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Chromosomal location of rDNA in *Allium*: in situ hybridization using biotin- and fluorescein-labelled probe

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Summary. A biotin- and fluorescein-labelled probe of *Helianthus argophyllus* has been used to map specific repeated rDNA sequences by in situ hybridization on mitotic chromosomes of *Allium cepa*, *Allium fistulosum*, a diploid interspecific (*Allium fistulosum* × *Allium cepa*) F_1 hybrid, and a triploid interspecific ($2\times = A.\ cepa$, $1\times = A.\ fistulosum$) shallot. Hybridization sites were restricted to satellited and smallest pairs of chromosomes in both *A. cepa* and *A. fistulosum*. The number, size, and position of the hybridization sites distinguish homologous chromosomes and identify the individual chromosomes carrying the nucleolus organizing region (NOR) at the secondary constriction, as well as the individual chromosomes carrying an additional NOR. This in situ hybridization technique is the first reported in a plant species and offers new cytogenetic markers in *Allium*.

Key words: Chromosomes – Introgression – Karyotypic analysis – Non radioactive labelling – NOR regions

Introduction

Specific DNA sequences have been detected often by in situ hybridization using radioactive-labelled probes. Biotin-labelled probes offer an alternative to radio-isotopic labels in both animal and plant mitotic chromosomes (Rayburn and Gill 1985; Ambros et al. 1986; Lapitan et al. 1986, 1987; Rayburn and Gill 1987). Fluorescein has provided an increase in resolving power in animal chromosomes (Baker and Wichman 1990). Biotin- and fluorescein-labelled probes offer advantages over conventional isotope-labelled probes, as they produce higher resolution of the hybridization sites and lower background interference. Signals can be detected in only a few hours compared to several days or even weeks of exposure time. The high resolution and chromosomal detail provided by fluorescein make in situ hybridization with biotin in combination with flourescein labelling an appealing technique for physical mapping of DNA sequences on metaphase chromosomes of plants. The biotin- and fluorescein-labelled probe of rDNA was used to identify hybridizing sequences of specific chromosomes. There are no reports of biotin and fluorescein labelling to visualize in situ hybridization sites in plants. In this investigation, two *Allium* species and their derivatives were analyzed using a ribosomal DNA (rDNA) clone from *Helianthus argophyllus*. Genes coding for rRNA consist of tandemly repeated rDNA units coding for the 5.8S, 18S, and 25S rRNA and separated by nontranscribed regions or external nontranscribed spacers (Choumane and Heizmann 1988). In situ hybridization sites using biotin- and fluorescein-labelled probes offer additional cytological markers in *Allium*.

Materials and methods

Plant material

Fast-growing roots of the following genotypes were harvested at 1–2 cm length: *Allium cepa* (cv 'New Mexico Yellow Grano' 2n=16), *A. fistulosum* (cv 'Heshiko' 2n=16), a diploid interspecific ('Heshiko' × 'New Mexico Yellow Grano') F_1 hybrid, 81215, and a triploid interspecific shallot ($2n=3\times=24$; $2\times = A.\ cepa$, $1\times = A.\ fistulosum$), 'Delta Giant' (Perkins et al. 1958). Root-tip chromosomes were used for karyotype analysis and in situ hybridization.

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Mitotic analyses

Root preparation was as previously described (Peffley and Mangum 1990). Squashes were photographed under 63 × magnification. Karyotypes were done as previously described (Peffley and Currah 1988) and with the squashes obtained from in situ hybridization slides.

Preparation of roots for in situ hybridization

Roots were treated with 1-bromonaphthalene (one drop in 10 ml ddH20) for 5 h at 4 °C, fixed in 3:1 ethanol:glacial acetic acid, and digested in 15% pectinase and 5% cellulase for 2 h at 37 °C in 0.1 *M* sodium citrate buffer, pH 4.7. Squashing was modified from Pijnacker and Ferwerda (1984). Slides were air-dried overnight.

In situ hybridization

All work was carried out under prevailing NIH guidelines for recombinant DNA work. The probe used was clone pHAR 1 obtained from Dr. M. Arnold from the University of Georgia, Athens. This clone consists of plasmid pBR322, which contains a 9.8-kb *Helianthus argophyllus* rDNA sequence. pHAR 1 was produced by *Hin*dIII digestion (Choumane and Heizmann 1988).

