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"IN SITU" HYBRIDIZATION ON SUNFLOWER METAPHASE CHROMOSMES

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SUMMARY

In situ hybridization on somatic metaphase chromosome from sunflower root tips, by detection system of biotin labeled DNA using streptavidin-alkaline phosphates conjugate, were performed at INRA-Dijon-France. The metaphase chromosomes slides were developed using 1.5 cm root tips of inbred lines CG-8034 and H-13116, from ICCPT Fundulea, România. After "*in situ*" hybridization during 24 hours of incubation with biotin labeled DNA (5 S ribosom DNA of 330 bp), the microscopical view showed the signals at the end of the chromosomes, associated with satelites. Repetitive DNA sequences detected directely on somatic metaphase chromosomes are localized at the heterochromatic regions in the satellites.

Key words: Sunflower, metaphase chromosomes, "*in situ*" hybridization.

INTRODUCTION

"*In situ*" hybridization techniques developed in recent years are important for the detection of specific nucleic acids sequences, directly on chromosomes. The methods for analysis of metaphase chromosomes by "*in situ*" hybridization, allow a study of the distribution of transcriptionally active regions along the lenght of cromosomes and a comparison of the intensity and pattern of labeling in different chromosomes (Kost, 1991). The active regions are important to determine the activity of the individual genes, groups of genes and whole chromosomes.

The application of nonradioactive "*in situ*" hybridization has facilitated the mapping of ribosomal RNA genes in several plant genomes (Matthew et al., 1991). Hueros et al., (1990), reported the use of biotinylated probes in genome characterization in Hordeum.

In soja, "*in situ*" localization using mitotic metaphase cells from aneuploid lines, is a potential alternative to examinate the progeny of wide species crosses, in an effort to generate and identify alien addition and substitution lines (Matthew et al., 1991).

"*In situ*" hibridization tehnique localize nonrepetitive cloned genes to a particular chromosome. Detection of the "waxy" gene on 48% of the chromosome 9 maize pachytene, was performed using tritium detection (Shen et al., 1987).

Repeated DNA sequences have been used as cytological markers in conjuction with nonradioactive "*in situ*" hybridization in wheat (Rayburn and Gill, 1987).

Near the whole repetative DNA can be izolated as satellites. Lewin considers that the DNA satellite is no more than 5% of the total DNA. Sometimes, DNA satellites are surrounding the centromere in heterochromatine, in mitosis, sugesting a possible stuctural function in the chromosome, during segregation (Lewin, 1988).

Biotin labelled probes give a resolution comparable to that obtained with radioactive probes. Lapitan et al., (1986) were able to identify the break point of certain wheat-rye translocations by analyzing the distribution of a dispersed highly repeated DNA sequences of rye.

"*In situ*" hybridization to plant metaphase chromosomes is often impeded due to their highly condensed nature. Sunflower contains 2n=34 small, morphologically similar mitotic metaphase chromosomes. Two pairs of submetacentric chromosomes have satellites (Georgieva – Todorova, 1972, Raicu et al., 1976).

In the present article, we report the first application of "*in situ*" hybridization directly on sunflower metaphase chromosomes.

MATERIAL AND METHODS

Chromosome preparation

Root tips from lines of *Helianthus annuus* L. (CG-8029, CG-8034, and H-13116) germplasm from ICCPT-Fundulea were used as the source for metaphase chromosomes. Root tips were collected from germinated seeds of 2-2.5 cm in petri dishes. Root tips were prefixed in α – bromonaphtalen 25% solution for 1-1.5 hours at 20°C, washed in distilated water, fixed in ethanol:acetic acid:chlorophorm (6:3:1) and stored at 4°C for 24 hours. Cell walls were digested in 5% Rapidase CX-a pectolytic enzyme, for 30 minutes. Squash preparations of metaphase chromosomes were visualized by contrast phase microscope. The cover slides were removed in liquid nitrogen. Siliconated cover slides were used.

