to control protoplasts was obtained. From these cell clusters, 2 weeks later, approximately 17% formed cell colonies (Fig. 3C).

After transfer to solid differentiation medium 3–4.5% of the microcalli (0.5–1 mm) showed further development forming about 18% mophogenic calli with green globular differentiation spots (Fig. 3D) after 2 weeks. Upon subcultivation for 4 weeks on medium without growth regulators, adventitious buds, with leaf primordia and small shoots, were regenerated (Fig. 3E). Elongated shoots (Fig. 3F) were cut and treated with NAA, yielding 32% plantlets (Fig. 3G). Three-weeks later, plants with well-developed roots were transplanted in pots and acclimatized, with a survival rate of more than 90%. Subsequently, the plants were transferred to the greenhouse for growth and seed production (Fig. 3H). The number of seeds produced varied from 1 to 16 in seed-producing plants.

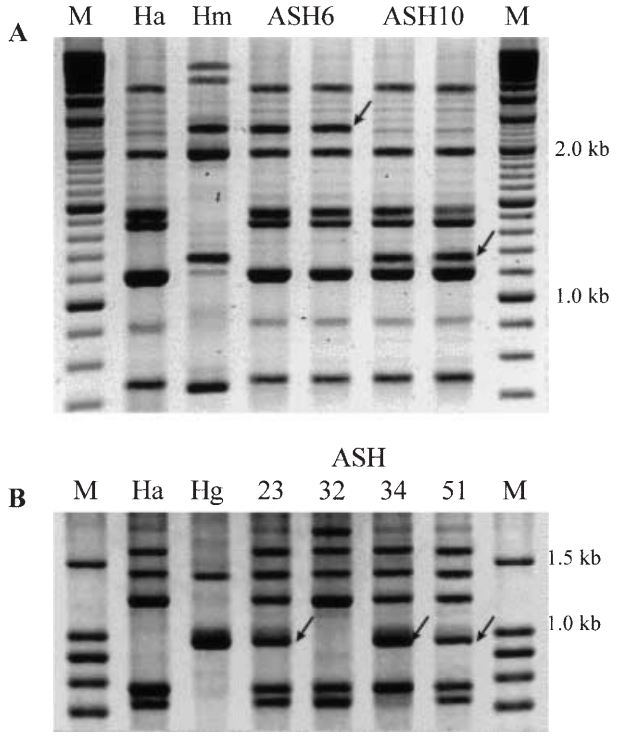

Fig. 4A, B RAPD analysis of regenerated asymmetric somatic hybrid (ASH) plants compared to the parent genotypes. **A** *Ha* – patterns of receptor genotype *H. annuus*, *Hm* – patterns of the donor species *H. maximiliani* and *ASH6* and *ASH10* with an additional band (*arrows*) of the donor species (Primer 160–6). *M* – size marker, 100-bp ladder. **B** *Ha* – patterns of the receptor genotype *H. annuus*, *Hg* – patterns of the donor species *H. giganteus* and respective ASH plants showing additional bands (*arrows*) of the donor species (Primer P1)

Genome analysis of regenerated plants

Twenty RAPD primers revealed polymorphisms between the recipient, the donors and the regenerated ASH plants. The presence of one or several specific bands of the donor species in the banding patterns of ASH plants indicated clearly the presence of an alien genome. Primer P160–6 gave rise to very different banding patterns for the parental plants *H. annuus* and *H. maximiliani* (Fig. 4A, lanes 2 and 3) and generated a banding pattern typical of the receptor genotype with an additional band (arrow) from the donor genotype for ASH6 (lanes 4 and 5). The same was true for ASH10 (lanes 6 and 7), although a different alien amplification product (arrow) was found. Figure 4B (Primer P1) shows the typical parental banding patterns of *H. annuus* and *H. giganteus* and of four regenerated ASH plants (ASH23, 32, 34 and 51). Comparison in these cases revealed that a *H. giganteus*-specific amplification product was obtained in the regenerated plants. Out of 53 regenerated plants, 28 (53%) were found to contain donor-specific amplification products with at least one of the tested primers.

Table 2 The mean of 2 C DNA values and the coefficient of variation (CV) of mesophyll nuclei of parents and RAPD-confirmed asymmetric somatic hybrids

Genotypes	Genome size 2 C (pg)	CV	Polymorphic bands (*n*)[a]
P. hybrida	2.85	2.69	–
H. annuus	7.36	2.49	–
H. giganteus	10.17	1.34	–
H. maximiliani	10.62	1.63	–
ASH3	7.86	1.28	1
ASH11	8.44	2.63	4
ASH13	7.96	4.21	n.d.
ASH19	8.01	1.85	6
ASH25	8.71	3.09	8
ASH27	8.13	2.04	3
ASH28	8.31	2.54	2
ASH31	8.22	1.93	4

