Characterization of interspecific asymmetric somatic hybrids and their progeny in the genus *Helianthus*

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Summary

Asymmetric somatic hybrid plants (ASH) 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 herbicides, amiprophos-methyl or oryzalin. Sub-diploid microprotoplasts were isolated by high speed centrifugation and the smallest fraction 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 investigations of metaphase cells of 16 hybrids revealed an addition of 2 to 8 extra chromosomes in these plants. Sexual transmissibility of the alien genome to the progeny could also be observed. Homoeologous chromosome pairing, observed in meiotic multivalent pairing indicated that ASH can be used as a bridge for alien gene transfer. These results indicate that micronuclei induction and asymmetric somatic hybridization seems to represent a potent tool for partial genome transfer aiming at specific transfer of economically important traits in breeding programs.

***Keywords*:** Sunflower; protoplasts; microprotoplasts; meiotic behavior; flow cytometry; RAPD

Introduction

Cultivated sunflower has narrow genetic variability which limits the development of advanced ideotypes adapted to different agroecological conditions and having important agronomic traits. The large variety and pronounced variability within wild *Helianthus* species offers opportunities to increase genetic variability in the cultivated sunflower by using methods which provide gene flow across interspecific sexual barrier (Seiler and Riesberg 1997). At the moment, the most important aims in sunflower breeding are, disease and insect resistance, drought tolerance, high heterosis for seed and oil yield, salt tolerance and a change in the habitus of the plants (Fick & Miller 1997).

One of the several methods that have been developed for gene transfer across sexually incompatible plants is symmetric somatic hybridization (Henn et al. 1998, Buiteveld et al. 1998) or asymmetric somatic hybridization (Ramulu et al. 1995, Binsfeld 1999). Stress-tolerance and many pathogen resistance traits in wild sunflower species are polygenic controlled and the respective genes remain to be characterized. In this case, somatic hybridization is very attractive and recommended. However symmetric somatic hybrids often exhibit anomalies that leads to chromosome instability and a high frequency of sterility or the plants present a high number of undesired traits. On the other hand, asymmetric somatic hybridization using selected microprotoplasts offers an alternative for gene transfer of a specific part of the donor genome (Ramulu et al. 1995, Rutgers et al. 1997). The main aim of the present investigation was the characterization of asymmetric somatic hybrids and their progenies to verify genome stability and suitability of these plants in breeding programs.

Material and methods

Production of asymmetric somatic hybrids (ASH)

The production of ASH was done as schematically summarized in Figure 1. Protoplasts of the perennial sunflower species *Helianthus giganteus* and *H. maximiliani* (2n = 34), were isolated and cultivated as described (Binsfeld et al. 1999). They were used as the donor source for micronuclei induction and isolation of microprotoplasts. As recipient of the microprotoplasts we used the highly regenerable annual sunflower genotype Florom-328 (*Helianthus annuus* L.). The hypocotyl protoplasts were isolated, as described by Schmitz and Schnabl (1989). Micronuclei induction, microprotoplast isolation, asymmetric fusion and plant regeneration was done as described by Ramulu et al. (1995) and Binsfeld (1999).

Characterization of asymmetric somatic hybrids (ASH)

The ASH plants and their progeny were analyzed for (1) phenotypic and meiotic behavior, (2) genome and chromosomal constitution, (3) ploidy and DNA content and (4) fertility analysis. The variable analysis were performed in order to verify the genome stability and sexual transmissibility of the transferred alien genome.

For genome analysis, total DNA was extracted from young fresh leaves as given by Binsfeld (1999). For the RAPD reactions, 30 ng of plant DNA was used as a template in a final volume of 15 µl reaction buffer, using a method of Williams et al. (1990). The PCR products were separated in 1.5% agarose gels containing ethidium bromide (2,5 µM) in Tris-borate-EDTA (TBE) buffer and were photographed under UV light (302 nm).

The ploidy level and relative DNA content of interphase nuclei of the parental and by RAPD confirmed, regenerated ASH plants, was determined by FCA based on the method described by Nagl and Treviranus (1995) and Ayele et al. (1996) and Binsfeld (1999).

For mitotic and meiotic analysis, root-tip cells and flower buds from RAPD confirmed ASH plants, their progeny and respective parent plants were collected and pretreated in a aqueous solution of 2.5 mM 8-hydroxyquinoline for 3 h at 4°C and fixed in 3:1 (v/v) ethanol-acetic acid for 36 h at 4°C, and stored in 70% ethanol at 4°C. Before the maceration, the root tips were digested with an enzyme mixture of 4% cellulase (Onozuka R-10, Serva) and 1% pectolyase (Y-23, Seishim Pharmaceutical) in 75 mM KCl, at pH 4.0 (Kakeda et al. 1991), for 30 min at 37°C. All the samples were squashed in 45% acetic acid and for the staining of the samples, DAPI or carmine acetic acid was used. Photographs were taken using a computer-assisted cooled CCD camera (Photometrics).