The in situ hybridization procedure was essentially that of Moyzis et al. (1987) with these modifications: 0.2 ml RNase (0.1 mg/ml 2 × SSC) (sodium chloride/sodium citrate) was added to each chromosome slide preparation, which was then covered with a cover glass (22 mm × 50 mm) and placed in a humidity chamber and incubated 1 h at 37 °C. Coverslips were removed, slides were rinsed in four changes of 2 × SSC at room temperature, dehydrated 2 min each in room temperature 70, 80, and 95% ETOH, and air-dried. The hybridization mixture was 9 µl formamide, 3 µl 20 × SSC, 10 µl H_2O, and 3 µl of *E. coli* carrier DNA; 5 µl aliquots of the probe (0.5–1 µl DNA) was added to the hybridization mixture. Slides were placed in BT buffer (0.084% sodium bicarbonate w/v, 0.1753% sodium chloride w/v, and 0.005% Tween 20 v/v) at room temperature, and drained but not dried. Each slide was incubated for 5 min at room temperature in 75 µl 5% BSA/BT (0.05 g of bovin serum albumin in 1 ml of BT buffer). Aliquots of 75 µls BT/BSA/avidin (0.1 ml of BSA/BT and 0.003% avidin) were added to each slide and incubated for 45 min in a humidity chamber at 37 °C. Slides were rinsed in four changes of BT buffer at 40 °C, then in room temperature BT buffer. Slides were drained but not dried. Aliquots of 75 µl 5% GS (1.9 ml BT in 0.1 ml goat serum) were added to each slide and incubated at room temperature for 5 min. To each slide 0.075 ml of avidin/antibody mixture (15 µl antibody and 1 ml of 5% GS) was added and incubated for 45 min at 37 °C. Slides were washed in four changes of BT buffer at 40 °C, then in room temperature BT buffer. To each slide was added 75 µl of BSA/BT; then incubated for 5 min at 37 °C. Slides were washed in four changes of BT buffer at 40 °C, then in room temperature BT buffer and drained but not dried.

One drop of an antifade mixture (antifade, 0.01% 4′6-diamidino-2-phenylindole and 0.1% propidium iodide) was added to each slide, which was then covered with a coverslip and stored in the refrigerator. Slides were counterstained with propidium iodide (Sigma) and 4,6-diamidino-2-phenylindole (DAPI; Sigma). Counterstaining with propidium iodide and viewing with ultraviolet light at 436 nm allowed simultaneous observation of the fluorescein-labelled hybridized probe (yellow) and total DNA (orange), while with DAPI and viewing at 365 nm total DNA can be observed. Visualization and photographs were taken with an Olympus Vanox Epi-fluorescent microscope using Ektor (Kodak) 1000 film exposed at E.I,2000.

Results

Metaphase chromosomes of each genotype are presented (Fig. 1). Figure 2 shows a schematic representation of biotin- and fluorescein-labelled chromosomes of each genotype. Karyotypes were used to identify the most likely homologues of the labelled chromosomes (Table 1). The symbols C (=*cepa*) and F (=*fistulosum*) are used when denoting the chromosome number of the respective species, as in chromosomal nomenclature systems of other cultivated plants, e.g. *Secale cereale* (Sybenga 1983).

rDNA sites

At least six metaphase cells each of two different individuals of both species were evaluated.

Allium cepa

The 9.8-kb repeated sequence of pHAR1 hybridized on the short arms of 4 of the 16 chromosomes (Fig. 1): the subtelocentric satellited chromosomes 6C′ and 6C″ and the shortest chromosomes 8C′ and 8C″ (Fig. 2). Subtelocentric chromosome 6C′ has three major hybridization sites: at the satellite, one on, and one below the secondary constriction; 6C″ has one major site on the secondary constriction and a weaker signal at the satellite. Submetacentric chromosome 8C′ has one major site on the terminal region; 8C″ revealed two minor sites at the telomeric position. The yellow signals of chromosomes 6C′ and 6C″ appeared stronger than those of chromosomes 8C′ and 8C″. In certain cells smears of fluorescent signal are observed in the short arms of chromosomes 6C″ and 8C′ below the primary hybridization site (not shown).

