Theor Appl Genet (1998) 97: 1240–1247

L. Natali · T. Giordani · E. Polizzi · C. Pugliesi
M. Fambrini · A. Cavallini

Genomic alterations in the interspecific hybrid *Helianthus annuus* × *Helianthus tuberosus*

Received: 22 May 1998 / Accepted: 4 June 1998

Abstract The genome of a *Helianthus annuus* (2n = 34) × *Helianthus tuberosus* (2n = 102) hybrid was studied at cytological, biochemical and molecular levels and compared to those of the parental species. Cytophotometric analyses showed that the hybrid has a 4C DNA content higher than expected and with a larger variability than in the parents. This high variability is probably not related to chromosome-number variations since the hybrid always had 2n = 68 chromosomes. Moreover, hybrid interphase nuclei showed lower heterochromatin condensation than the parental ones. Thermal denaturation of genomic DNAs indicated that quantitative variation of some DNA families occurred in the hybrids compared to parents. Finally, molecular analyses of DNAs restricted with different enzymes, after Southern blotting and hybridization with HR probes, showed restriction patterns in the hybrid different from those observed in parents. These results indicate that interspecific hybridization between *H. annuus* and *H. tuberosus* may determine quantitative variation of some DNA families and differential DNA methylations that probably modify the nuclear structure. These phenomena are probable responses to a "genomic shock" following the interspecific cross.

Key words Chromatin structure · Genetic variability · Genomic shock · *Helianthus* · Interspecific hybridization

Communicated by F. Mechelke

L. Natali · E. Polizzi · C. Pugliesi
M. Fambrini · A. Cavallini (✉)
Dipartimento di Biologia delle Piante Agrarie,
Sezione di Genetica, Via Matteotti 1/B, I-56124 Pisa, Italy
Tel.: +39 50 571567
Fax. +39 50 576750
E-mail: acavalli@agr.unipi.it

T. Giordani
Scuola Superiore di Studi Universitari e Perfezionamento "S. Anna",
Via Carducci, I-56100, Pisa, Italy

Introduction

When genetic material of one species is inserted into another, i.e. into a different nucleo-cytoplasmic background, absence of gene expression may often be observed, due either to gene inactivation or even to elimination of the alien gene, chromosome or genome. A classical example is the interspecific cross between *Hordeum vulgare* and *Hordeum bulbosum*, where a progressive elimination of *H. bulbosum* chromosomes occurs during hybrid embryo development (Kasha and Kao 1970). Another example concerns the hybrid *Crepis capillaris* × *C. neglecta* where only NORs from *C. capillaris* are active (Wallace and Landgridge 1971). More recent experiments have shown that genes transferred through genetic transformation are sometimes inactivated by base methylation (Martin-Tanguy et al. 1996; Park et al. 1996). The introduction of alien genetic material into a new genetic background may be defined "genomic shock" (McClintock 1984).

Genomic shock occurring in conjunction interspecific hybridization is particularly important both in plant breeding programs (Carter 1978) and in the evolution of cultivated plants since many cultivated species (wheat, tobacco, cotton, etc.) are allopolyploid, originating from the formation of an interspecific hybrid (Lewis 1979). Both cultivated and wild species are the result of a complex evolution that has conferred a high degree of variation on the original genome, as is the case of polyploids showing a nuclear DNA content lower than the expected multiple of the diploid ancestor (Bennett and Smith 1976; Marchi et al. 1983), or in the number of genes belonging to particular gene families (Feldman 1976; Rieseberg et al. 1995 a). Has all

genome differentiation from the ancestor species been occurring during the course of evolution or was part of it established during the early generations of the interspecific hybrid? It is known that during the early generations of such a hybrid chromosome elimination and rearrangement may occur due to imprecise meiotic pairing (Lacadena 1978; Sharma and Gill 1983) or to the combination of epigenetic variations in the two species (Jablonka and Lamb 1989). Less known is the occurrence, in the interspecific cross, of processes that can be defined as "cryptic" involving quantitative and/or qualitative variations of particular DNA families. In the present paper we have studied the genome of the interspecific hybrid *Helianthus annuus* × *Helianthus tuberosus*, compared to those of parental species, to determine whether genomic shock following interspecific hybridization has given rise to genetic variation.

Materials and methods

Plant material

The 'HA89' line of *H. annuus* and the clone 'S. Pietro' of *H. tuberosus* were crossed as female and male parent, respectively. HA89 is a male-sterile line propagated in our Department through crosses to its maintainer isogenic line for at least 10 years. Plants of the S. Pietro clone were maintained exclusively through vegetative tuber propagation, ensuring high genetic stability. Sunflower seeds were germinated in damp vermiculite under sterile conditions at 25°C. The seedlings were cultivated in the greenhouse until maturity in Jiffy pots containing mould which had been sterilized by autoclaving. *H. tuberosus* plants were developed from tubers and cultivated in the same greenhouse. At maturity, flowers of *H. annuus* were fertilized by *H. tuberosus* pollen and seeds were collected. The hybrid nature of the collected seeds was verified by chromosome counts on secondary roots of plantlets germinated as above. Hybrids were cultivated in the greenhouse until maturity. Immature leaflets of hybrid or parental plants were used as experimental material for cytological and cytophotometric analyses. DNA extraction and analysis was carried out on adult leaves of the three genotypes.

