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Chromosomal differentiation in *Helianthus annuus* var. *macrocarpus*: heterochromatin characterization and rDNA location

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The $2n = 34$ chromosomes of the inbred line HA89, and the Flamme and Mirasol hybrids of *Helianthus annuus* var. *macrocarpus* possess centromeric heterochromatin as established by Giemsa C-banding. This heterochromatin can not be differentiated by fluorochromes such as DAPI or Chromomycin A3, with selective affinity for specific DNA base pairs. This situation probably results from either a balanced AT/GC composition of the involved repeat or the existence of alternating repetitive sequences of opposite base pair composition in these heterochromatic areas. However, there is also heterochromatin associated with the secondary constrictions on three pairs of chromosomes. This heterochromatin appears to be GC-rich according to its response to the fluorochrome treatments, thus indicating heterochromatin heterogeneity in *H. annuus*. Silver staining reveals the existence of active NORs associated with these secondary constrictions. *In situ* hybridization with an rDNA probe confirms these results and makes the existence of other inactive rDNA sites unlikely. These results are relevant to evolutionary and breeding studies on sunflowers.

Keywords: chromosome banding, FISH, heterochromatin, NORs, sunflower cytogenetics.

Introduction

Although the genus *Helianthus* is of clear economic and evolutionary interest, the karyotypic status of the 50 or so species that make up the genus (Schilling & Heiser, 1981) has not been analysed to any great extent (Chandler, 1991). Sunflower chromosomes have not been individually identified and numbered, so there are no cytological markers and no work on chromosome banding has been reported. Although some genetic linkage groups have been identified, they have yet to be localized to specific chromosomes (Rieseberg *et al.*, 1993; Gentzbittel *et al.*, 1995). Most of the karyological information about this taxon has come from the analysis of meiotic pairing in interspecific hybrids in the study of hybrid fertility, introgression and genome relationships (Chandler *et al.*, 1986; Chandler, 1991). Our limited knowledge is a consequence of the intrinsic difficulties of studying an organism which has a relatively large number of comparatively small chromosomes. Furthermore, their occurrence in an oily cytoplasm makes good quality preparations difficult to obtain.

On the other hand, the use of banding techniques is a general procedure by which markers for individual chromosomes may be obtained, thereby permitting discrimination of species and varieties at the karyological level. In the sunflower the lack of such markers has made it difficult to analyse the introgression of economically interesting traits and the evolutionary relationships of the genus are not well understood. In fact, it has even been proposed that the recognized species of *Helianthus* are ecotypes of a single ecospecies (Kulshreshtha & Gupta, 1979). As we show in this study, the use of pectinase treatment improves the quality of sunflower chromosomal preparations. This has allowed us to characterize the chromosomes of one of the commonest inbred lines and two varieties of the

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cultivated sunflower species *Helianthus annuus* L. We describe the distribution of the constitutive heterochromatin, its response to specific DNA base pair ligands such as 4′-6-diamidino-2-phenylindole (DAPI) or Chromomycin A3, and the number and position of the Nucleolar Organizer Regions (NORs), as demonstrated by silver staining and fluorescence *in situ* hybridization (FISH) with an rDNA probe.

Materials and methods

Seeds from the inbred line HA89 of *H. annuus* var. *macrocarpus* and the commercial hybrids Mirasol and Flamme were grown in the dark at 25°C. To obtain mitotic chromosomes arrested at metaphase, roots were collected after 3 days and placed in a 0.04 per cent solution of colchicine for 90 min, fixed in 3:1 absolute ethanol:glacial acetic acid and stored at 4°C. Roots were pretreated with a 0.5 per cent pectinase solution in standard pH 4.2 citrate buffer for 1 h at 37°C. The meristems were squashed in 45 per cent glacial acetic acid on microscope slides using a coverslip, the coverslip was removed with a razor blade after immersion in liquid nitrogen and the slides were air dried. Additionally, some root tips were stained employing the standard Feulgen technique and squashed on slides, according to Darlington & La Cour (1976, pp. 125–127). A minimum of 15 roots per line or variety was studied for each technique employed.

C-banding and silver staining treatments were carried out according to Schwarzacher *et al.* (1980) and Lacadena *et al.* (1984), respectively, with minor modifications. Air-dried slides were stained after a minimum of 3 days with 4′-6-diamidino-2-phenylindole (DAPI) or Chromomycin A3 (CMA3) and counterstained with Distamycin-A (DA) or Actinomycin-D (AD), as described by Schweizer (1976, 1980).