Allium fistulosum

In like manner, the probe hybridized to the short arm of chromosomes 5F′, 5F″, 8F′, and 8F″ (Fig. 2). The terminal region of the pair of subtelocentric chromosomes 5 reveals no label. A very clear signal is visible as three dots on chromosome 5F′: on the proximal portion of the satellite, on and below the secondary constriction. Chromosome 5F″ has two major sites: on the proximal portion of the satellite and on the NOR region at the secondary constriction. The secondary constriction is more or less stretched according to the degree of condensation of the chromosomes. The signal is much less intense on the smallest metacentric chromosomes 8 than on subtelocentric chromosomes 5. Two minor sites are labelled at the terminal region of chromosome 8F′. Chromosome 8F″ has two minor interstitial signals.

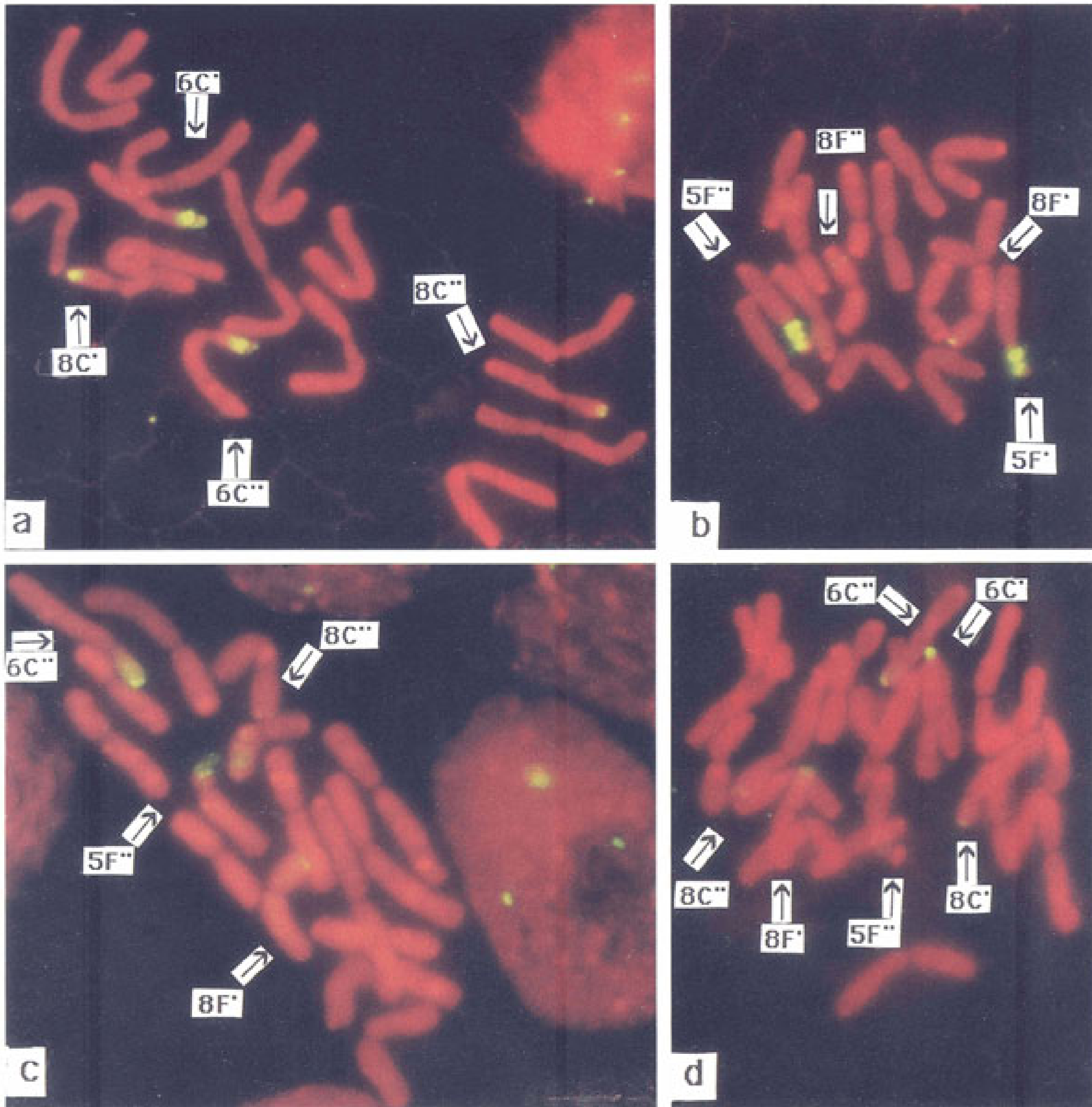

Fig. 1 a–d. In situ hybridization of *Allium* metaphase chromosomes. Yellow indicates areas hybridized with rDNA probe. Orange chromosomal material is hybridized and visualized with propidium iodide. *Arrows* with a number show the labelled chromosomes of each genotype as presented in Table 1. **a** *Allium cepa* cv 'New Mexico Yellow Grano'; **b** *Allium fistulosum* cv 'Heshiko'; **c** a diploid interspecific '81215'; **d** a triploid interspecific 'Delta Giant'

Interspecific derivatives

A. cepa has 27% more total chromosome volume and correspondingly larger chromosomes than *A. fistulosum* (Jones and Rees 1968). The satellites of *A. fistulosum* are larger than in *A. cepa* (Peffley and Currah 1988). In *A. fistulosum* × *A. cepa* F_1 hybrid 81215, 11 metaphase cells each of six different individuals were evaluated. Of the 16 chromosomes, 4 have sites that are labelled (Fig. 1). According to the karyotype (Table 1), the labelled chromosomes are subtelocentric chromosome 6C″ (one major site on terminal region), submetacentric chromosome 8C″ (two minor sites, terminal region), subtelocentric chromosome 5F″ (two major sites, terminal region not labelled), and the smallest metacentric chromosome 8F′ (two minor sites on the terminal region) (Fig. 2).

