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Molecular and cytogenetic constitution of plants obtained via two different somatic hybridization methods

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Abstract Somatic hybrids between *Helianthus annuus* and *H. maximiliani* obtained through two different hybridization methods were investigated. Random amplified polymorphic DNA markers of symmetric somatic hybrids (SSH) showed typical profiles of full somatic hybrid plants, while in asymmetric somatic hybrids (ASH) a small amount of alien genome was identified. Flow cytometric analysis of the genome size showed a strong correlation (r=0.92) between chromosome number and relative DNA content of the hybrid plants. Chromosome analysis of SSH and ASH showed a variable number of extra chromosomes in addition to the normal parental set ($2n$=34). In SSH the number of added chromosomes ranged from 26 to 31, while in ASH two to six additional chromosomes could be observed. Although the SSH and ASH plants varied largely in their genome constitution, both hybrid plant groups, to different degrees, were a genomic recombination of *H. maximiliani* and *H. annuus*, and some of these plants will be of use for practical breeding purposes.

Keywords Flow cytometry · Microprotoplasts · Protoplast fusion · RAPD · Sunflower

Introduction

The increased production and expanded cultivation of sunflower (*Helianthus annuus* L.) as an important oil crop is required, but these goals are often limited by numerous diseases and pests that cause serious yield reduction (Gulya et al. 1997). The transfer of such polygenic traits as pathogen resistance is of major interest in the breeding of this crop (Seiler and Rieseberg 1997; Henn et al. 1998). However, conventional breeding using sexual crossing is often limited due to sexual incompatibility, especially if the gene of interest is only present in the wild species (Jan 1997; Guo and Deng 2001). An alternative way to overcome crossing barriers, to transfer polygenic traits and to generate new unexpected alleles is provided by somatic hybridization (Wolters et al. 1994). Symmetric somatic hybridization (SSH) between protoplasts of wild and cultivated species has been achieved successfully for several species (Krasnyanski and Menczel 1995; Krasnyanski et al. 1998; Henn et al. 1998; Guo and Deng 2001; Patra et al. 2001). However, in addition to having desirable traits, some of these wild-type plants also have many undesirable traits, some of which are linked to fertility or complete sterility (Yamada et al. 1998). This disadvantage may be overcome by partial genome transfer using asymmetric somatic hybridization (ASH; Ramulu et al. 1995). In this method, only a part of the wild genome is transferred to a receptor protoplast (Rasmussen et al. 1997), thereby reducing the number of undesirable traits incorporated in the receptor genome.

In the investigation reported here, we chose the wild sunflower species *H. maximiliani* for the production of somatic hybrid plants because of its superior resistance against the most economically damaging sunflower pathogen, the fungus *Sclerotinia sclerotiorum*. Our main aim was to analyze SSH and ASH plants between *H. annuus* and *H. maximiliani* in order to obtain useful information with respect to their use in specific breeding programs as a way to overcome sexual barriers and transfer desirable traits from related wild species to cultivated ones.

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Materials and methods

Plant material

Five symmetric somatic hybrid plants (SSH-1, 2, 3, 4, 5), obtained from experiments carried out by Henn et al. (1998), were compared with five asymmetric somatic hybrid plants (ASH-1, 2, 3, 4, 5) obtained from the fusion of microprotoplasts and protoplasts, as reported by Binsfeld et al. (2000). The parental plants used for the hybridization process were the wild perennial *Helianthus maximiliani* (Hm) (2*n*=34) and cv. Florom-328 of *H. annuus* L. (Ha) (2*n*=34).

Molecular analysis

The genomic DNA of five SSH and five ASH plants and that of their parental plants was extracted from young leaves as described by Binsfeld et al. (1999). For random amplified polymorphic DNA (RAPD) amplification, 30 ng DNA and 10-mer primers P1 (5′-CGGCCACTG-3′) and P2 (5′-CGGCCACTGT-3′) from Pharmacia (Biotech, Germany) and P3 (5′-ACAACGCGA-3′), P4 (5′-CTACTACCCG-3′) from Roth Random Primer (Roth, Germany) were used as described by Binsfeld et al. (1999). The amplification products were electrophoretically separated on 1.5% agarose gels containing ethidium bromide (2.5µ*M*) in Tris-Borate-EDTA buffer and subsequently photographed under UV light (302 nm).