The 9 kbp *Eco*RI rDNA fragment of wheat containing the 18S, 5.8S and 26S rRNA coding regions (plus the spacers), obtained from the pTA71 clone (Gerlach & Bedbrook, 1979) was nick translated with digoxigenin-11-dUTP and *in situ* hybridized using the method of Pendás *et al.* (1993). Hybridization sites were detected with antidigoxigenin antibody conjugated to fluorescein isothiocyanate (FISH). Some slides were counterstained with propidium iodide (2.5 μg mL^{-1}) and mounted in Vecta Shield antifade solution (Vector Laboratories).

Preparations were examined at 100× magnification under a microscope equipped with a 100 W epifluorescence system and the appropriate filters for the observation of DAPI- and CMA3-stained chromosomes, and a double filter for the simultaneous observation of fluorescein and propidium iodide in the *in situ* hybridized chromosomes. Light field photographs were taken with Kodak Imagelink HQ film and Kodak Plus-X film was used for fluorescence and *in situ* hybridization photographs.

Results

The techniques used revealed no chromosomal differences among the line HA89 and the two hybrids of *H. annuus* studied.

Feulgen staining

Helianthus annuus has $2n = 34$ chromosomes of similar size which, following Al-Allaf & Godward (1979), can be grouped as four pairs of metacentric (M1–M4), eight pairs of submetacentric (SM5–SM12) and five pairs of subtelocentric (ST13–ST17) chromosomes (Fig. 1a). The similarity in size and morphology of the chromosomes within each group makes further differentiation difficult. However, in all the cases considered, two pairs of submetacentric (SM7 and SM10) and one pair of subtelocentric (ST13) chromosomes show secondary constrictions (Figs 1a,b) which allow them to be distinguished.

C-banding

All the chromosomes possess small amounts of centromeric constitutive heterochromatin, and the three pairs of chromosomes bearing secondary constrictions (satellite chromosomes) also have associated heterochromatin (Fig. 1b). Distal or interstitial C-heterochromatin has not been revealed in any member of the complement. Variation in size between different individuals of these heterochromatic regions has not been observed.

Fluorochrome staining

DA-DAPI staining shows no positive response, but instead the chromosomes stain uniformly (Fig. 2a). Similarly, AD-DAPI does not reveal any clear differentiation, although some centromeric regions yield a diffuse, slightly positive response, although this cannot be reproduced photographically. However, in both cases the heterochromatin associated with the secondary constrictions appears negative, in contrast with the positive response it shows with DA-CMA3.