Fertility was determined through pollen viability test, seed production and germination test. Pollen viability was determined using a method described by Alexander (1980), based on differential staining of viable and

nonviable pollen grains. The viable pollen were counted, and expressed as a percentage of the total pollen grains. Statistical analysis was performed using SANEST (Statistical analysis system) (Zonta et al. 1984).

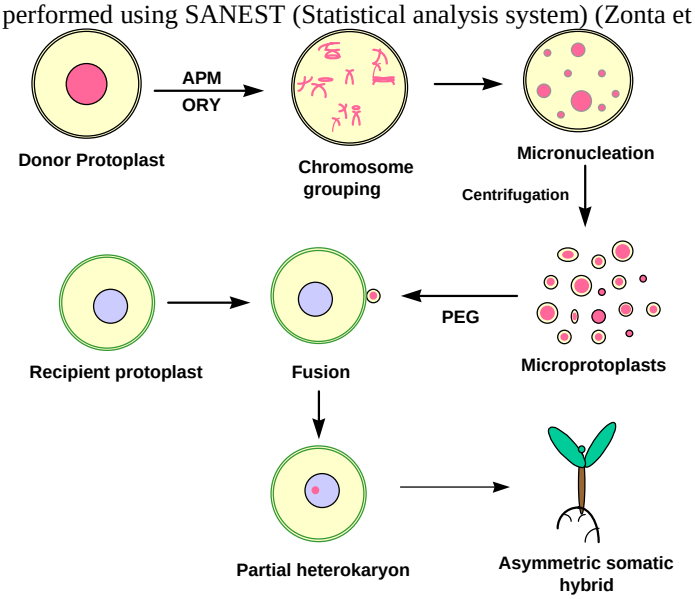

Figure 1. Schematic diagram, showing the basic steps in asymmetric somatic hybridization using microprotoplast strategy for partial genome transfer. After inducing micronuclei by treatment of donor cells with anti-mitotic toxins (APM, ORY), microprotoplasts can be isolated from micronucleated protoplasts and fused with the recipient protoplast order to regenerate asymmetric somatic hybrids.

Results and Discussion

The results obtained in this study show that microprotoplast fusion makes the production of ASH feasible, even for recalcitrant *Helianthus* species, aiming partial genome transfer. Pre-requisites for successful asymmetric somatic hybridization, using microprotoplasts, include an efficient micronuclei induction, isolation of microprotoplasts, appropriate fusion method and an efficient regeneration procedure. If these four pre-requisites are fulfilled, transgenic plants for mono- or polygenically controlled traits can be produced using, unidentified or uncloned genes from sexually incompatible species. As also reported by Ramulu et al. (1995), this technique allows the production of chromosome addition lines especially between plant species that present sexual incompatibility.

The results of micronuclei induction and microprotoplast isolation generally agreed with results obtained on *Nicotiana*, *Solanum* and *Lycopersicon* species (Verhoeven et al. 1991, Ramulu et al. 1995). This might suggest that micronuclei induction can be generally achieved in plant species that exhibit a high binding affinity to spindle toxins leading to inhibition of spindle system formation during metaphase (Fig 2A).

The sensitivity of microprotoplasts to DMSO and PEG was very high, this led to severe damage which was also true for the receptor hypocotyl protoplasts. Inspite of at 18-20% of fusates were recovered (Fig 2B) and cultured in agarose droplets (Fig 2C) in which a division rate of 50-55% was achieved after 10 days. The regeneration efficiency (2D), calculated from the total embedded fusates, was comparable to the results reported by Wingender et al. (1996) for different *H. annuus* cultivars but lower than that reported by Henn et al (1998) by symmetric somatic hybrids, this might suggest that the regeneration capacity is positively affected by the heterocarion protoplasts. The higher regeneration potential of the symmetric fusion products can probably be attributed to the wild genome, which might lead to a superior regeneration potential. Similar heterotic effects were reported for symmetric hybrids of onion and leek (Buiteveld et al. 1998).

Figure 2. Plant regeneration of asymmetric somatic hybrids (ASH) after fusion between donor microprotoplasts with receptor protoplasts of *H. annuus*. (A) several micronuclei in the cell, stained with the DNA fluorochrome DAPI, (B) asymmetric fusion with one micronucleus in the receptor protoplast, stained with DAPI (C) microcalli development in agarose droplets after 4 weeks in culture, (D) regenerated ASH, (E) flowering ASH, with varied form of the ray flowers, (F) ASH plant with seed production, (G) Seedling from ASH progeny, (H) diakinesis of ASH showing multivalent and univalent chromosomes, (I) metaphase chromosomes of a ASH, stained with DAPI, showing the presence of 4 alien chromosomes, (J) flow cytometric analysis of DNA content (2C) of mesophyll nuclei from (1) *Petunia hybrida* (Standard), (2) *Helianthus annuus*, (3) ASH plant and (4) *H. giganteus*, (L) RAPD analysis of ASH plants compared to the parent genotypes, Ha - patterns of receptor genotype *H. annuus*, Hg - patterns of the donor specie *H. giganteus* and respective ASH showing additional bands (arrow) of the donor specie. M - Size Marker.