Cytophotometric determinations

Root apices of hybrid plants and immature leaflets of the three genotypes were fixed in ethanol/acetic acid 3:1 (v/v) and then treated in 4% pectinase (Sigma Chemical Co.) for 30 min at 37°C. Squashes were made in a drop of 45% acetic acid and cover slips were removed by the dry ice method. Slides were then hydrolysed in HCl under different conditions: either 1 N at 60°C for 8 min or 5 N at 20°*C* for 10–60 min. After hydrolysis, slides were Feulgen-stained in 0.5% basic fuchsin for 1 h at room temperature, washed twice in SO_2 water for 15 min, dehydrated and mounted in D.P.X. balsam (Fluka Chemie). The chromosome number of the hybrid plants was counted analyzing root apices. Nuclear DNA content was estimated on nuclei squashed from leaflets of the three genotypes by a Barr and Stroud, GN5-type, integrating cytophotometer at a wavelength of 550 nm. Slides to be directly compared were concurrently stained. When simultaneous processing was not possible, due to the large number of preparations to be analysed, squashes made with root tips of a single plantlet of *Vicia faba* (4C = 53.31 pg, Bennett and Smith 1976) were concurrently stained for each group of slides and used as standards to make all results comparable.

Chromatin analyses

On the same slides used for DNA content determinations (using a Leitz MPV3 integrating microdensitometer) the Feulgen/DNA absorption of chromatin fractions with differing condensation were determined by measurements on one and the same 2C (G_1) interphase nucleus, after selecting different thresholds of optical density in the instrument (Cavallini et al. 1989). The instrument read all parts of the nucleus where optical density is greater than the preselected limit: at low thresholds all the nucleus (euchromatin plus heterochromatin) was measured, while at high thresholds only heterochromatin was read. Absolute values of the first-derivative curve of the absorption at different thresholds allow one to discriminate easily between differently condensed chromatin fractions. All absorption values were normalised (absorption = 100, at the minimum optical density threshold) to facilitate comparisons. Optical density curves were also reported as first-derivative curves (in absolute values) to readily distinguish the differently condensed chromatin fractions. For each genotype 20 nuclei × 5 individuals were measured.

DNA isolation and fractionation

DNA was isolated according to the protocol reported by Cavallini et al. (1996a) from adult leaves of the three genotypes and purified by ethidium bromide – CsCl density gradients. Adult leaves were homogenized in liquid nitrogen and lysed at 60° for 15 min in 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.15 N NaCl, 2% sodium dodecyl-sarcosinate, 100 mM diethyl-dithio-carbamic acid. After incubation at 37°C for 3 h in proteinase K (Boehringer; final concentration 250 µg/ml), the mixture was centrifuged at 20 000 g for 15 min and then solid CsCl and ethidium bromide were added to the supernatant up to final concentrations of 0.8 g/ml and 300 µg/ml, respectively. The solution was centrifuged at 44 000 rpm in a Beckman L5-65 ultracentrifuge using a 50 Ti rotor and the DNA band, visualized under long-wave UV illumination, was collected and re-centrifuged. Ethidium bromide was then removed by gentle inversion of the solution with n-buthanol. For fractionating DNA at different C_ot values it was solubilized in 0.12 M Na phosphate buffer pH 7.0 and sheared by sonication in an MSE sonicator at medium energy output for 5 × 5 s with 10-s intervals at 4°C. The DNA was then denatured for 15 min at 103°C, allowed to reassociate according to Britten et al. (1974) up to the desired C_ot values, and fractionated by elution through a hydroxylapatite column equilibrated in the same buffer as above. Single-strand DNA was eluted with this same buffer and reassociated sequences were recovered by elution with 0.5 M Na phosphate buffer. C_ot values for the reassociation of highly repetitive (HR) sequences were 5×10^{-2} for *H. annuus* (Cavallini et al. 1996a), 7×10^{-2} for *H. tuberosus* and 7×10^{-2} for the hybrid. C_ot values for both *H. tuberosus* and the hybrid were determined by a preliminary experiment (data not shown).

Thermal denaturation kinetics

Thermal denaturation of genomic DNA or the highly repetitive DNA fractions was performed in 0.1 × SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) using a Shimadzu UV-2101PC spectrophotometer equipped with a temperature programme controller, and the increase in hyperchromicity at 258 nm was continuously followed. Melting profiles were made by using 40 µg of DNA for each sample.

Restriction endonuclease digestion and gel electrophoresis

Genomic DNA was digested with different restriction endonucleases in a five-fold excess according to the instructions of the suppliers

(Boehringer and New England BioLabs). That digests went to completion was checked by including unmethylated bacteriophage lambda DNA, which, when digested with *Eco*RI plus *Hin*dIII (DNA molecular size-marker III; Boehringer), was also used as a fragment size marker. DNA fragments were separated by electrophoresis performed on horizontal 1.0% agarose slab gels in 0.05 M Tris, 20 mM sodium acetate, 18 mM NaCl and 2 mM EDTA (pH 8.0) at 30 V overnight.

Southern-blot hybridization and densitometric evaluation

Southern blotting of digested DNAs and hybridization were each performed according to standard protocols (Sambrook et al. 1989; Brown et al. 1991). Repetitive fractions of genomic DNA isolated as above were used as probes after having been labelled with digoxigenin-dUTP using a random primed DNA labelling kit (Boehringer). To identify specific HR bands, hybridizations were performed by using labelled HR sequences of one parental species in the presence of an excess (35 : 1) of unlabelled HR sequences of the other species. In some cases, in order to achieve the same labelling intensity in each lane, more genomic restricted DNA of the species to which the unlabelled HR sequences belong was loaded than of the other species. After hybridization, filters were washed sequentially in $2\times$ and $0.3\times$ SSC containing 0.05% SDS at 60°C. Hybridization was detected with a DIG-DNA detection kit (Boehringer) by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin-alkaline phosphatase conjugate).

