Theor Appl Genet (2001) 102:1280–1285

P.C. Binsfeld · R. Wingender · H. Schnabl

Cytogenetic analysis of interspecific sunflower hybrids and molecular evaluation of their progeny

Received: 16 July 2000 / Accepted: 25 September 2000

Abstract Meiotic cells of transgenic asymmetric somatic hybrid (ASH) plants obtained by fusion of microprotoplasts of the donor species *Helianthus giganteus* or *Helianthus maximiliani* and recipient protoplasts of *Helianthus annuus* were investigated. Over 85% of the ASH meiocytes showed regular bivalent chromosome pairing; however, several anomalies like anaphase bridges, laggard chromosomes, univalent and multivalent pairing were also observed. Pollen viability of the ASH plants ranged from 79.2 to 95% with a strong negative correlation to chromosome number which varied between 34 and 42. Molecular investigation of ASH progeny using RAPD markers revealed the presence of donor genotype markers in 68% of the offspring. These results suggest that asymmetric somatic hybridization offers an efficient alternative method to overcome sexual barriers for gene flow and the genetic improvement of *H. annuus* by introgression of economical important traits from wild *Helianthus* species.

Keywords Chromosome transfer · *Helianthus* · Meiotic behavior · Pollen viability · RAPD

Introduction

The forty nine wild annual and perennial *Helianthus* species represent a vast reservoir of useful agronomic traits for improvement programs of cultivated sunflower (*Helianthus annuus* L.). This secondary gene pool is extremely important for the transfer of traits like disease and insect resistance, herbicide resistance, salinity and drought tolerance, fatty acid variation, CMS and fertility restoration (Seiler 1992; Jan 1997; Yemets et al. 2000). However, sexual incompatibility between most wild and cultivated species, as well as self-incompatibility and low fertility of the resulting F_1 hybrids, limits their use in certain breeding methods (Atlagic et al. 1995; Rieseberg et al. 1995; Jan 1997; Sukno et al. 1999). Successful interspecific hybridization of wild species and cultivated sunflower has been achieved by embryo rescue (Sukno et al. 1999) and somatic hybridization (Krasnyanski and Menczel 1995; Henn et al. 1998; Binsfeld et al. 2000) promoting genetic exchange between homoeologous chromosomes at meiotic pairing.

Symmetric or asymmetric somatic hybridization by protoplast or microprotoplast fusion is an efficient method to overcome crossing barriers between sexually incompatible species and to increase the genetic variability of higher plants (Ramulu et al. 1995; Gaikwad et al. 1996; Barbosa and Vieira 1997; Miranda et al. 1997; Buiteveld et al. 1998; Henn et al. 1998; Yemets et al. 2000; Binsfeld et al., 2000). The combination of unrelated genomes with differences between homeologous chromosomes frequently leads to reduced pairing or unbalanced gametes in somatic hybrids. These hybrids are often semi-sterile or sterile (Rieseberg et al. 1995). Chromosome elimination in somatic hybrids has been observed, but this elimination is usually incomplete for related species (Samoylov et al. 1996). Therefore, the production of addition lines, chromosome substitution, or the introgression of certain genetic traits into the cultivated species, are all possible (Sybenga 1992; Buiteveld et al. 1998; Sukno et al. 1999; Chetelat and Meglic 2000). The occurrence of meiotic recombination between homoeologous chromosomes is essential for introgression success in somatic hybrids. The production of fully stable and fertile plants through introgression can occur in the form of whole-chromosome substitution or small chromosome segments via crossing-over (Sybenga 1992; Rieseberg et al. 1995; Lashermes et al. 2000). Frequently, if sufficient affinity of the parental genomes exists, or pairing control is lax enough to permit the meiot-

Communicated by K. Glimelius

P.C. Binsfeld (✉) · R. Wingender · H. Schnabl
Institute of Agricultural Botany,
Department of Physiology and Biotechnology of Plants,
University of Bonn, Karlrobert-Kreiten-Strasse 13,
D-53115 - Bonn, Germany
e-mail: ulp50b@uni-bonn.de
Fax: 0049-228-731696

ic pairing of homoeologous chromosomes, meiotic stability will decrease because of the formation of multivalents and, consequently, fertility may be reduced. But, in this sense, small introgressions from the donor to the receptor species are possible (Sybenga 1992; Atlagic et al. 1995; Rieseberg et al. 1995; Chetelat and Meglic 2000; Henn et al. 1998).