Probe and labelling methods

The Petunia 5 S rDNA probe of 330 pb was supplied by the laboratory of INRA-Dijon. The DNA probe was labelled directly by biotin 14-d ATP, following the protocol.

The 5 μl 10x d-NTP Mix., 1 μl 5 S r DNA, 45 μl H_2O ultra pure and 5 μl 10x Enzyme Mix. were incubated at 16°C for one hour. The reaction was stopped by 5 μl stop buffer. The probe precipitated in 100 μl ethanol 100° and 1 μl glycogen at minus 70°C centrifugated at 13300 rmp during 10 minutes and recuperated in 30 μl TE, was stored at minus 20°C.

"*In situ*" hybridization

The slides were treated with ARN-ase (10 μg/ml) in SSC 2x (SSC 1x is 0.5 M NaCl, 0.015 M Na citrare) at 37°C for 1 hour. After washing in SSC 2x for 5 minutes and dehydratation in an ethanol series of 80°, 95° and 100° at room temperature, the slides were denaturated in formamide 70% in SSC 2x at 70°C for 3 minutes. The slides, dehydrated again in 4°C ethanol series of 80,95 and 100, were hybridized at 37°C for 15 hours.

The hybridization solution contains dextran sulphate 10%, formamide 50% in SSC 2x, DNA salmon sperme 5 μg/l, ultrapure distilled water and 5 S r DNA labelled probe (10%). The probe was denaturated for 5 minutes at 99°C and then immediately introduced in hybridization solution. The slides were incubated in a humid chamber at 37°C for one night. After hybridization, the slides were washed 2 times in SSC 2x, in PBS (phosphate buffered saline) and saturated in PBS, pH 7.4, BSA (bovine serum albumine)