Results

The interspecific cross between *H. annuus* used as the female parent ($2n = 34$) and *H. tuberosus* ($2n = 102$) was analyzed at the genomic level. Every hybrid plant showed $2n = 4x = 68$ chromosomes. Chromosome-number variability in root apices was very low and possibly related to errors in counting. Because of the chromosome structure of the genus *Helianthus* (small, numerous and very similar chromosomes), it was not possible to detect small chromosome abnormalities.

Cytophotometric analyses

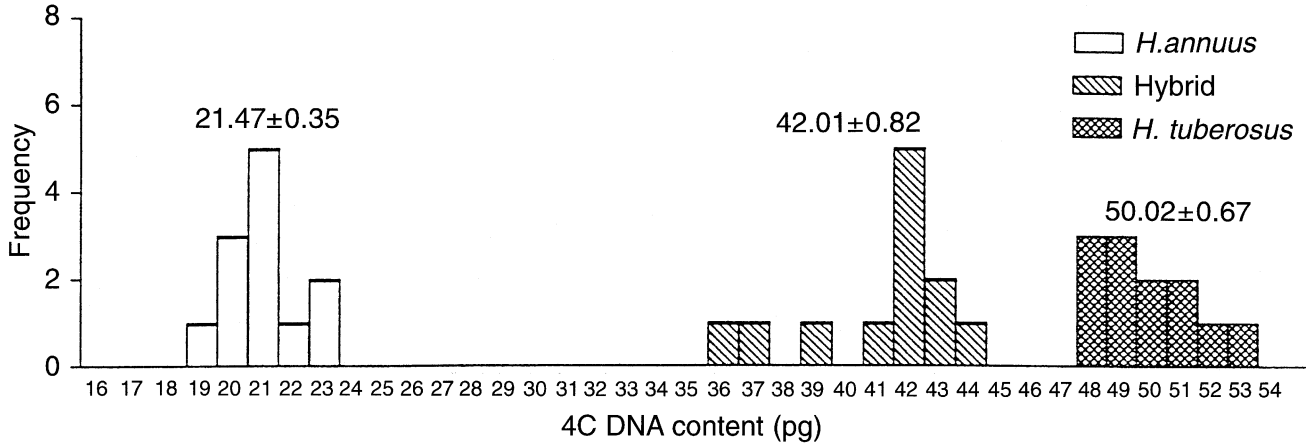
The basic nuclear DNA content ($=4C$) of the parental species and the hybrid plants was measured (see Fig. 1); *H. annuus* plants ranged from 19.91 to 23.05 arbitrary units (a.u.) (15.77% variability), *H. tuberosus* from 48.61 to 53.23 a.u. (9.50%) and their hybrid from 36.98 to 44.90 a.u. (21.42%). Statistical analysis shows that the mean 4C value of the hybrids is higher than the expected mean between the parental 4C values ($P \leqslant 0.001$). The same result was obtained with either hot or cold hydrolysis prior to the Feulgen reaction.

The chromatin conformation in nuclei of parents and the hybrid was measured after Feulgen staining (Cavallini et al. 1996 b). Chromatin structure was studied cytophotometrically in interphase nuclei of the three genotypes by analysing Feulgen absorption at different thresholds of optical density (Fig. 2 a). The presence of differently condensed chromatin fractions may be observed in the first-derivative curves of absorption profiles (Fig. 2 b): values at a 1–3 threshold indicate less condensed chromatin (euchromatin), while values at a 8–12 threshold indicate more-condensed chromatin (heterochromatin). *H. tuberosus* nuclei show a large peak of much-condensed chromatin, which is missing in the *H. annuus* nuclei; hybrid nuclei show a peak of condensed chromatin, but it appears at lower thresholds, i.e. the heterochromatin in the hybrid is less condensed than in *H. tuberosus*. This result is also obtained when comparing first-derivative curves of the profile of hybrid nuclei to the expected profile of the same nuclei, calculated through the weighed mean of the parent profiles ($3 \times H.\ tuberosus + 1 \times H.\ annuus/4$; Fig. 2 c).

Biochemical analyses

Thermal denaturation profiles of DNAs extracted from the three genotypes are reported in Fig. 3. In the first-derivative curve of the denaturation profile, *H. annuus* and *H. tuberosus* DNAs show some heavy shoulders

Fig. 1 4C DNA content (pg) of single plants of *H. annuus*, *H. tuberosus* and the hybrid *H. annuus* × *H. tuberosus*, as determined cytophotometrically after hot hydrolysis, Feulgen staining, and measuring 20 prophase nuclei per plant. Mean 4C DNA content ($\pm$SE) is also reported for each genotype