[a] No. of polymorphic RAPD markers verified on the ASH plants in relation to the band profile of the receptor plant (cv Florom-328)

Nuclear DNA measurements

The lowest genome size was measured in *H. annuus* with a mean of 7.36 pg per nucleus, and the highest values were found in *H. giganteus* and *H. maximiliani* with 10.17 and 10.62 pg per nucleus respectively (Table 2). Although the number of chromosomes is the same for *H. annuus* and the perennial *Helianthus* species the latter contained about 40% more DNA per nucleus than cultivated sunflower. The DNA content of the regenerated ASH plants was significantly (according to the *t*-test) different from *H. annuus* and ranged from 7.86 to 8.71 pg. Out of the ASH plants analyzed ASH3 had the lowest genome size (7.86 pg) and, in addition, it exhibited the lowest number of polymorphisms with respect to *H. annuus*.

Chromosome analysis and morphological characterization

The somatic chromosome number in root-tip cells of 22 ASH plants, confirmed by RAPD analysis, could be clearly determined. Six of these plants showed 2n = 34 chromosomes, i.e., the same number as the control (*H. annuus*). In 16 ASH plants, 2–8 additional (alien) chromosomes were found in the genome, and the most frequent chromosome number was 38 (34+4), an addition of four alien chromosomes. The cell shown in Fig. 3L, contained 2n = 36 chromosomes, with the full chromosome set of the receptor plant and two chromosomes in addition.

Several ASH plants exhibited morphological abnormalities: reduced growth, leaf-shape alteration, serrated leaves, early flowering, more than one flower bud per plant or variation of the ray flowers (Fig. 3I). The seeds (Fig. 3J) resembled those of *H. annuus*. A correlation between the presence of a high number of alien chromosomes (2n = 34+8) and morphological alterations could

be observed. Some ASH plants were more robust and differed from the growth habit of *H. annuus*.

Discussion

Production of asymmetric somatic hybrids (ASH) plants

In this study, we show that partial genome transfer by microprotoplast fusion could be achieved even in a recalcitrant genus like *Helianthus*. Pre-requisites for successful asymmetric somatic hybridization, using microprotoplasts, include micronuclei induction, isolation of microprotoplasts, an appropriate fusion method and an efficient regeneration procedure. For micronucleation not only a high mitotic index and metaphase arrest with well-scattered single chromosomes but also the formation of stable nuclear membranes around these chromosomes is important (Ramulu et al. 1994). As has been shown for *N. plumbaginifolia* (Verhoeven et al. 1990) both ORY and APM were well suited for metaphase arrest and micronucleation in *Helianthus* suspension cells. APM treatment was, however, superior to ORY resulting in a higher percentage of micronucleated cells and a lower number of chromosomes per micronucleus. In addition it was found to be less toxic than ORY, which seems to be a common phenomenon (potato, tobacco, Ramulu et al. 1995; *Haemanthus katherinae,* carrot, Morejohn et al. 1987). While micronuclei induced by ORY tended to fuse and formed restitution nuclei, those induced by APM persisted much longer suggesting a retarding effect of APM on the cell cycle. In accordance with Verhoeven et al. (1990) and Ramulu et al. (1993) the application of CB was indispensable for a satisfactory induction and maintenance of micronuclei in *Helianthus* cells. So far no cold treatment or spermidine supplementation has been reported for microprotoplast isolation; these treatment did not contribute to direct micronuclei induction, but they might have an important impact on condensation and stabilization of the micronuclei and their membrane system. Unlike other reports the cell suspensions used for this study originated from cultivated protoplasts (Binsfeld et al. 1999a) which were found to represent a good source for mitotic, active cells. Established cell cultures were less suited since they tended to polyploidization (unpublished).

Our results support the suggested conclusions of Ramulu et al. (1993) and Krioutchokova and Onishchenko (1998) that CB disrupts the microfilaments, while anti-mitotic toxins inhibit the re-formation of microtubules in micronucleated cells, and spermidine acts on membrane stabilization. This treatment increased the yield of isolated microprotoplasts two-fold and maintained the micronuclei in a stable state without fusion or restitution of the nucleus. Due to the action of APM and CB, the protoplasts were completely devoid of a cytoskeleton providing the basis for the isolation of microprotoplasts in a gravity field. According to their specific density, microprotoplasts, consisting of one micronucleus and a thin layer of cytoplasm surrounded by a plasma membrane, separated upon centrifugation. Flow-cytometric and cytological analysis revealed that sequential filtration through nylon sieves of decreasing pore size separated small microprotoplasts from large ones, as already reported by Ramulu et al. (1993).

The sensitivity of microprotoplasts and receptor hypocotyl protoplasts to DMSO and PEG was very high leading to severe damage. The use of lower DMSO (6%) and PEG (8%) concentrations than reported for symmetric fusion (Krasnyanski and Menczel 1995; Henn et al. 1998) favoured cell viability with concomitant doubling of the yield of fusates. It remains to be tested whether even lower PEG and DMSO concentrations might result in less damaged cells, and concomitantly in a higher recovery of fusates as well as a better division potential.