Characterization of ASH plants

The variability expressed for some morphological traits by the regenerated plants could be confirmed by nuclear DNA analysis based on RAPD amplification. More than half (53%) of the regenerated plants were ASH containing additional genomic DNA of the donor species. The most expressive morphological differences were: reduced growth, leaf shape alteration, serrated leaves, early flowering, more than one flower bud per plant or variation of the ray flowers (Fig. 2E). The seeds were more similar to the receptor species (*H. annuus*). A correlation between the presence of a high number of alien chromosomes (2n = 34 + 8) and morphological alterations could be observed. Similar morphological differences could also be observed on the progeny of the ASH plants, suggesting sexual transmissibility.

The presence of additional alien genome in the regenerated ASH could also be confirmed by chromosome counting and by flow cytometric analysis. On the basis of flow cytometric analysis, no variation at the ploidy level of examined ASH could be detected. Additional genomic DNA was reflected in an increment of the total amount of DNA in the respective ASH (Fig 2J). These results were similar of those reported for *Brassicaceae* somatic hybrids (Fahleson et al. 1988), they showed a high correlation between chromosome number and DNA content of the somatic hybrids. Mitotic chromosome counting of ASH plants, also facilitates the identification of addition lines, aneuploids and cytochimeras. On this basis, mitotic chromosome analysis of RAPD confirmed ASH plants, revealed in 75% of the cases, a presence of 2 or more alien chromosomes in addition (Fig 2I). Even plants without additional chromosomes, might be ASH, resulting from recombinant chromosomes due to reciprocal, interstitial translocation or introgression.

Meiotic analysis of ASH showed that more than 85% of the analyzed meiocytes were normal with a bivalent chromosome pairing. Less than 15% showed irregular chromosomal behavior, with univalent or multivalent pairing (Fig 2H), chromosome bridges or laggard chromosomes. This behavior is probably a result of different factors. The high percentage of bivalent chromosomes suggests either an existence of a high homology between the chromosomes of the ASH (Jan 1997), or the presence of transferred alien chromosome in the ASH is reduced. The fact that some of the bivalents were heteromorphic, open (rod bivalents) or chromosomes paired end-to-end, suggests that the bivalents did not possess complete chromosome homology, but showed only partial chromosome pairing (Sybenga 1992, Singh 1992, Jan 1997). On the other hand, the formation of multivalents in ASH, is also indicative of intergenomic homoeology, which would help in introgressing genes from perennial *Helianthus* species to *H. annuus*, as reported for somatic hybrids between *Brassica* (+) *Sinapis* (Gaikwad et al. 1996). In this way, gene flow from wild perennial *Helianthus* species to the cultivated form might be possible.

The RAPD analysis showed that the regenerated ASH plants contained part of the donor genome (Fig 2L). The method is easy, quick, needs only a small amount of tissue for DNA isolation and can be applied during early stages of plant development (Krasnyanski et al. 1998). The only restriction for a secure ASH identification, in this study, was the high number of primers needed. While Takemori et al. (1994), Krasnyanski et al. (1998) and Henn et al.

(1998) needed only a small number of primers to characterize their somatic hybrids we had to use more primers to identify the smaller amount of DNA transferred from the donor microprotoplasts to the receptor protoplasts of *H. annuus*. Since we found polymorphic bands in the regenerated ASH that carry the same cytoplasm of *H. annuus*, this difference can only be ascribed to nuclear origin from the donor microprotoplast. Genomic composition of the progenies showed a polymorphic band profile. We could identify the characteristic bands of the donor genome in the progeny of ASH plants. It confirms the sexual transmissibility of the alien genome (microprotoplast) to the progenies.

Pollen viability and plant fertility are directly related to the presence of meiotic abnormalities caused by defective pairing, non-disjunction or unequal chromosome distribution (Singh 1992). We found in ASH plants, that decreased pollen viability was strongly negative correlated (r = -0.96) with the chromosome number in root-tip cells. The lowest pollen viability was found in the ASH plants with the highest number of chromosomes in root-tip cells. These results agree with many scientists who have attempted to correlate cytological behavior and meiotic irregularities with pollen viability, plant fertility or seed production (Sybenga 1992, Singh 1992, Gaikwad et al. 1996, Jan et al. 1997).

In conclusion, it appears to be possible to obtain transgenic plants by direct transfer of a single chromosome via microprotoplasts technique, between sexually incompatible *Helianthus* species. Besides this, it might be a practicable tool to transfer polygenetically determined traits or alien genes, for improvement and gene pool extension of *H. annuus.* The unique advantages of the microprotoplast fusion is that it allows the transfer of traits which are polygenetically controlled or with unknown molecular background; it also allows the production of monosomic or dissomic addition lines for the transfer or introgression of economically important traits between sexually incompatible species.

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