In the present study we report the meiotic behavior of asymmetric somatic hybrid (ASH) plants obtained through the fusion of microprotoplasts of perennial species with hypocotyl protoplasts of cultivated sunflower and a molecular analysis of their progeny. Knowledge of the behavior of the alien genome in the receptor species provides useful information for planning a more-efficient use of these ASH plants as a bridge for the transfer of desirable agronomic traits between wild perennial and cultivated sunflower. The goals of the experiments were: (1) meiotic characterization of ASH plants, (2) evaluation of their pollen viability and (3) analysis of the sexual transmissibility of the alien genome to the progeny.

Materials and methods

Plant material

Asymmetric somatic hybrid (ASH) plants were obtained by chemically induced fusion between microprotoplasts of the perennial sunflower species *Helianthus giganteus* (Hg) or *Helianthus maximiliani* (Hm) (2n=34), and hypocotyl protoplasts of the cultivar Florom-328 [*H. annuus* L. (Ha), 2n=34] as reported by Binsfeld et al. (2000).

Meiotic analysis

Flower buds of 12 ASH plants (confirmed hybrids by RAPD markers) were collected and fixed in 15 ml of 3/1 (v/v) ethanol/glacial acetic acid for 36 h at room temperature and stored at 4°C in 15 ml of 70% ethanol. Anthers were digested in a 100-µl enzyme mixture [4% cellulase, Onozuka R-10, Serva and 1% pectolyase (Y-23, Seishim Pharmaceutical) in 75 mM KCl, pH 4.0, Kakeda et al. (1991)], for 20 min at 37°C and then squashed in 45% acetic acid. The samples were stained with a drop (5µl) of 10-µM DAPI for fluorescence microscopic analysis or with a drop 50% carmine acetic acid for light microscopy. All phases of meiosis were investigated to identify anomalies during chromosome migration, such as laggard chromosomes, anaphase bridges and their segregation at diakinesis, to verify univalents, bivalents and multivalents. Photographs were taken using a computer-assisted cooled CCD camera (Photometrics).

Pollen viability

Pollen viability of six flowering ASH plants and their respective parents (Ha, Hg and Hm) was determined by differential staining of viable and nonviable pollen grains as described by Alexander (1980). Pollen grains from five anthers, collected from different flowers of the head, were suspended in a drop (20 µl) of staining solution and distributed on four slides. After 30 min the viable pollen was counted. Statistical analysis was performed using SANEST (Statistical analysis system) (Zonta et al. 1984).

RAPD analysis

The genomic DNA of 25 progeny and their parental plants was extracted from young leaves as described by Binsfeld et al. (1999). For RAPD amplification 30 ng of DNA and different 10-mer primers (P1, P2, Pharmacia; I11, B12, D13, P160–6, Roth FRG) were used in a final volume of 15 µl as described by Binsfeld et al. (1999). The amplification products were electrophoretically separated in 1.5% agarose gels containing ethidium bromide (2.5 µM) in Tris-Borate-EDTA buffer and were photographed under UV light (302 nm).

Results

Meiotic analysis

Cytological analysis of meiosis in the donor (*H. giganteus* or *H. maximiliani*) and receptor species (*H. annuus*) revealed only a few abnormalities. The average number of 17 bivalents per meiocyte confirmed the diploid character of the receptor and donor species (2n=34). Less than 3% of the meiocytes were found to contain chromosome bridges and laggard chromosomes, which was correlated with the low frequency of micronuclei (less than 3%) in the analyzed tetrads (Table 1).