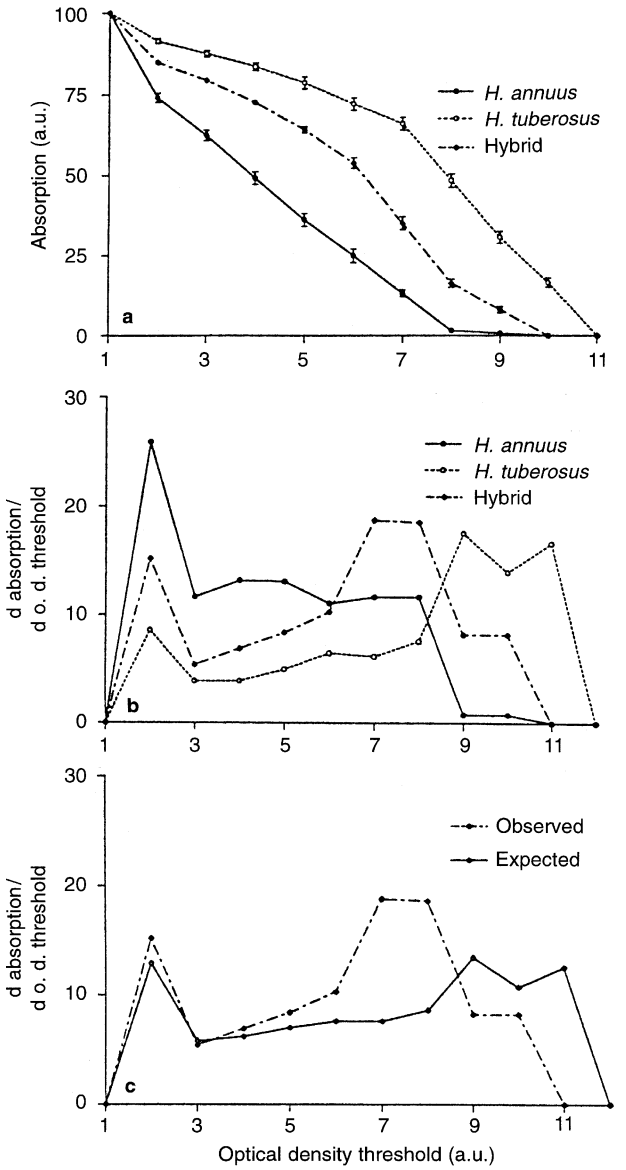


Fig. 2 Optical-density profiles of Feulgen-stained interphase nuclei (G_1) from root meristems of *H. annuus*, *H. tuberosus* and *H. annuus* × *H. tuberosus* (**a**), their first-derivative curves (in absolute values, **b**) and expected profiles, calculated by the weighed mean of parental nuclei curves (three genomes of *H. tuberosus* + one genome of *H. annuus*/4, **c**). For each genotype 30 nuclei were measured (five nuclei × six plantlets) and means ± SE are reported; values at minimal optical-density threshold were normalized (= 100) to facilitate comparisons

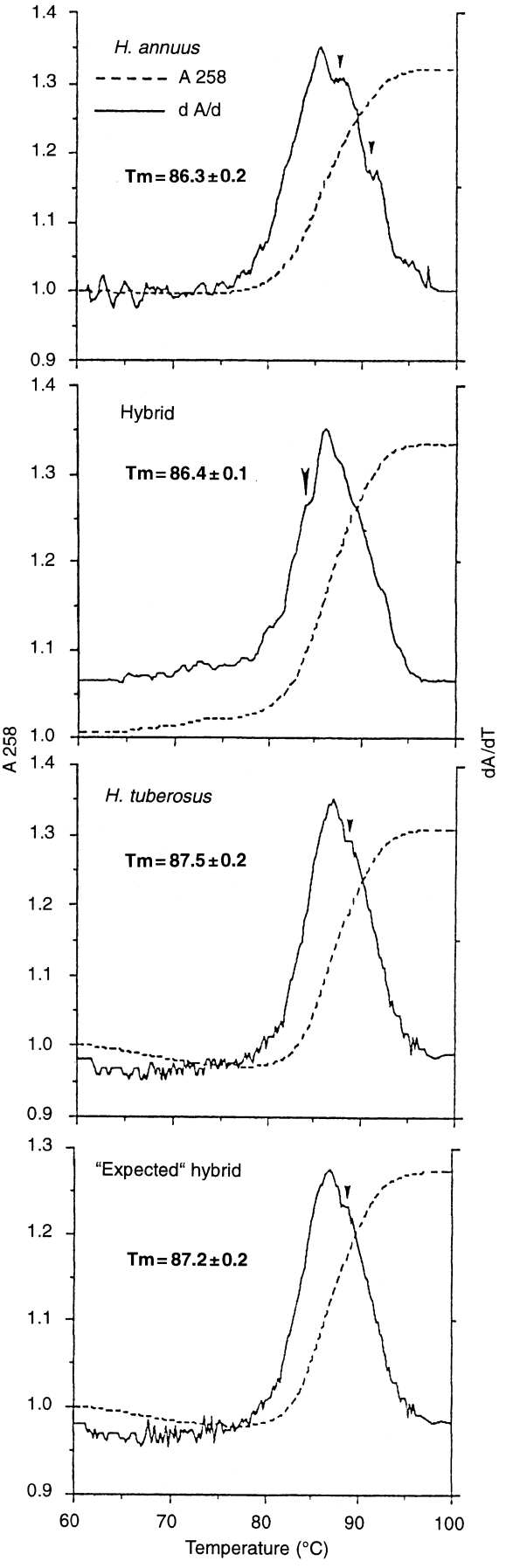


Fig. 3 Melting profiles and first-derivative curves of DNA extracted from leaves of *H. annuus*, *H. tuberosus* and the hybrid *H. annuus* × *H. tuberosus*. Graphs are obtained from the averaged values of four repetitions, two for each of two different DNA extractions. The expected (three genomes of *H. tuberosus* + one genome of *H. annuus*/4) profile for the hybrid *H. annuus* × *H. tuberosus* is also shown. For each genotype the mean (± SE) of the Tm_{50} is reported. *Arrows* indicate the position of particular A-T- or G-C-rich DNA families

(G-C-rich families) while their hybrid does not; moreover, in the hybrid DNA at least one light shoulder (A-T-rich family) appears that is not detectable in the parental DNAs. Because of the nuclear DNA-content variability found cytophotometrically among hybrid plants, we analyzed DNA thermal denaturation of single hybrids: only minor variations were observed (data not shown). From the data reported in Fig. 3 it is evident that the Tm50 of hybrid DNA is lower than expected.