In the triploid interspecific 'Delta Giant', six metaphase cells each of seven different individuals were evaluated (Table 1). The 9.8-kb repeated sequence was observed to hybridize on 6 of the 24 chromosomes: four

Table 1. Chromosome morphology of *Allium cepa* cv 'New Mexico Yellow Grano', *A. fistulosum* cv 'Heshiko', interspecific F_1 diploid 81215, and interspecific triploid 'Delta Giant'

Genotype	Chromosome	Arm ratio (SA/SA+LA)	Relative chromosome length (%)
'New Mexico Yellow Grano'	1	0.463±0.058	7.61±0.31
	2	0.400±0.037	7.16±0.11
	3	0.399±0.034	6.48±0.20
	4	0.396±0.033	6.18±0.26
	5	0.459±0.019	6.07±0.26
	6	0.262±0.033	5.62±0.34
	7	0.466±0.022	5.48±0.58
	8	0.390±0.020	4.91±0.06
'Heshiko'	1	0.462±0.019	7.76±0.08
	2	0.403±0.024	7.46±0.06
	3	0.402±0.017	6.78±0.17
	4	0.437±0.032	6.30±0.03
	5	0.314±0.105	6.05±0.08
	6	0.488±0.015	5.72±0.11
	7	0.410±0.004	5.04±0.11
	8	0.427±0.038	4.85±0.10
F_1 81215	1	0.458±0.008	8.91±0.25
	2	0.408±0.006	8.56±0.31
	3	0.358±0.042	7.93±0.29
	4	0.405±0.011	7.35±0.10
	5	0.481±0.003	7.24±0.16
	6	0.300±0.008	6.87±0.01
	7	0.453±0.018	6.06±0.11
	8	0.456±0.044	5.09±0.86
	9	0.603±0.103	6.45±0.08
	10	0.381±0.048	6.19±0.03
	11	0.581±0.146	5.39±0.41
	12	0.399±0.010	5.13±0.37
	13	0.354±0.021	5.04±0.15
	14	0.368±0.060	4.73±0.45
	15	0.492±0.092	4.65±0.23
	16	0.413±0.060	4.34±0.54
'Delta Giant'	1	0.476±0.033	5.16±0.35
	2	0.416±0.023	5.10±0.21
	3	0.386±0.018	4.83±0.28
	4	0.548±0.202	4.24±0.74
	5	0.464±0.003	4.14±0.15
	6	0.238±0.008	3.82±0.17
	7	0.430±0.021	3.26±0.02
	8	0.386±0.019	3.13±0.03
	9	0.500±0.001	5.08±0.24
	10	0.423±0.020	5.06±0.16
	11	0.365±0.004	5.01±0.15
	12	0.466±0.065	4.75±0.05
	13	0.454±0.017	4.32±0.27
	14	0.282±0.040	3.74±0.08
	15	0.420±0.031	3.64±0.15
	16	0.428±0.001	3.23±0.04
	17	0.461±0.012	4.41±0.07
	18	0.336±0.029	4.06±0.19
	19	0.404±0.033	3.90±0.14
	20	0.458±0.010	3.80±0.18
	21	0.380±0.033	3.56±0.14
	22	0.500±0.001	3.36±0.10
	23	0.422±0.106	3.21±0.02
	24	0.569±0.146	2.88±0.05