Flow cytometric analysis

The relative DNA content of interphase nuclei of five SSH and five ASH plants and their parental genotypes was determined by flow cytometric analysis (FCA) as described by Binsfeld et al. (2000). For the isolation of nuclei, 10 µl freshly isolated protoplasts was incubated for 5 min in 1 ml chopping solution (solution A of the plant nuclei isolation kit; Partec, Münster, Germany) and stained with 2 ml of DAPI solution (solution B of the plant nuclei isolation kit; Partec) for 5 min. As a standard, nuclei of *Petunia hybrida* cv. F_1 hybrid Hit parade blau were used (2C=2.85 pg; Nagl and Treviranus 1995). Three repetitions of the respective samples were measured on a Partec CA-III flow cytometer (Partec) equipped with an HBO-100 mercury high pressure lamp using the UG1 excitation filter, TK420, TK560 dicroic mirrors and CG435 longpass filter. The software DPAC (Data pool application for cytometry; Partec) was used for calculating the variation coefficient values and evaluating diagrams of relative DNA content.

Mitotic and meiotic analysis

For the mitotic analysis, root-tip cells from SSH and ASH plants and their parental plants were collected and pre-treated in 2 ml of an aqueous solution of 2.5 m*M* 8-hydroxyquinoline for 3 h at 4°C, fixed in 2 ml ethanol/glacial acetic acid (3:1, v/v) for 36 h at 4°C and stored in 2 ml 70% ethanol at 4°C. For the meiotic analysis, flower buds of the same plants 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. Before maceration, the root tips or anthers were digested in 100 µl enzyme mixture [4% cellulase (Onozuka R-10; Serva) and 1% pectolyase (Y-23; Seishim Pharmaceutical) in 75 m*M* KCl, pH 4.0 (Kakeda et al. 1991)] for 20 min at 37°C and then squashed in 45% acetic acid. They were then stained with 50% carmine acetic acid. Photographs were taken using a computer-assisted cooled CCD camera (Photometrics).

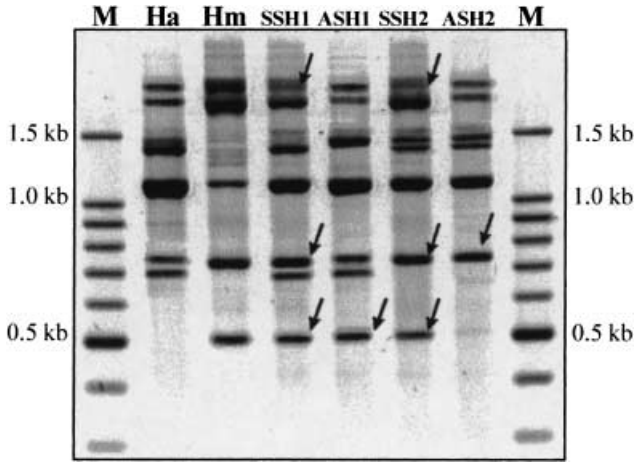

Fig. 1 RAPD-PCR banding profile of ASH and SSH plants and their parental genotypes for primer P4. *M* Molecular weight, *Ha* profile of *Helianthus annuus*, *Hm* profile of *H. maximiliani*, *ASH-1*, *-2* asymmetric somatic hybrid plants with additional bands of the Hm genome (*arrows*), *SSH-1*, *-2* symmetric somatic hybrid plants showing the presence of multiple bands of the Hm genome

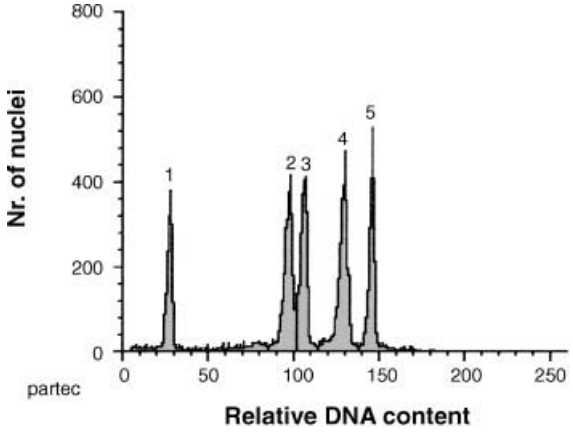

Fig. 2 Flow cytometric analysis of relative DNA content (2C) of mesophyll nuclei from plants of *Petunia hybrida* (standard) (*peak 1*), *H. annuus*, (*2*) ASH1 plant (*3*) *H. maximiliani* (*4*), SSH1 (*5*)