Molecular analyses

Molecular analyses of genomes of parental and hybrid plants were performed on either pooled or individual plants. The use of pools of plants facilitates the detection of any variability possibly related to heterozygosity in the *H. tuberosus* clone; on the other hand, analyses on single individuals establish the frequency of variations.

The experiments included DNA digestion with restriction enzymes differently sensitive to cytosine methylation (*Msp*I and *Hpa*II, *Bst*NI and *Eco*RII), gel electrophoresis, Southern blotting and hybridization using as probes digoxygenin-labelled HR sequences of *H. annuus* or *H. tuberosus*, respectively, in order to detect bands specific to one or the other species.

When using pooled plants, no variation from the expected was observed in *Msp*I-digested DNAs probed with sunflower HR sequences (Fig. 4 a). The use of *Hpa*II shows that the vast majority of HR sequences are methylated at these sites in all three genotypes (Fig. 4 b). However, a sequence that is unmethylated in sunflower is absent in the hybrid, probably due to cytosine methylation of the restriction site after the interspecific cross.

After digesting with *Msp*I and probing with *H. tuberosus* HR sequences (Fig. 4 c) a ladder can be observed, indicating the presence of a tandem repeated sequence (approximately 480-bp long, and absent in sunflower DNA) which is differently methylated at the restriction site. A less extended ladder is observed in the hybrid as compared to the parents, indicating a hypo-methylation of this sequence after the interspecific cross. After digestion with *Hpa*II and probing with *H. tuberosus* HR sequences, all sequences were shown uncut, and hence methylated, with no variation among the three genotypes (data not shown).

When DNA was digested with *Bst*NI it was observed that: (1) after probing with sunflower HR sequences, one sunflower and two *H. tuberosus* DNA bands were missing in the pooled hybrids (Fig. 4d), while (2) after probing with *H. tuberosus* HR sequences, no variations occurred among the three genotypes (data not shown). The use of *Eco*RII showed no difference among genotypes and indicated that all *Eco*RII sites in the HR sequences are methylated (data not shown).

Twenty single hybrids were then analyzed to establish if the observed variations were common. Even though this number of individuals is not very large, no difference was observed among hybrids. Figures 5 and 6 illustrate the labelling patterns of 5–6 of the 20 hybrids.

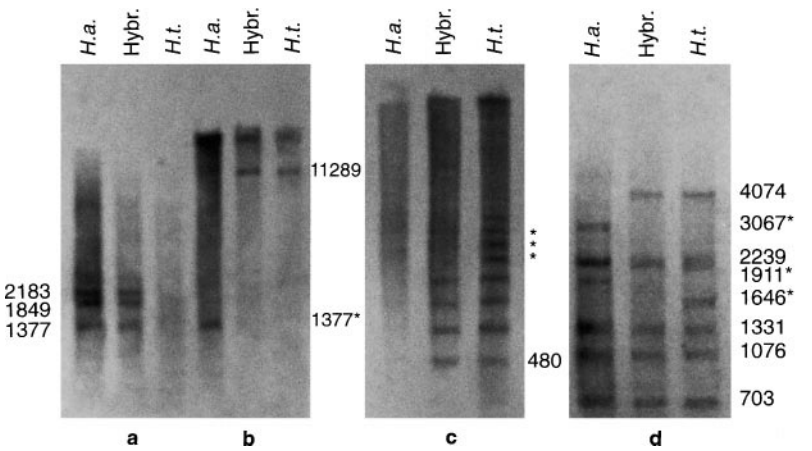


Fig. 4 Southern blot of DNA of *H. annuus*, *H. tuberosus* and the hybrid *H. annuus* × *H. tuberosus*, restricted by (**a**) *Msp*I or (b) *Hpa*II and probed with digoxygenin-labelled HR DNA of *H. annuus*, (**c**) *Msp*I and probed with digoxygenin-labelled HR DNA of *H. tuberosus*, (**d**) *Bst*NI and probed with digoxygenin-labelled HR DNA of *H. annuus*. Hybridizations were performed in the presence of an excess (35 : 1) of unlabelled HR DNA of *H. tuberosus* or *H. annuus* as a competitor, to highlight specific repeated sequences. DNA samples were extracted from leaves of 30 pooled plants. *Asterisks* indicate bands showing an unexpected pattern in the hybrid DNA. Molecular weights were calculated by co-running molecular marker-III DNA (Boehringer) and the integrity of genomic DNAs was checked by co-running uncut DNA (data not shown)

Discussion

The different experiments reported in this paper indicate that interspecific hybridization between *H. annuus* and *H. tuberosus* may be accompanied by quantitative DNA sequence variations and by DNA structural

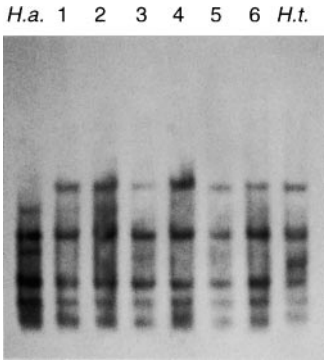


Fig. 5 Southern blot of DNA of *H. annuus*, *H. tuberosus* and six individuals of the hybrid *H. annuus* × *H. tuberosus*, restricted by *Bst*NI and probed to digoxygenin-labelled HR DNA of *H. annuus*. Hybridizations were performed in the presence of an excess (35 : 1) of unlabelled HR DNA of *H. tuberosus* or *H. annuus* as a competitor, to highlight specific repeated sequences. Double quantities of DNA were loaded for hybrids and *H. tuberosus* in order to achieve similar labelling intensities. Molecular weights as in Fig. 4 d; the integrity of genomic DNAs was checked by co-running uncut DNA (data not shown)