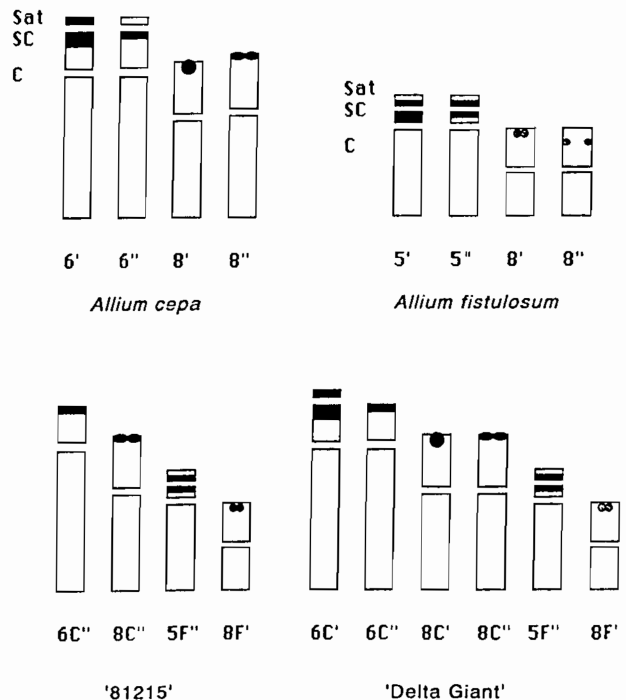

Fig. 2. Schematic representation of in situ hybridization sites on chromosomes of *Allium cepa* cv 'New Mexico Yellow Grano', *Allium fistulosum* cv 'Heshiko', a diploid interspecific '81215', and a triploid interspecific 'Delta Giant'. (Sat = satellite; SC = secondary constriction; C = centromere)

A. cepa and two *A. fistulosum* (Fig. 1). These are the *A. cepa* subtelocentric chromosomes 6 (two to three major sites in 6C′, one major site on terminal region of 6C″), the *A. cepa* submetacentric chromosomes 8 (one major site on terminal region in both homologues but with different strength of the signal), the subtelocentric chromosome 5F″ (two sites, nonlabelled terminal region), and the metacentric chromosome 8F′ (two minor sites on the terminal end) (Fig. 2).

Discussion

Biotin- and fluorescein-labelled in situ hybridization sites were observed on the short arms of chromosomes 6C, 8C, 5F, and 8F. Signals were observed at the terminal regions of all chromosomes except 5F homologues and 8F″. Our results are in agreement with those obtained by in situ hybridization using a radioactive rDNA probe (Schubert and Wobus 1985). *A. cepa* individuals showed four labelled chromosomes, two at the satellited nucleolar chromosomes (6C′ and 6C″) and two at the end of the short arm of the smallest submetacentric chromosome pair (8C′ and 8C″). However, hybridization with