The general features of the meiotic behavior of the ASH plants are summarized in Table 1. Irrespective of the parental genotypes, meiotic cells of ASH plants exhibited more abnormalities. The widespread occurrence of chromosomal abnormalities, and the impossibility of a clear identification of homoeologous pairing, have focused our attention on anaphase-I bridges (Fig. 1A), lag-

Table 1 Meiotic behavior of asymmetric somatic hybrid (ASH) plants and the respective parent genotypes *H. annuus* (Ha), *H. giganteus* (Hg) and *H. maximiliani* (Hm)

Genotypes	% Meiocytes with			Chromosome bridges (%)	Lagging chromosomes (%)	Tetrads with micronuclei (%)
	Univalent	Bivalent	Multivalent			
Ha	0	99.2	0.8	1.2	2.9	2.3
Hg	0	100	0	0.8	2.4	1.8
Hm	0	100	0	1.7	1.6	2.1
ASH[a]	5.1	87.6	7.3	8.8	9.1	10.1
ASH[b]	8.6	86.2	5.2	7.4	8.9	13.3

[a] Mean of five ASH plants from *H. annuus* (+) *H. giganteus*
[b] Mean of seven ASH plants from *H. annuus* (+) *H. maximiliani*

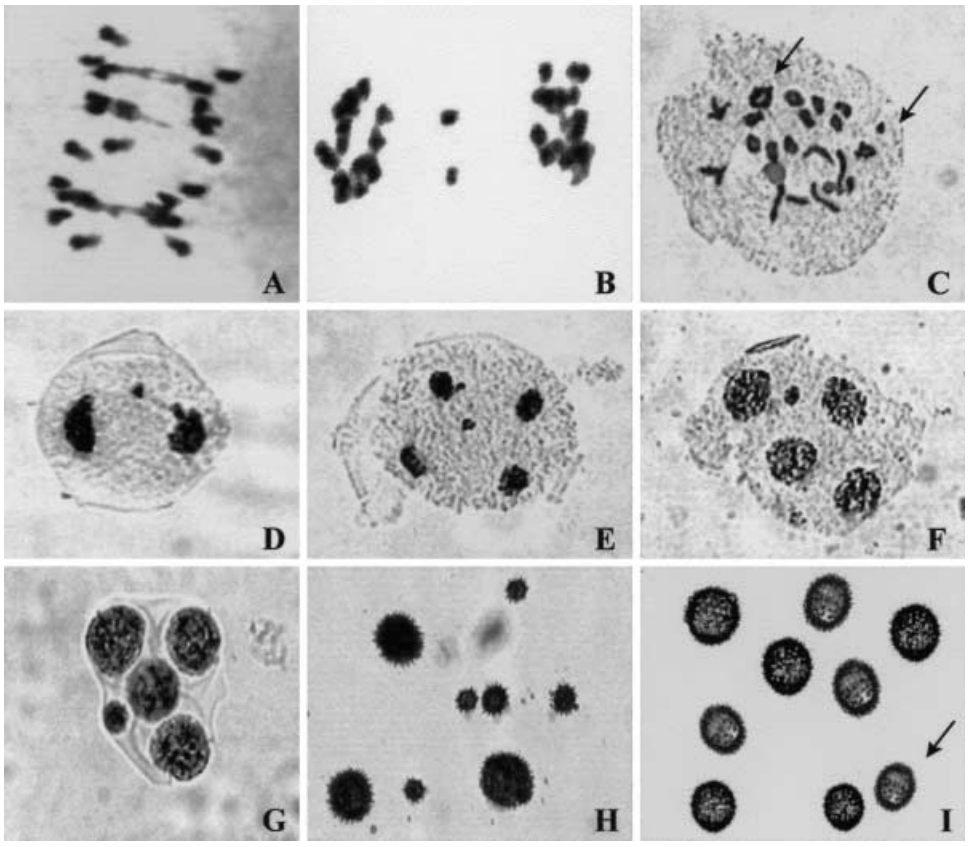

Fig. 1A–I Photomicrographs of microsporogenesis of ASH plants (*H. annuus* fusions with *H. giganteus* or *H. maximiliani*). **A** Chromosome bridges, **B** laggard chromosome, **C** chromosome pairing at diakinesis showing multivalent and univalent chromosomes (*arrows*), **D** interphase with lagging chromosome, **E** telophase-II lagging chromosome, **F** the small nuclei resulting from condensed laggard chromosomes, **G** a tetrad with a small micronucleus, resulting in chromosome elimination, **H** the presence of small sterile pollen grains, **I** a pollen viability test showing viable (*dark*) and nonviable (*less dark*, *arrow*) pollen grains

Table 2 Mean comparison (*t* test) of pollen grain viability (%) between asymmetric somatic hybrid (ASH) plants and the parent genotypes *H. annuus* (Ha), *H. giganteus* (Hg) and *H. maximiliani* (Hm) in relation to the respective chromosome number in root-tip cells (NCRT). [ns] The means are not significantly different