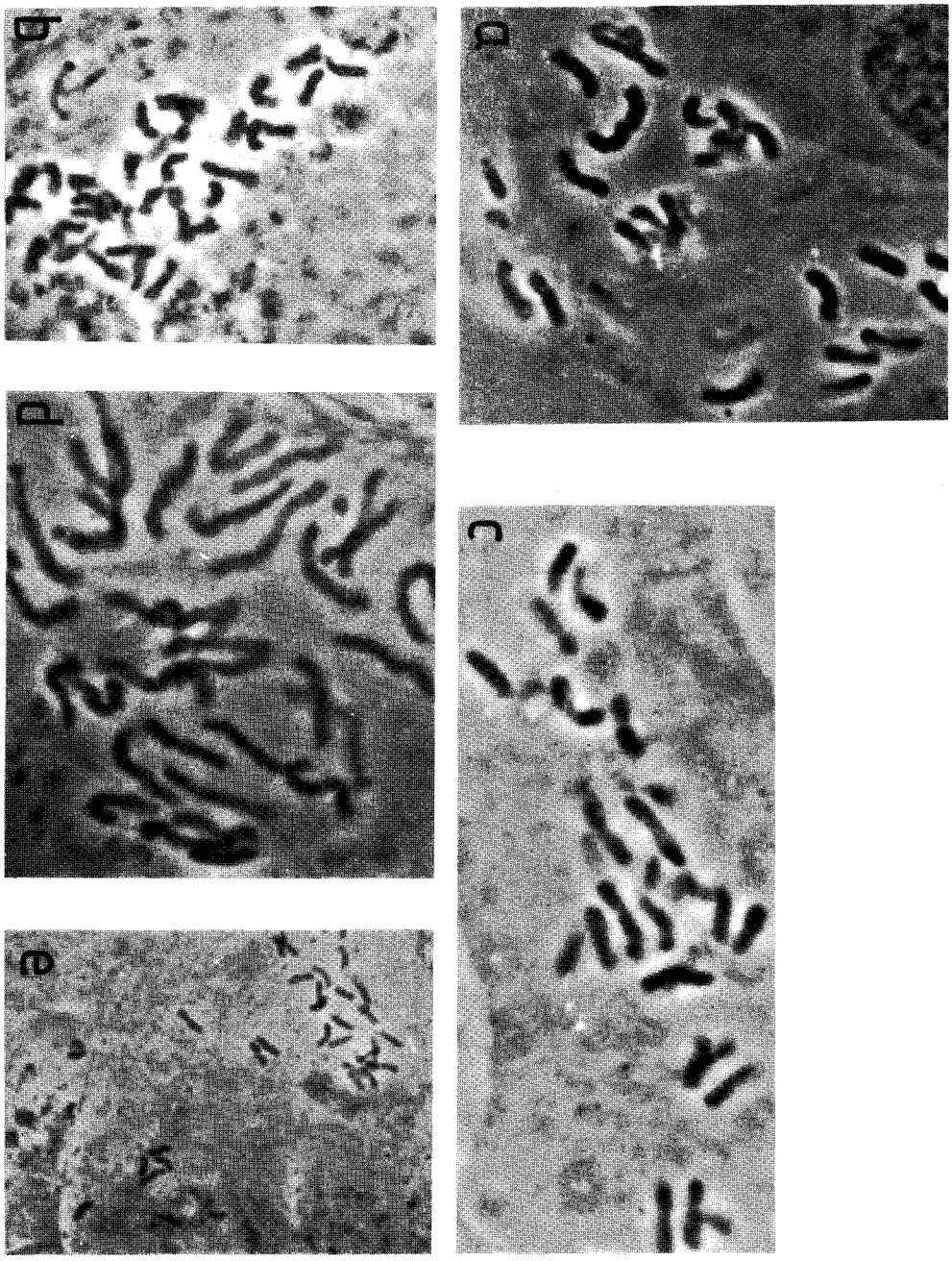

Figure 1. Sunflower metaphase chromosomes stained by Scheef reagent. CG-8029 (a and b) H-13166 (c), CG-13116 (e) and prometaphase H-13116 (d). (a, b, c, d - 100x; e - 40x)

3% and Na azide 1.2 μg/ml. Following the protocol (Kreatech Biotin Detection System), the slides were incubated in streptavidin 20 μg/ml for 45 minutes, washed 2 times in PBS, incubated in phosphatase alkaline 20 mg/ml for one hour, washed 2 times in PBS, saturated in staining buffer, and stained in Giemsa 3%. After washing in phosphate buffer and H_2O ultra pure for 20 minutes, the slides were dried and covered by Eukit for microscope detection of hybridization points.

RESULTS

Chromosome spreading for "*in situ*" hybridization

The preparation of metaphase chromosomes of sufficient quality for fluorescent "*in situ*" hybridization, involved three major steps, including: arrest of cells at mitotic metaphase by chemical pretreatment, digestion of cell walls to obtain clean metaphase cells and a good spreading of the chromosomes.

In sunflower, the best chemical pretreatment to arrest the metaphase chromosomes is using α – bromonaphtalen 25% at room temperature. Figure 1 presents the spreading of sunflower chromosomes by squash method, after 2.5 hours treatment in α – bromonaphtalen, fixation in ethanol-acetic acid 3:1, hydrolysis in HCl 1N for 10 minutes at 62°C and coloration by Scheef reagent.

The sunflower chromosomes are small and many, 2n=34 (68 and 102 in polyploids) and in addition it is difficult to squash them well, in spite of a very good wall cell digestion.

For *in situ* hybridization purpose, the preparations were not hydrolizated and stained.