biotinylated and fluoresceinated probe revealed differences between the homologous chromosomes of the two labelled pairs: *A. cepa* subtelocentric satellite chromosomes 6C′ has three signals, whereas 6F″ has only one signal at the telomeric region and a weak signal at the terminal end suggesting an inactive NOR. *A. cepa* 8C′ has a major site at the telomeric region suggesting an additional, small, active NOR; *A. cepa* 8C″ has two minor sites possibly inactive at the telomeric region. Moreover, the signals in 8C are significantly weaker than those at the NOR sites of the usual nucleolar chromosomes 6C′ and 6C″. Using N-banding, Cortes and Escalza (1986) showed that NORs were restricted to the distal, secondary constrictions present in the short arms of *A. cepa* 6 and 8 homologues. Other researchers working with Giemsa C- and G-banding, Feulgen and silver staining found an extra NOR in the smallest submetacentric 8C′ in addition to the NORs in satellite chromosomes in *A. cepa* (Noda 1953; Maggini et al. 1978; Sato 1981). The size and the number of in situ hybridization sites observed in *A. cepa* using biotin- and fluorescein-labelled probe may allow the same conclusion.

The presence of a major in situ hybridization site at the telomeric region of 8F′ suggests the presence of an NOR site in addition to those in the progenitor satellite chromosomes. An intercalary signal on the distal portion of the short arm of 8F″ was observed in all cells. Inversions have been observed in meiosis of *A. fistulosum* × *A. cepa* interspecific F_1 hybrids (Emsweller and Jones 1938; Levan 1936). These results may provide evidence that chromosomes 8F′ and 8F″ are heterozygous for an inversion in the short arm.

Allium cepa and *A. fistulosum* normally contain one pair of satellite chromosomes, each with an active NOR at the secondary constriction (Hizume et al. 1980; Schubert et al. 1983). By silver staining, only one homologous satellite chromosome of *A. fistulosum* shows an active NOR (Schubert 1984). Chromosome 5F′ has two in situ hybridization sites on the NOR region, whereas chromosome 5F″ may have only one in situ hybridization site on the NOR region suggesting an inactive NOR. Schubert (1984) suggests that most (or all) rDNA sequences are deleted in one of the satellite chromosomes, the chromosome we have designated 5F″.

Intercalary heterochromatin regions are organized in such way that certain genes can be easily inserted into them and can be easily lost as well (Ilyin et al. 1978). This may be caused by local amplification or by replication in NOR sites, followed by insertion or breaks in heterochromatin. The shape and intensity of hybridization sites may reflect rRNA gene number (Sato 1981) or gene inactivation by methylation. Repetitive DNA families (i.e., rDNA) are widespread in genomes of higher organisms (Sano and Sano 1990). Differences in NORs due to varying amounts of rDNA have been observed by quantitative comparison of silver-(AgNO3)-stained regions and of silver grains after in situ hybridization with radioactively labelled rRNA between different rDNA sites of humans (Warburton and Henderson 1979) and Chinese hamster (Jhanwar et al. 1981); similar differences in extension of secondary constriction after Feulgen staining and after Giemsa banding have been reported (Schubert 1984). The weaker signal of the intercalary rDNA hybridization site in 8F″ probably consists of fewer rDNA sequences than in the 8F′. In our preparations, smears were observed along the short arm of certain chromosomes in certain cells, which may suggest that some repeated rDNA sequences in a small number of copies are extending proximally toward the primary constriction. Their visualization may depend on the degree of condensation of the chromosomes (i.e., more condensed chromosomes have less smear and a stronger signal).

The chromosomes transmitted to 81215 and 'Delta Giant' can be discerned by their in situ hybridization patterns: 6C″ and 5F″ and 8C″ and 8F′ in 81215; and in 'Delta Giant', 5F″ and 8F′ and the four *A. cepa* chromosomes. *A. fistulosum* chromosomes 5F″ and 8F′ are apparently preferentially transmitted in interspecific individuals.

The use of biotin- and fluorescein-labelled probes provides more precise and more reliable data than those labelled with ^{125}I. Thus, we were able to distinguish homologous satellited chromosomes of 6C and 5F by the number of fluorescent signals. Also, remarkably, both species have NORs on the smallest metacentric chromosomes of their genomes, 8C and 8F, distinguishable by the size and/or the position of labelled sites.

We also infer from this work that rDNA sites of *A. cepa* and *A. fistulosum* homologous to *Helianthus argophyllus* are restricted to the heterochromatic ends of short chromosome arms. Our results agree with the data obtained in *A. cepa* by Durante et al. (1985) and confirm a general hypothesis of a predominant localization of highly repetitive DNA at the telomeric (heterochromatic) regions in the monocotyledons.

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