	Ha	Hg	Hm	ASH_1[a]	ASH_2[a]	ASH_3[a]	ASH_4[b]	ASH_5[b]	ASH_6[b]
Ha	96.2[c]	0.8[ns]	0.9[ns]	8.2[d]	13.6[d]	1.2[ns]	17.0[d]	15.0[d]	6.9[d]
Hg		95.4[c]	1.7[ns]	7.4[d]	12.8[d]	0.4[ns]	16.2[d]	14.2[d]	6.1[d]
Hm			97.1[c]	9.1[d]	15.5[d]	2.1[ns]	17.9[d]	15.9[d]	7.8[d]
ASH_1[a]				88.0[c]	5.4[d]	7.0[d]	8.8[d]	6.8[d]	1.3[ns]
ASH_2[a]					82.6[c]	12.4[d]	3.4[ns]	1.4[ns]	6.7[d]
ASH_3[a]						95.0[c]	15.8[d]	13.8[d]	5.7[d]
ASH_4[b]							79.2[c]	2.0[ns]	10.1[d]
ASH_5[b]								81.2[c]	8.1[d]
ASH_6[b]									89.3[c]
NCRT	34	34	34	38	40	34	40	42	36

[a] ASH plants from *H. annuus* (+) *H. giganteus*
[b] ASH plants from *H. annuus* (+) *H. maximiliani*
[c] Pollen viability expressed in percentage
[d] The means are significantly different ($p<0.05$)

gard chromosomes (Fig. 1B) and univalent or multivalent chromosome pairing at diakinesis (Fig. 1C, arrow). Partial chromosome disjunction (Fig. 1D) and laggard chromosomes in telophase-II (Fig. 1E) were also observed. Individual condensation of the separated chromosomes (Fig. 1F) finally led to the formation of micronuclei in the tetrads (Fig. 1G) resulting in small and sterile pollen grains (Fig. 1H). Despite the wide spectrum of meiotic abnormalities, more than 85% of the ASH meiocytes were normal, with the formation of a full synapsis achieving normal bivalent pairing, so that no systematic trend for chromosome elimination was evident. This suggests that the added (alien) chromosomes were included in the normal chromosome set of the receptor genome in subsequent mitotic and meiotic generations.

Pollen analysis

The pollen viability of the donor (Hg or Hm) and receptor (Ha) species, was over 95% without significant differences between them (Table 2). The pollen viability among ASH plants was, however, lower and ranged from 79.2 to 95% being significantly different ($p<0.05$)