The plating efficiency, calculated from the total embedded fusates, was 3–4%; these rates were comparable to the results reported by Wingender et al. (1996) for different *H. annuus* cultivars and by Henn et al. (1998) for symmetric somatic hybridization between *Helianthus* species. The percentage of shoot differentiation was comparable to Wingender et al. (1996) but lower than that reported by Henn et al (1998), suggesting that the regeneration capacity is positively affected by the heterokaryon protoplasts.

Characterization of asymmetric somatic hybrid (ASH) plants

Based on RAPD analysis, more than half (53%) of the regenerated plants were found to represent asymmetric somatic hybrids containing additional genomic DNA from the microprotoplast donor species. Taken into account that the fusion rate ranged between 12.7 and 17.9% the question arises how such a high percentage of hybrids could be obtained without applying any selection. This interesting phenomenon may be due to a heterotic effect, observed in somatic hybrids of some species like *Solanum* (Cardi et al. 1993), and *Helianthus* (Henn et al. 1998) as well as leek (+) onion somatic hybrids (Buitenveld et al. 1998). Compared to other reports on the identification of fusion products by RAPD analysis (Takemori et al. 1994; Henn et al. 1998; Krasnyanski et al. 1998), in the present study more primers had to be used to identify the smaller amount of DNA transferred from the microprotoplasts. The selection of primers was such that they generated complementary polymorphic bands for both parents, permitting an easy confirmation of the hybrid character of the regenerated plants. In our study, the RAPD markers fulfilled the criteria to identify regenerated partial hybrids; it can not however be ruled out that even more ASH plants could have been detected using more primers.

The presence of an alien genome in the regenerated ASH plants could also be confirmed by chromosome counting and by flow-cytometric analysis. No variation at the ploidy level of the eight examined ASH plants was detected but the presence of additional genomic DNA was

apparent by an increment of the total amount of DNA measured in the respective plants. Similar results were reported for *Brassica* somatic hybrids (Fahleson et al. 1988), showing a high correlation between chromosome number and the DNA content of the somatic hybrids. The precise detection of differences in DNA content between two different plants depended on the peak resolution or variation (coefficient of variation = CV) (Ulrich and Ulrich 1991) with a high CV requiring a large difference in DNA content (Fahleson et al. 1988; Nagl and Treviranus 1995). For all analyzed ASH plants, however, the additional DNA content was higher than 0.18 pg (CV of *H. annuus*) and thus differences in DNA content were significant (Table 2). These results demonstrate that FCA is a suitable procedure for the identification of somatic hybrids, even with a small amount of additional genomic DNA.

Mitotic chromosome counting of ASH plants, enabled the identification of addition lines, aneuploids and cytochimeras. On this basis 75% ASH plants contained two or more added chromosomes. The six ASH plants with 2n = 34 chromosomes, might have resulted from recombinant chromosomes due to reciprocal, interstitial translocation, or introgression. The occurrence of intergenomic translocations is a common phenomenon in somatic hybrids (Cardi et al. 1993), asymmetric hybrids obtained by irradiation of the donor cells (Piastuch and Bate 1990), and in microprotoplast hybrids (Ramulu et al. 1996). Out of the 24 ASH plants analyzed, two cytochimeric ones were identified. As has been previously discussed (Sybenga 1992; Cardi et al. 1993), different mechanisms can probably give rise to mitotic chromosome pairing or unequal chromosome elimination during mitotic cell division. The even chromosome number, observed for most of the ASH plants, might be due to duplication of the donor chromosome during the first mitosis of the receptor protoplast. The donor microprotoplast originates from a cell in the G_2 phase (Ramulu et al. 1995), thus containing a perennial *Helianthus* chromosome with sister chromatids, while the recipient protoplast is in the G_1 phase. During the first DNA replication of the fusate, the alien chromosome is also duplicated giving rise to four chromatids per chromosome, followed by a centromere disjunction leading to two copies of a given chromosome.

One can speculate that, in the near future, partial genome transfer might be an elegant method to obtain transgenic plants. Direct transfer of a single chromosome via the microprotoplast technique between sexually incompatible *Helianthus* species might be a relevant tool to transfer polygenetically determined traits or alien genes, for the improvement and gene pool-extension of *H. annuus*.

Acknowledgments We thank Dr. O. Schrader for advice on *Helianthus* chromosome analysis; the Institute of Cereal and Industrial Plant Research, Fundulea, Romania, for providing *H. annuus* seeds; Bayer Mobay Corporation, Kansas, USA, for kindly providing the sample of amiprophos methyl; the DowElanco Chemical Corporation, Indianapolis, USA, for kindly providing the sample of oryzalin; and to the Deutsche Forschungsgemeinschaft for financial support.

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