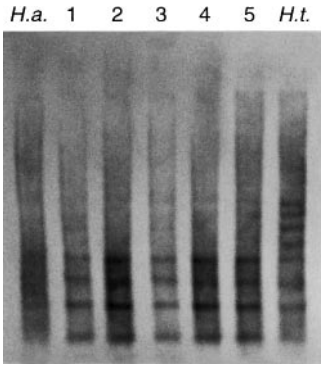


Fig. 6 Southern blot of DNA of *H. annuus*, *H. tuberosus* and five individuals of the hybrid *H. annuus* × *H. tuberosus*, restricted by *Msp*I and probed to digoxygenin-labelled HR DNA of *H. annuus*. Hybridizations were performed in the presence of an excess (35 : 1) of unlabelled HR DNA of *H. tuberosus* or *H. annuus* as a competitor, to highlight specific repeated sequences. Double quantities of DNA were loaded for hybrids and *H. tuberosus* in order to achieve similar labelling intensities. Molecular weights as in Fig. 4 c; the integrity of genomic DNAs was checked by co-running uncut DNA (data not shown)

modifications (as, for example, differential base methylations) probably affecting nuclear structure.

In particular, cytophotometric analyses show that the mean 4C nuclear DNA content of the hybrid is higher than the mean between the parents (Fig. 1). A similar behaviour was reported for an interspecific hybrid between two other *Compositae* species, *Microseris douglasii* and *M. bigelovii* (Price et al. 1985).

Part of the 4C DNA-content variability among individual hybrids could be due to variability in the parental species. For example, in *H. annuus* intraspecific genome-size variation has been reported within progenies of pure lines (Cavallini et al. 1986; Spencer-Johnston et al. 1996). In our lines, this variability was shown to be related to seed position in the inflorescence: seeds collected at the periphery showed a higher DNA content than seeds collected from the centre of the head (Cavallini et al. 1989; Natali et al. 1993). In the present experiments, we used *H. tuberosus* pollen on pistils of ovaries located on at the periphery of one and the same head, where quantitative DNA variations, if any, should be only minor. For *H. tuberosus*, it cannot be excluded that, as a consequence of the high heterozygosity of this allogamous and vegetatively propagated species (Heiser 1976), the DNA content in pollen grains may be different. However, the range of variability measured among individuals of the clone used in our experiments is very small (9.50%) compared to those observed in *H. annuus* (15.77%) and in the hybrid (21.42%). This indicates that, at least in part, genome size variation among hybrids is related to the interspecific cross.

DNA-content variation could be due to changes in chromosome number, as documented in other interspecific hybrids, for example *Hordeum vulgare* × *H. bulbosum* where a progressive elimination of *H. bulbosum* chromosomes is observed during development (Kasha and Kao 1970). In the present case, the possibility that the DNA increase and variability depend on chromosome- number variation cannot be excluded. However, some hybrids were analyzed during development, and before meiosis, and all showed the expected chromosome number ($2n = 4x = 68$); therefore, the variation in 4C DNA content should be due to processes of differential DNA replication (amplification, under-replication and under representation), whose importance in species differentiation is commonly accepted (Flavell 1982; Nagl 1990; Cavallini and Natali 1991).

The occurrence of DNA amplification processes is also supported by biochemical analyses: thermal denaturation of the genomic DNA of the hybrid shows at least one A-T-rich shoulder that is absent in the parental species (Fig. 3). Differential DNA replication after an interspecific cross was reported for the hybrid between *Nicotiana rustica* and *N. tabacum* (Neelam and Narayah 1994) and between *Triticum aestivum* and *Secale cereale* (Lapitan et al. 1988). In those cases, however, analyses were performed on plants regenerated in vitro: hence, variation might depend also on tissue culture-stress. It is known, in fact, that in vitro culture may determine a genomic shock in plant tissues (Peschke and Phillips 1992).

As to nuclear structure, the presence of differently condensed chromatin was investigated in the three genotypes by cytophotometric determination of nuclear optical-density profiles. Chromatin condensation may in fact reflect different gene activities of the DNA involved, possibly due to processes of base methylation (Heslop-Harrison and Bennett 1990; Monk 1990; Selker 1990).

Differences in chromatin conformation are observed in the nuclei of the hybrid plant compared to those of its parents (Fig. 2): the condensed chromatin (heterochromatin) is less condensed than expected. This reduced condensation might result in a higher gene activity of DNA sequences interspersed therein. In this sense, the reduced heterochromatin condensation observed in hybrid plant nuclei might be related to a different degree of base methylation of the DNA sequences involved: thus, the Tm value of genomic DNA in the hybrid is significantly lower ($P \leqslant 0.01$) than expected (Fig. 3). As discussed above, this may be due to amplification of A-T-rich sequences, but also to reduced DNA methylation. It is known, in fact, that base methylation increases the thermal stability of double-stranded DNA (Pivec et al. 1974): consequently, reduced base methylation might determine both Tm reduction and heterochromatin de-condensation.