Analysis of hybridization results

The yellow fluorescent signals are visible on separate metaphase chromosomes (Figures 2-5). In the photos on the figures, the signals are hardly visible. Sometimes, using

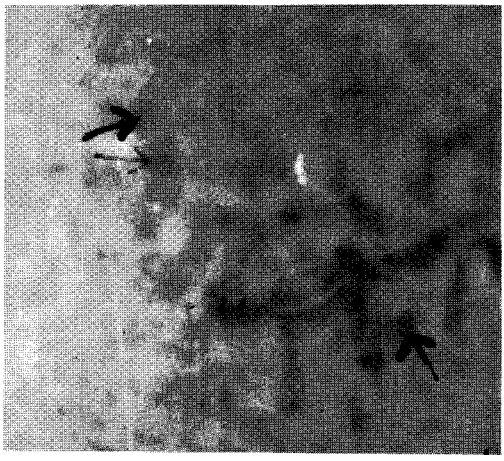

Figure 2. Metaphase chromosomes of H. annuus (CG-8031) hybridized by Biotin 5 S rDNA probe (Slide 30), (100x)

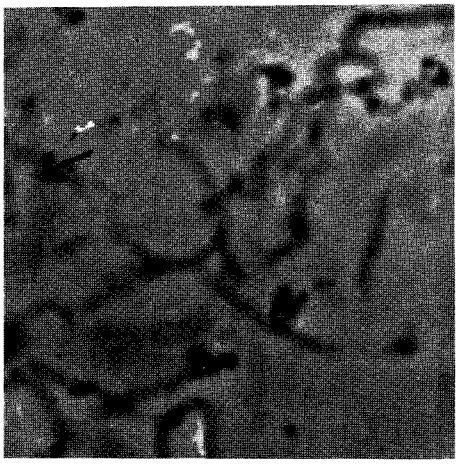

Figure 3. Prometaphase chromosomes of H. annuus (H13116) hybridized by Biotin 5 S rDNA probe (Slide 40), (100x)

biotinilated probes, therecould apear almost similar signals with unspecifically cell wall components, distrubing the hybridizations signals. So, it is necessary to make many metaphase chromosomes hybridizations, to identify the signals in satellites, where repetitive DNA sequences are present.

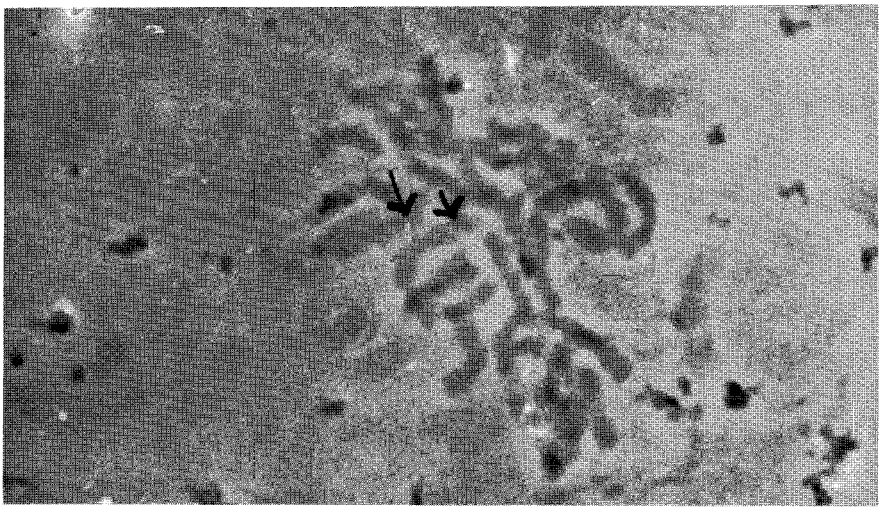

Figure 4. Metaphase chromosomes of H. annuus (CG-8031) hybridized by Biotin 5 S rDNA probe (Slide 52), (100x)