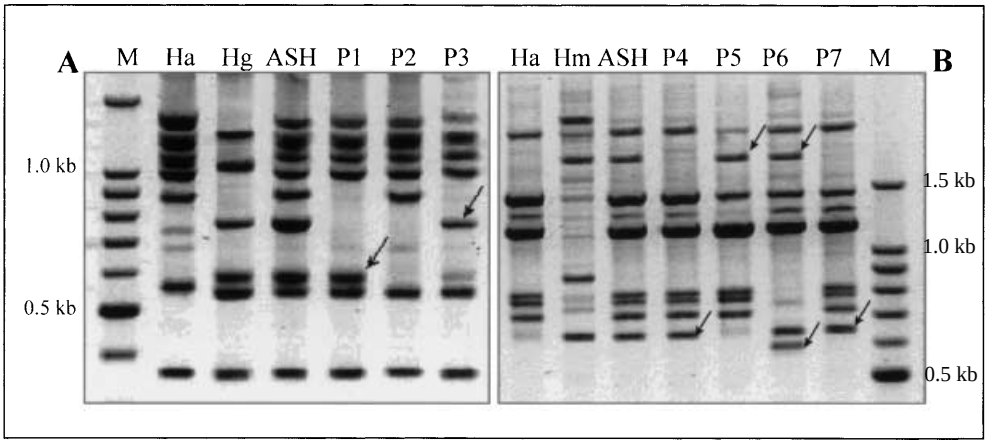

Fig. 2 RAPD-PCR patterns of ASH plants, their progeny (*P*) and the parental genotypes using primer D13 (**A**) and primer I11 (**B**). **A** *M* size marker, *Ha* (*H. annuus*), *Hg* (*H. giganteus*), *ASH* mother plant of the progeny *P1*, *P2* and *P3*, bands corresponding to the donor species are marked by an *arrow*; **B** *Ha* (*H. annuus*), *Hm* (*H. maximiliani*), *ASH* mother plant of the progeny *P4*, *P5*, *P6* and *P7*, bands corresponding to the donor species are marked by an *arrow*, *M* size marker

except for ASH3. ASH plants having a high chromosome number had the lowest viability (ASH_2, ASH_4 and ASH_5, Table 2). A strong negative correlation (r=–0.93) between pollen viability and chromosome number was observed. A higher number of univalent or irregular multivalent chromosome pairing results in the degeneration associated with a reduction of pollen-grain viability (Fig. 1I).

Genomic analysis of ASH progeny

ASH plants displayed a combination of parental bands, confirming their hybrid character (Fig. 2A, B). In several cases, however, not all parental genome patterns were obtained (Fig. 2B), suggesting either a loss of the recipient or the donor genome during the mitotic process. Upon selfing, a genetically heterogeneous offspring of the ASH plants was obtained. For example P1, P2 and P3 (Fig. 2A) represent different genotypes. This is also true for the progeny of ASH plants between Ha (+) Hm (Fig. 2B). Irrespective of the parental genomes, the presence of donor markers (Fig. 2, arrows) could be verified in the progeny indicating sexual transmission of the alien genome, although complete loss of donor markers was observed (Fig. 2A, P2). A further observation was the appearance of new markers (Fig. 2B, P6, second arrow) in some of the progeny of ASH plants.

Discussion

The meiotic behavior displayed by the ASH plants reveals that over 85% of the analyzed meiocytes exhibited normal bivalent chromosome pairing. Less than 15% showed irregular chromosome behavior, with univalent or multivalent pairing, chromosome bridges or laggard chromosomes. Meiotic abnormalities and spontaneous chromosome elimination in somatic hybrids are a common phenomenon, especially when protoplasts of distantly related species are fused. Similar observations were made in meiotic cells of somatic hybrids of *Brassica* (+) *Sinapis* (Gaikwad et al. 1996), tomato (+) eggplant (Samoylov et al. 1996), passionfruit (Barbosa and Vieira 1997), citrus (Miranda et al. 1997), potato (+) tomato (Garriga-Calderé et al. 1998; Chetelat and Meglic 2000) and interspecific hybrids obtained by sexual crosses between *Helianthus* species by Singh (1992), and Atlagic et al. (1995).