The occurrence of differential base methylation and, probably, differential DNA replication is also supported by Southern bloting and HR hybridization (Figs. 4–6). HR bands usually behave as common molecular markers (with incomplete dominance): bands occurring in one or both parental DNAs are observed also in the hybrid DNA. However, in some cases restriction patterns are different in the hybrid as compared to the parental DNAs. In particular, one sunflower and two *H. tuberosus* bands, revealed after digestion with *Bst*NI and probing with *H. annuus* HR sequences, are absent in the hybrid pattern, though they are present in at least one parental species (Fig. 4 d). This result may be explained by DNA rearrangements occurring in the hybrid, possibly related to DNA amplification or under- representation in the hybrid genome. Analogous results were reported in the hybrid *Oryza alta* × *Oryza sativa* (Mao et al. 1995), in the subsequent generations of a synthetic allopolyploid of *Brassica* (Song et al. 1993, 1995; Heath and Earle 1996) and, as evidenced by RAPD analysis, in the hybrid *H. annuus* × *Helianthus petiolaris* (Rieseberg et al. 1995 b) and in natural hybrids of *Cyrtandra* (Smith et al. 1996). In all such cases (including our hybrid) the observed variability could be due to heterozygosity in the parental plants. In our case, however, the *H. annuus* line is highly inbred (it does not show any phenotypic segregation); consequently, the observed variations concerning *H. annuus* bands should be related to real rearrangements. The *H. tuberosus* clone is probably highly heterozygous, but, since some experiments were performed on DNA isolated by using pooled plants, the restriction pattern observed for *H. tuberosus* or for the hybrid should include all the existing variability.

Southern-blot experiments also identified variations in the cytosine methylation of HR sequences in the hybrid compared to the parents. In particular, in the parental DNA restricted with *Msp*I, *H. tuberosus* HR sequences show a ladder that is much reduced in the hybrid, indicating that sites methylated in the hexaploid parent are not methylated in the hybrid (Fig. 4 c).

A reduction in repetitive DNA methylation in hybrid plants is in complete agreement with the cytophotometric (showing a reduced heterochromatin condensation) and biochemical (showing a reduction of Tm value) data. However, probably not all repeated sequences are hypo-methylated in the hybrid: for example, Fig. 4 b indicates that a sunflower HR sequence is hyper-methylated.

It is worth recalling that foreign DNA in eukaryotic cells is frequently methylated and inactivated (Doerfler 1991): for example, variations in DNA base methylation have been reported in DNA sequences transferred via genetic transformation (Martin-Tanguy et al. 1996; Park et al. 1996). Since, however, there is no correlation between transgene expression and methylation, it is conceivable that methylation is not the only mechanism of inactivation: epigenetic inactivation can also result from the activity of DNA-binding proteins (Jablonka and Lamb 1995). Indeed, the observed variations in chromatin conformation in our hybrid may possibly depend on the combination of DNA-binding proteins from the two species.

Molecular analyses on single hybrid plants did not show any difference between individuals (Figs. 5, 6). This indicates that the observed genomic alterations occurring after the interspecific cross are, if not general, at least very common.

In conclusion, the present experiments indicate that, under our experimental conditions in the interspecific hybridization between *H. annuus* and *H. tuberosus*, DNA modifications occur, probably altering nuclear structure and giving rise to quantitative DNA variation. Unexpected genome changes in interspecific hybrids were recently reported to occur in F_2 or later generations of synthetic allopolyploids of *Brassica* (Song et al. 1993, 1995). In this case, changes in the genome of hybrid progeny might be due to meiotic abnormalities in the F_1 or to the segregation and selection of recombinant gametes. In our experiments, since variations were observed directly in the F_1, they probably should be considered as related to the "genomic shock" due to interspecific hybridization. The nature and consequences of these variations have still to be evaluated, but they are possibly important during plant evolution. Studies are in progress to determine if these phenomena occurr also in other genotypes of these two species or other species and to ascertain if the variations observed are, or are not, maintained in either selfed or backcrossed progenies of these hybrids.

Acknowledgements We thank Prof. Francesco D'Amato for critical reading of the manuscript. This work was supported by funds of University of Pisa, Italy.

References

Bennett MD, Smith JS (1976) Nuclear DNA amounts in angiosperms. Phil Trans Royal Soc Lond B 274 : 227–274

Britten RJ, Graham DE, Neufeld BR (1974) Analysis of repeated DNA sequences by reassociation. Methods Enzymol 29: 363–405

Brown PTH, Gobel E, Lörz H (1991) RFLP analysis of *Zea mays* callus cultures and their regenerated plants. Theor Appl Genet 81 : 227–232

Carter JF (1978) Sunflower science and technology. Am Soc Agronomy, Madison, Wisconsin, USA

Cavallini A, Natali L (1991) Intraspecific variation of nuclear DNA content in plant species. Caryologia 44 : 93–107

Cavallini A, Zolfino C, Cionini G, Cremonini R, Natali L, Sassoli O, Cionini PG (1986) Nuclear DNA changes within *Helianthus annuus* L.: cytophotometric, karyological and biochemical analyses. Theor Appl Genet 73 : 20–26

Cavallini A, Zolfino C, Natali L, Cionini G, Cionini PG (1989) Nuclear DNA changes within *Helianthus annuus* L.: origin and control mechanism. Theor Appl Genet 77 : 12–16

Cavallini A, Natali L, Giordani T, Durante M, Cionini PG (1996 a) Nuclear DNA changes within *Helianthus annuus* L.: variations in the amount and methylation of repetitive DNA within homozygous progenies. Theor Appl Genet 92 : 285–291

Cavallini A, Natali L, Cionini G, Castorena-Sanchez I (1996 b) Cytophotometric and biochemical analyses of DNA in pentaploid and diploid *Agave* species. Genome 39 : 266–271