Results

Genomic constitution

All of the SSH and ASH plants displayed a combination of parental RAPD bands, thereby confirming their hybrid character (Fig. 1). Since at least one specific band of each parental species appeared on the banding profile of the somatic hybrid plants, it was evident that these were hybrid plants. A high number of variable polymorphisms between SSH and ASH plants could be identified. Variability was more pronounced in SSH, where in general there were four polymorphisms for each polymorphism in ASH. For example, in Fig. 1 (arrows), a typical RAPD marker of Hm is visible in the profiles of ASH plants, while in the SSH plants three Hm markers are present.

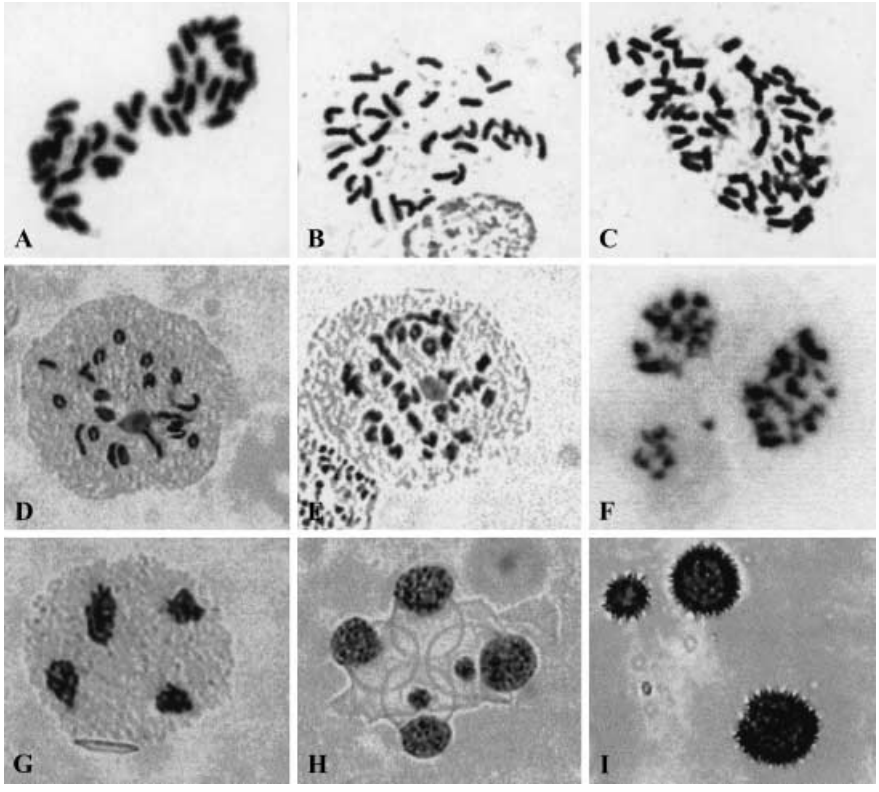

Fig. 3A–I Photomicrographs of cytological comparison of ASH and SSH plants obtained from microprotoplast and protoplast fusion between *H. annuus* (Ha) and *H. maximiliani*. **A** Root-tip cell of Ha with 34 chromosomes, **B** root-tip cell of ASH1 with 36 chromosomes, **C** root-tip cell of SSH1 with 63 chromosomes, **D** chromosome pairing at diakinesis of ASH1, **E** diakinesis of SSH1 showing normal bivalent, multivalent or univalent chromosome pairing, **F** interphase with elimination of a group of chromosomes in SSH1, **G** normal telophase II meiocytes of ASH1 plant, **H** tetrad of SSH1 with two small micronuclei, as a result of chromosome elimination, **I** different sizes of pollen grains in SSH1

The different profiles of the RAPD markers may have their origin from the variable genomic combinations of the respective somatic hybrid plants. These results support the methodological differences between SSH and ASH in terms of plant variability and their practical applicability in breeding programs.