The high percentage of bivalent chromosomes suggests either high homology between the chromosomes of the ASH plants (Jan 1997), or a low number of alien chromosomes. The fact that some of the bivalents were heteromorphic, open rod bivalents, or else that chromosomes paired end-to-end, suggests partial chromosome homology (Singh 1992; Sybenga 1992; Jan 1997). According to Sossey-Alaoui et al. (1996) amphidiploidization in the genus *Helianthus* led to different genomic structures in the various sections. Based on RAPD fragments common to the species of one section or to all sections, this suggests the following genomic structures: HC for sect. *Helianthus*, DC for sect. *Atrorubens* and CC for sect. *Ciliares* (autopolyploid origin). With *H. annuus* belonging to sect. *Helianthus*, and *H. giganteus* as well as *H. maximiliani* to the sect. *Atrorubens*, the parental genomes of the ASH plants shared at least one set of chromosomes of common origin, probably favoring correct pairing. Also the formation of multivalents in the ASH plants might be indicative of intergenomic homology, which would help introgressing genes from perennial *Helianthus* species to *H. annuus*, as reported for somatic hybrids between *Brassica* (+) *Sinapis* (Gaikwad et al. 1996) and *Nicotiana* species (Yemets et al. 2000). The different mechanisms of alien chromosome integration, such as homoeologous chromosome pairing, recombination at meiosis, or somatic transfer mechanisms occurring in pre- or post-meiotic cells, have been discussed previously (Sybenga 1992; Ramulu et al. 1996; Samoylov et al. 1996; Chetelat and Meglic 2000). Although we have no evidence for the impact of the amphidiploid character of the parental genomes, chromosome pairing in the ASH plants indicates homology between the genomes, enabling gene flow from wild perennial *Helianthus* species to the cultivated form.

Pollen viability and plant fertility are directly related to the meiotic abnormalities caused by defective pairing, non-disjunction or unequal chromosome distribution (Singh 1992). In this study, decreased pollen viability was strongly negative correlated (r=–0.93) 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, and might be a common phenomenon in interspecific hybrids (Singh 1992; Sybenga 1992; Gaikwad et al. 1996; Sukno et al. 1999; Yemets et al 2000). These results show that pollen viability can be used as a direct indicator of meiotic abnormalities.

In the present investigation, RAPD-PCR markers were appropriate to identify characteristic banding patterns of the parents Ha and Hg or Hm, as well as ASH plants and their progeny (Fig. 2A, B). Monitoring of the sexual transmission of the alien genome revealed that 68% of the analyzed ASH progeny plants contained donor RAPD markers. The absence of such markers might result from chromosome elimination or rearrangements during meiosis. These findings correlate with the cytological results corresponding to a rather unstable genomic constitution of the ASH plants, which is further substantiated by the appearance of new markers in the progeny (Fig. 2B, P6, second arrow). Although the nature of this band is unknown, one might speculate that it reflects chromosomal rearrangement. In order to monitor the introgression of desirable traits, a fine-mapping of the respective locus such as QTL mapping, would be needed (Bernacchi et al. 1998). Thus, somatic hybridization, and especially partial hybrids, might present a powerful tool for sunflower breeding programs based on the high genomic heterogeneity obtained in the offspring of ASH plants, thus displaying a vast genetic variability.

In conclusion, we have demonstrated here that: (1) despite meiotic abnormalities, ASH plants presented regular chromosome pairing in the great majority of their analyzed meiocytes, (2) they produced a considerable high-pollen viability, that permitted (3) sexual transmissibility of the added (alien) genome to the progeny in most of the plants analyzed. These results suggest that asymmetric somatic hybridization provides a useful and efficient method for the transfer of economically important traits (e.g. *Sclerotinia sclerotiorum* resistance) from wild *Helianthus* species to the cultivated species.

Acknowledgments The authors are grateful to Dr. O. Schrader, Quedlinburg, for advice on *Helianthus* chromosome and meiotic analysis, to the Institute of Cereal and Industrial Plant Research, Fundulea, Romania, for providing *H. annuus* material, and to the Deutsche Forschungsgemeinschaft for financial support to H.S.

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