Doerfler W (1991) Patterns of DNA methylation – evolutionary vestiges of foreign DNA inactivation as a host defense mechanism. Biol Chem Hoppe-Seyler 372 : 557–564

Feldman M (1976) Wheats. In: Simmonds NW (ed) Evolution of crop plants. Longman, London, pp 120–128

Flavell RB (1982) Sequence amplification, deletion and rearrangement: major sources of variation during species divergence. In: Dover AG, Flavell RB (eds) Genome evolution. Academic Press, New York, pp 301–323

Heath DW, Earle ED (1996) Re-synthesis of rapeseed (*Brassica napus* L.): a comparison of sexual versus somatic hybridization. Plant Breed 115 : 395–401

Heiser CB Jr (1976) The sunflower. University of Oklahoma Press, Norman, Oklahoma, USA

Heslop-Harrison JS, Bennett MD (1990) Nuclear architecture in plants. Trends Genet 6 : 401–405

Jablonka E, Lamb MJ (1989) The inheritance of acquired epigenetic variations. J Theor Biol 139 : 69–83

Jablonka E, Lamb MJ (1995) Epigenetic inheritance and evolution. Oxford University Press, Oxford, UK

Kasha KJ, Kao KN (1970) High-frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225 : 874–876

Lacadena JR (1978) Interspecific gene transfer in plant breeding. Proc 8th Eucarpia Congr, Madrid, Spain, pp 45–62

Lapitan NLV, Sears RG, Gill BS (1988) Amplification of a repeated DNA sequence in wheat × rye hybrids regenerated from tissue culture. Theor Appl Genet 75 : 381–388

Lewis WH (1979) Polyploidy in angiosperms: dicotyledons. In: Lewis WH (ed) Polyploidy, biological relevance. Plenum Press, New York, pp 241–269

Mao L, Zhou Q, Wang X, Hu H, Zhu L (1995) RFLP analysis of the progeny from *Oryza alta* Swallen × *Oryza sativa* L. Genome 38 : 913–918

Marchi P, Illuminati O, Macioce A, Capineri R, D'Amato G (1983) Genome evolution and polyploidy in *Leucanthemum vulgare* Lam. Aggr. (*Compositae*). Karyotype analysis and DNA microdensitometry. Caryologia 36 : 1–18

Martin-Tanguy J, Sun LY, Burtin D, Vernoy R, Rossin N, Tepfer D (1996) Attenuation of the phenotype caused by the root-inducing, left-hand, transferred DNA and its *rolA* gene. Correlations with changes in polyamine metabolism and DNA methylation. Plant Physiol 111 : 259–267

McClintock B (1984) The significance of responses of the genome to challenge. Science 226 : 792–801

Monk M (1990) Variation in epigenetic inheritance. Trends Genet 6 : 110–114

Nagl W (1990) Gene amplification and related events. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 11. Somaclonal variation in crop improvement I. Springer, Berlin, pp 153–210

Natali L, Cavallini A, Cionini G, Sassoli O, Cionini PG, Durante M (1993) Nuclear DNA changes within *Helianthus annuus* L.: changes within single progenies and their relationships with plant development. Theor Appl Genet 85 : 506–512

Neelam A, Narayah RJ (1994) Studies on backcross progeny of *N. rustica* × *N. tabacum* interspecific hybrids. II. Genomic DNA analyses. Cytologia 59 : 385–391

Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret MA, Matzke AJM, Matzke MA (1996) Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant J 9 : 183–194

Peschke VM, Phillips RL (1992) Genetic implications of somaclonal variation in plants. Adv Genet 30 : 41–75

Pivec L, Horska K, Vitek A, Doskocil J (1974) Plurimodal distribution of base composition in the DNA of some higher plants. Biochem Biophys Acta 340 : 199–206

Price HJ, Chambers KL, Bachmann K, Riggs J (1985) Inheritance of nuclear 2 C DNA content variation in intraspecific and interspecific hybrids of *Microseris* (Asteraceae). Am J Bot 70 : 1133–1138

Rieseberg LH, Randal-Linder C, Seiler GJ (1995 a) Chromosomal and genic barriers to introgression in *Helianthus*. Genetics 141 : 1163–1171

Rieseberg LH, Van Fossen C, Desrochers AM (1995 b) Hybrid speciation accompanied by genomic reorganization in wild sunflowers. Nature 375 : 313–316

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA

Selker EU (1990) DNA methylation and chromatin structure: a view from below. Trends Biol Sci 15 : 103–107

Sharma HC, Gill BS (1983) Current status of wide hybridization in wheat. Euphytica 32 : 17–31

Smith JF, Burke CC, Wagner WL (1996) Interspecific hybridization in natural populations of *Cyrtandra* (*Gesneriaceae*) on the Hawaiian Islands: evidence from RAPD markers. Plant Syst Evol 200 : 61–77

Song KM, Tang KL, Osborn TC (1993) Development of synthetic *Brassica* amphidiploids by reciprocal hybridization and comparison to natural amphidiploids. Theor Appl Genet 86 : 811–821

Song KM, Lu P, Tang KL, Osborn TC (1995) Rapid genome change in synthetic polyploids of *Brassica* and its implications for polyploid evolution. Proc Natl Acad Sci USA 92 : 7719–7723

Spencer-Johnston J, Jensen A, Czeschin DGJr, Price HJ (1996) Environmentally induced nuclear 2 C DNA content instability in *Helianthus annuus* (*Asteraceae*). Am J Bot 83 : 1113–1120

Wallace H, Landgridge WHR (1971) Differential amphiplasty and the control of ribosomal RNA synthesis. Heredity 27 : 1–13