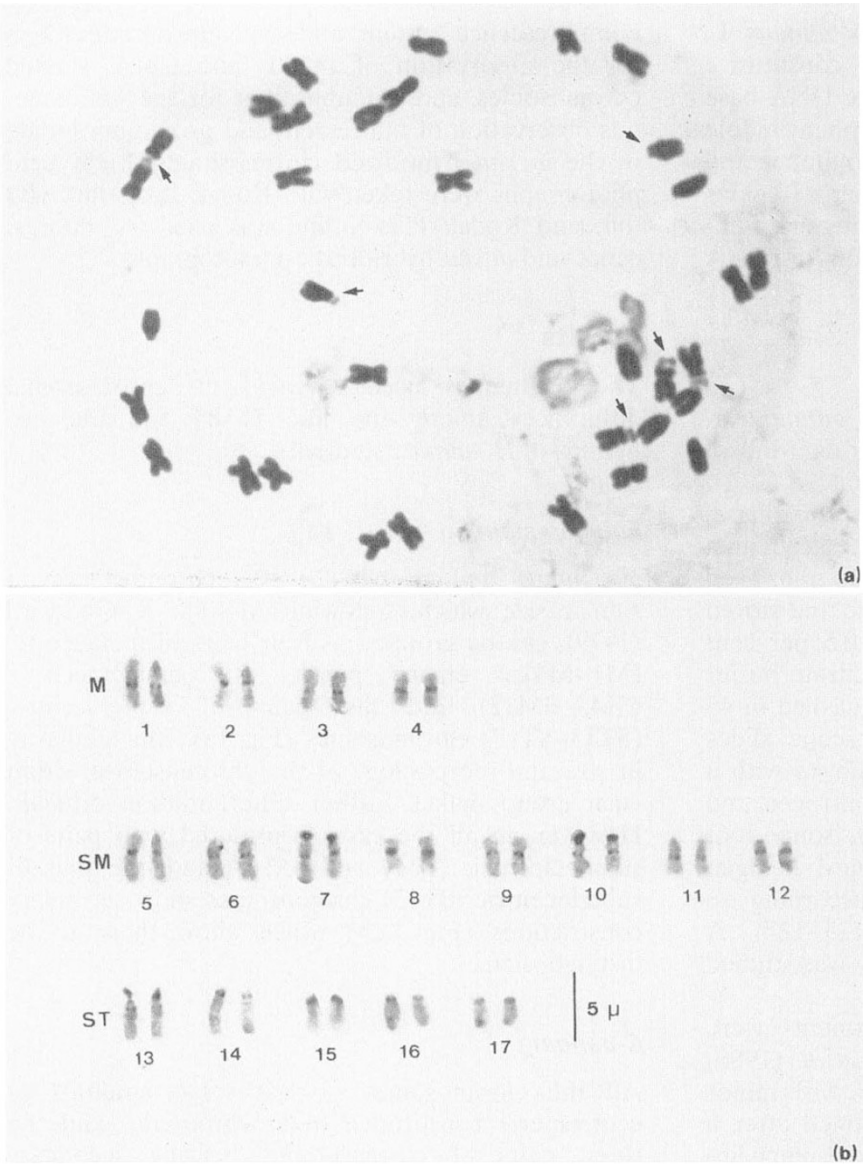

Fig. 1 (a) Feulgen-stained mitotic metaphase chromosomes of the hybrid Flamme of *Helianthus annuus*. Note the existence of three pairs of chromosomes bearing secondary constrictions (arrows). (b) C-banded karyotype of the inbred line HA89. The heterochromatin is restricted to the centromeric areas and the secondary constrictions of the satellite chromosomes (pairs SM7, SM10 and ST13).

No other differentiation is revealed with this latter treatment (Fig. 2b). None of these fluorescence techniques has differentiated non-C-heterochromatic regions.

Silver staining

Silver precipitates attached to the secondary constrictions are shown by the three pairs of satellite chromosomes: SM7, SM10 and ST13 (Fig. 3a). Interphase nuclei show between one and six spots of silver. In a sample of 537 silver-stained interphase meristem cells, 39.8 per cent had one nucleolus, 25.6 per cent had two, 28.3 per cent had three, 4.4 per cent four, 1.1 per cent five and 0.5 per cent six nucleoli.

FISH

The chromosomal *in situ* hybridization of the rDNA probe confirms the existence of ribosomal clusters associated with the secondary constrictions of the three pairs of chromosomes (Fig. 3b). Hybridization does not occur in any other location and interphase nuclei consistently show six hybridization sites (close to 99 per cent nuclei in a sample of 214 cells).

Discussion

Despite the economic and evolutionary importance of the genus *Helianthus* its cytogenetics has hitherto not been investigated in any great detail. This is partially because of the intrinsic difficulty of obtain-