Nuclear DNA constitution

The relative DNA content of the parental plants was quite different, although the chromosome number of *H. annuus* and *H. maximiliani* in both species was the same (2*n*=34). The genome size of *H. maximiliani* (10.62 pg DNA per nucleus) was around 40% higher than that of *H. annuus* (7.36 pg DNA per nucleus). While in ASH plants the DNA content ranged from 7.98 pg to 8.37 pg per nucleus, in SSH plants it was almost twice as much and ranged from 13.41 pg to 15.11 pg DNA per nucleus, a difference which is significant at $P<0.01$. The genome size of genotype ASH1 (peak 3) was intermediate between that of *H. annuus* (peak 2) and that of *H. maximiliani* (peak 4) but closer to the genome size of *H. annuus* (Fig. 2). On the other hand, genotype SSH1 (peak 5) showed the largest genome size, indicating the presence of a higher number of chromosomes and resulting in nuclei with almost double the DNA content of *H. annuus*. The strong correlation (r=0.92) between genome size and chromosome number indicates that genome size can be used for a direct estimation of additional genomic DNA in somatic hybrid plants.

Mitotic and meiotic analysis

Cytological analysis of mitosis revealed the presence of extra chromosomes in the somatic hybrid in addition to those of the normal chromosome set (2*n*=34) of the parental genotypes (Table 1, Fig. 3A). In ASH plants, the chromosome set ranged from 2*n*=36 to 2*n*=40 (Fig. 3B), corresponding to the addition of two to six chromosomes. For the SSH plants the chromosome set ranged from 2*n*=60 to 2*n*=65 (Fig. 3C), corresponding almost to the sum of the chromosome sets of the parental plants.

Cytological analysis of meiosis in the donor (*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 (2*n*=34). The general features of meiotic behavior of the SSH and ASH plants are summarized in Table 1. Irrespective of the parental genotypes, meiotic cells of the SSH and ASH plants exhibited more meiotic abnormalities. The presence of a low number of alien chromosomes in ASH led to an elevated level of regular bivalent pairing (Fig. 3D), while in SSH plants the combination of almost both full sets of the parental chromosomes led to a high frequency of abnormal univalent and multivalent chromosomes pairing (Fig. 3E). A strong correlation (r=0.93) between the number of chromosome bridges and the chromosome number of the analyzed plants was observed. A similar correlation level (r=0.88 and r=0.90, respectively) between lagging chromosomes and the presence of micronuclei in the tetrads was also observed. Another common

Table 1 Mean number of chromosomes in root-tip cells and meiotic behavior of asymmetric somatic hybrid (*ASH*) plants, symmetric somatic hybrid (*SSH*) plants and their parental genotypes *Helianthus annuus* (*Ha*) and *H. maximiliani* (*Hm*)

Variables analyzed:	Genotypes			
	Ha	Hm	ASH[a]	SSH[a]
Number of chromosomes in root-tip cells	34	34	36–40	60–65
Meiocytes with univalents (%)	0	0	5.2	14.9
Meiocytes with bivalents (%)	99.2	100	85.4	67.4
Meiocytes with multivalents (%)	0.8	0	9.4	17.7
PMCB (%)[b]	1.2	1.7	10.1	15.6
PMLC (%)[c]	2.9	1.6	7.4	45.8
Tetrads with micronuclei (%)	2.3	2.1	10.9	41.3

[a] Mean of the results from two repetitions of five somatic hybrid plants each
[b] Percentage of meiocytes showing chromosome bridges
[c] Percentage of meiocytes showing lagging chromosomes

anomaly observed in SSH plants was the presence of trinucleated meiocytes in telophase I (Fig. 3F). In telophase II, about 90% of the meiocytes of ASH plants were normal (Fig. 3G). On the other hand, in SSH tetrads the presence of one or more small micronuclei was observed in at least 41.3% of the tetrads analyzed (Fig. 3H). These micronuclei finally resulted in small and sterile pollen grains (Fig. 3I). This phenomenon of chromosome elimination was significantly ($P<0.01$) higher in SSH plants than in ASH plants.

Discussion

A quite large variability could be produced using symmetric and asymmetric somatic hybridization; this variability extended to traits for breeding programs, which are presently under evaluation. Analysis of genomic DNA, genome size and cytological behavior of the SSH and ASH plants showed that almost a full genome of both parents was present in the recombined SSH plants, while in ASH plants, just a small part of *H. maximiliani* genomic DNA was identifiable.

Since somatic hybridization is a random genomic recombination process and the genome composition of the somatic hybrids is not well-known, RAPD markers are necessary for an estimation of hybridity (Henn et al. 1998; Krasnyanski et al. 1998; Binsfeld et al. 2000; Patra et al. 2001). Well-defined RAPD markers are also useful for monitoring the stability of the offspring of somatic hybrid plants, and they can also be applied as efficient and easy selection markers in early growth stages of the plants in advanced backcross experiments to control the genomic stability or gene flow from SSH or ASH to the following generations.

Meiocytes of SSH and ASH plants displayed a high bivalent chromosome pairing, suggesting a high homology between chromosomes of the hybrid plants (Table 1). The fact that some of the bivalents were heteromorphic, open-rod bivalents or that the chromosomes paired end-to-end, suggests partial chromosome homology (Sing 1992; Jan 1997). Multivalent chromosome pairing also might be indicative of intergenomic homology, which helped to introgress genes from *H. maximiliani* to *H. annuus*, as reported for somatic hybrids between *Brassica* (+) *Sinapsis* (Gaikwad et al. 1996) and *Nicotiana* species (Yemets et al. 2000). If this assumption is correct, a more significant impact in the generation of genetic variability in SSH plants could be expected. Evidence of this is the pronounced variability in the RAPD markers of the SSH plants (4:1 respectively for SSH:ASH). The different mechanisms of alien chromosome integration, such as homoeologous chromosome pairing, recombination in meiosis or somatic transfer mechanisms occurring in pre- or post-meiotic cells, have been discussed previously (Sybenga 1992; Chetelat and Meglic 2000).

Meiotic abnormalities (univalent chromosomes, chromosome bridges or lagging chromosomes) and spontaneous chromosome elimination in somatic hybrid plants are common phenomena and are especially prevalent in distantly related parental plants. Shepard et al. (1983) reported somatic chromosome elimination in potato (+) tomato hybrids plants. In the present investigation, we find somatic chromosome elimination in the SSH plants, in which the number of chromosomes varied between 60 and 65 (Table 1) instead of the sum of the parental sets 68 (34+34), as could be expected. Jacobsen et al. (1995) reported abnormalities and chromosome elimination in meiotic cells of somatic hybrids in *Brassica* (+) *Sinapsis*, Barbosa and Vieira (1997) in Passion fruit and Yamada et al. (1998) in potato interspecific hybrids. As summarized in Table 1 and shown in Fig. 3H, our results confirm the presence of several meiotic abnormalities in SSH plants, resulting in genome instability and chromosome elimination and leading to low fertility or even sterility. Despite this common phenomenon in somatic hybrids, many scientists have reported a stable genomic transfer through this technique, especially using ASH (Wolters et al. 1994; Rutgers et al. 1997; Binsfeld et al. 2000; Chetelat and Meglic 2000; Varoto et al. 2001). Furthermore, the latter technique has been successfully used for alien chromosome transfer, the production of addition lines and for the introgression of genes between sexually incompatible species (Ramulu et al. 1995; Rasmussen et al. 1997; Rutgers et al. 1997; Binsfeld et al. 2000).

Since our knowledge of interspecific hybridization in sunflower has been improved (Krasnyanski and Menczel

1995; Henn et al. 1998; Binsfeld et al. 2000), this procedure might be a useful tool for producing variability for breeding programs based on wild *Helianthus* species. The advantages or limitations of such hybrids in breeding programs have not yet been systematically investigated and require more data for further analysis. Chromosome elimination can apparently be overcome by transferring small parts of the genome, preferably one or few chromosomes, to produce specific addition lines, to promote introgression or to favor the expression of new alleles in the recombined hybrid genome.

In conclusion, (1) SSH as well as ASH plants can be used in different breeding programs; (2) SSH plants appear to produce a wide range of variability in genomic controlled traits, in cytoplasmic controlled genes and in species in which vegetative propagation is usual; (3) in contrast, ASH plants appear to be more applicable for limited or specific gene transfer – i.e. the introgression of gene groups or gene families, chromosome parts or chromosomes for the production of addition lines. ASH plants are also associated with a low presence of anomalies and higher plant fertility.

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