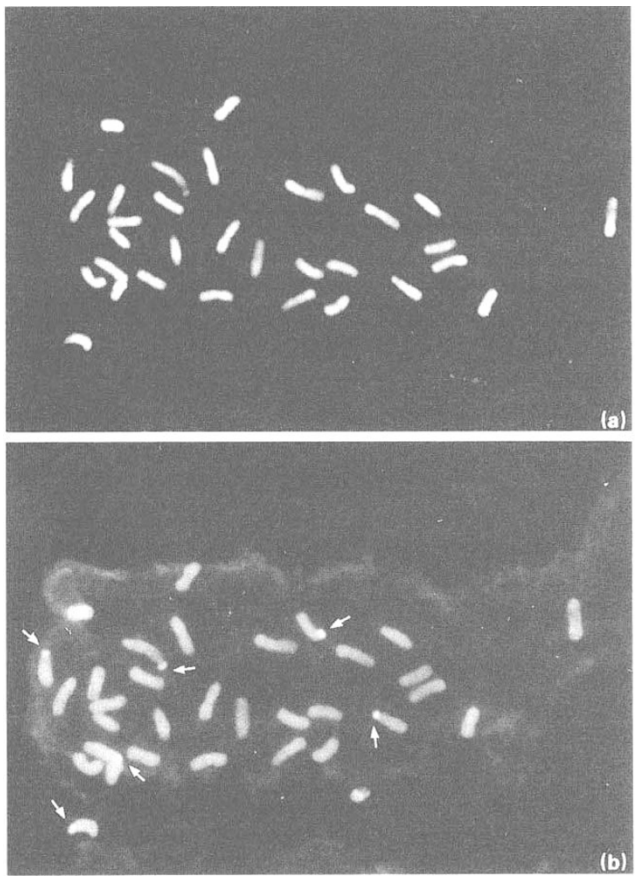

Fig. 2 Fluorescent tri-staining of the mitotic metaphase chromosomes of the commercial hybrid Flamme of *Helianthus annuus*. The DA-DAPI treatment (a) does not show clear differentiation whereas there is positive response to the DA-CMA3 staining of the heterochromatin associated with the secondary constrictions (b).

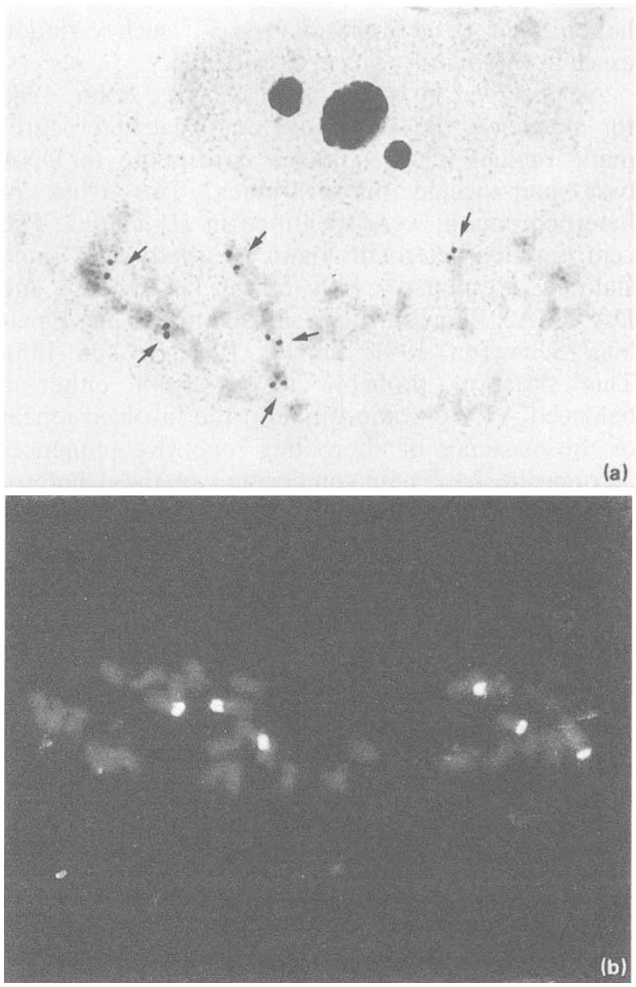

Fig. 3 In the inbred line HA89 of *Helianthus annuus* the silver staining (a) shows six silver spots (NORs) attached to the secondary constrictions of pairs SM7, SM10 and ST13 (arrows) in the mitotic metaphase chromosomes. These results are confirmed by FISH with an rDNA probe (b). Note also the three nucleoli in the interphase nucleus in (a).

ing mitotic chromosome preparations of sufficient quality. The enzymatic pretreatment of the meristematic root tissues with pectinase helps to solve this problem, as is commonly observed in other plants. The chromosome number and karyomorphology of the line and varieties of *H. annuus* var. *macrocarpus* studied here agree with those described by other authors for other varieties (Al-Allaf & Godward, 1979; Chandler, 1991). However, there have been no previous studies of heterochromatin distribution, chromosomal response to specific DNA sequence fluorochromes, or rDNA activity and location.

The absence of variation in the C-heterochromatin distribution within and between the inbred line and varieties studied here suggests that this is very probably the standard heterochromatin pattern of the *macrocarpus* variety, which is the most commonly cultivated sunflower. However, this does not imply that this pattern is invariable within *H. annuus*. It is reasonable to expect that in natural populations of *H. annuus* there exist polymorphisms for the distribution, size or even composition of the heterochromatin. The lack of karyological information concerning this genus precludes comparison of the C-band distribution reported here with the banding pattern of other *Helianthus* species and varieties. C-band variation can provide valuable cytotaxonomic information as has been shown in work on the related Anthemidea (Schweizer & Ehrendorfer, 1983). It would therefore be useful to survey a

larger range of germplasm to see if such variation exists in *H. annuus*.

As observed in other organisms (see John, 1988 for a review), the *H. annuus* equilocal heterochromatic regions have an identical response to DNA base pair-specific fluorochromes. Two types of heterochromatin were identified in *H. annuus*. The centromeric heterochromatin showed the same staining response with the DA-DAPI and DA-CMA3 fluorochromes, indicating no base pair bias (Schweizer, 1981; Sumner, 1990, pp. 155–185). This situation probably results from either a balanced AT/GC composition of the involved repeat or the existence of alternating repetitive sequences of opposite base pair composition in these heterochromatic areas. However, the heterochromatin in the secondary constrictions of the satellited chromosomes was DA-CMA3-positive, showing that it was GC-rich. In any case, the two types of heterochromatin revealed by these treatments confirm, at the cytological level, the coexistence of distinct families of DNA repetitive sequences in the genome of *H. annuus* that have also been distinguished by molecular analysis (K. Sossey, personal communication). DNA content within the genus *Helianthus* exhibits a four-fold range of variation largely because of polyploidy; however, there is also considerable variation in DNA content and chromosome size among sunflower species with the same chromosome number. For example, *H. annuus* (HA89) has the fourth lowest DNA content of the 19 species with $2n = 34$ chromosomes (Sims & Price, 1985). Intraspecific variation in DNA content amongst cultivated varieties and inbred lines of *H. annuus* has also been described (Cavallini *et al.*, 1986; Chandler, 1991; Michaelson *et al.*, 1991). To what extent different amounts of heterochromatin may be involved in this DNA content variation remains to be elucidated, but from our results we know that HA89 has little constitutive heterochromatin, which is confined to small bands in the centromeric areas and secondary constrictions. Further molecular and cytological studies will investigate this relationship.

Silver staining reveals active ribosomal genes (Hubbell, 1985). In root meristem cells from HA89 and the two hybrids of *H. annuus*, the three pairs of satellite chromosomes show silver deposits attached to the secondary constrictions, revealing the position of active rDNA clusters. This coincidence with secondary constrictions as well as the GC richness of the associated heterochromatin are common features of NORs (Schweizer, 1980). The *in situ* hybridization with an rDNA probe confirms the number and location of the rDNA clusters and makes the existence of other inactive ribosomal sites unlikely. The existence of one to three silver spots in most of the interphase nuclei of root meristem cells (93.7 per cent) is probably the result of nucleolar fusion (Giménez-Martín *et al.*, 1977) although FISH with the rDNA probe shows six clearly separated fluorescent dots in the majority of the interphase cells. The six chromosomes with rDNA clusters show silver deposits attached to them at metaphase, indicating that all of them were active during the preceding interphase (Miller *et al.*, 1976).

The search for chromosomal markers in other species of the genus *Helianthus* is helpful in a taxon where (i) there are diploid, tetraploid and hexaploid species, all of which have a basic chromosome number of $x = 17$ and are probably of polyploid origin (Jackson & Murray, 1983; Chandler *et al.*, 1986); (ii) different races of the same *Helianthus* species may be chromosomally distinct, although successful crosses may be produced between perennial and annual species, and between diploid and polyploid species (Kulshreshtha & Gupta, 1979); (iii) the cytogenetical information comes almost exclusively from the analysis of interspecific hybrid meiotic configurations (Chandler, 1991). These chromosomal markers could be useful either from the taxonomic point of view to identify chromosomes from different genomes or to follow the introgression of alien chromosomes in cultivated sunflowers, as happens in other plant species (Gustafson & Dille, 1992; Werner *et al.*, 1992). In any case, the existence of linkage maps in the sunflower (Rieseberg *et al.*, 1993; Gentzbittel *et al.*, 1995) where ribosomal gene polymorphisms have been described (Choumane & Heizmann, 1988) might permit the chromosomes with NORs to be assigned to linkage groups. Current work on sunflower RFLP maps may also yield *in situ* hybridization probes of use in assigning linkage groups to chromosomes.

The study of the constitutive heterochromatin distribution and fluorochrome response, as well as the number and location of the rDNA sequences in other species and other varieties of *Helianthus* will provide complementary information on their evolutionary status and, hopefully, suitable markers for breeding studies. Combined research on breeding, cytogenetics and molecular techniques are required in the sunflower to understand natural and artificial interspecific gene transfer (introgression) and the selective forces involved (Belhassen *et al.*, 1994), as well as to provide new tools to evaluate variability in this taxon.

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