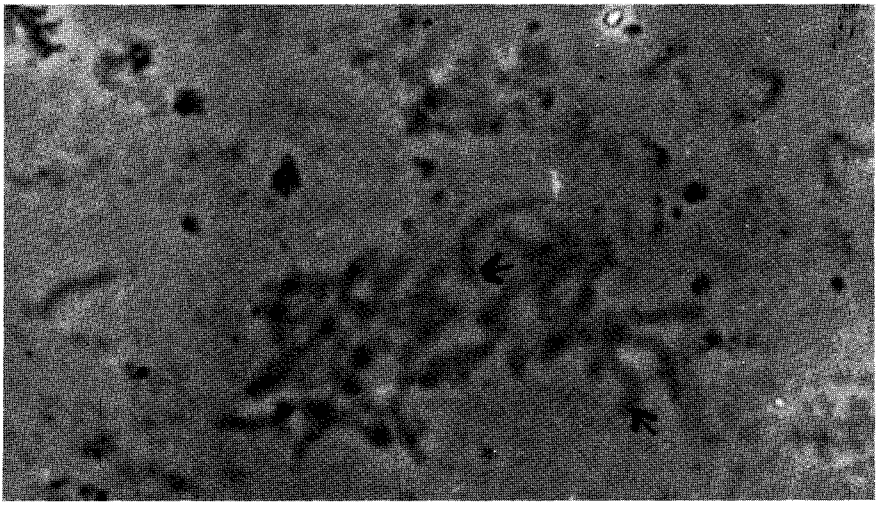

Figure 2. Metaphase chromosomes of H. annuus (CG-8031) hybridized by Biotin 5 S rDNA probe (Slide 58), (100x)

DISCUSSION

Despite its considerable economic importance, sunflower lacks a cytogenetic map. There is no significant banding patterns for chromosomes identification.

In situ hybridization tehniques are important for detection of specific nucleic acid sequences directly on chromosomes. These sequences can account for a considerable proportion of the total nuclear DNA in some plants. Repetitive DNA is a noncodant concentrated DNA in particular zones of the chromosomes as repetitive tandem DNA sequences, like satellites.

Repetitive DNA sequences were detected directly on somatic metaphase chromosomes from sunflower root tips, using "*in situ*" hybridization. These DNA sequences are localised at the heterochromatic regions in the satellites.

The repeated units of each family can diverge considerable during evolution, and so, there are often large differences among the repeated species. The middle and highly repeated fractions of DNA have been used to detect the presence of the genome under investigation in the DNA of hybrids, amphiploids or derived plants.

The use of molecular markers promises to be a powerful tool in the characterization of hybrid plants and recombinant chromosomes.

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HIBRIDACION "IN SITU" CON CROMOSONATE DE GIRASOL EN METAEASE

RESUMEN

La hibridación *in situ* con cromosomas somáticos de girasol en metafase procedentes de extremos de raices mediante un sistema de detección utilizando ADN marcado con biotina y el conjugado de la estreptavidina-alcalina fosfatasa se llevó a cabo en el INRA en Dijon Francia.

Los preparaciones de los cromosomas metafásicos se llevaron a cabo utilizando extemos de raices de las líneas puras CG-8034 y H-13116 procedentes de ICEPT Fundulea Rumania. Desputés de la hibridación "in situ" durante 24 horas de incubación con ADN marcado con biotina la observación microscópica mostró señales en el extremo de los cromosomas, asociadas con los satélites metafásicos están localizados en las regiones heterocromáticas en los satélites.

HYBRIDATION *IN SITU* SUR DES CHROMOSOMES MÉTAPHASIQUES DE TOURNESOL.

RÉSUMÉ

Nous avons réalisé à l'INRA Dijon – France – une hybridation *in situ* sur des chromosomes somatiques en métaphase provenant d"extrémités de racines de tournesol. Le système de détection consistait en ADN merqué à la biotine utilisant une liaison streptavidin-alkaline phosphate. Les coupes des chromosomes métaphasique ont été développées à partir de fragments de 1,5 cm de long de racines prélevées sur les lignées CG-8034 et H-13116 (ICPT Fundulea, Roumanie). Après hybridation *in situ* pendant 24 heures d'incubation avec de l'ADN marqué à la biotin (ADN du ribosome 5S – 330 bp), les coupes microscopiques ont montré des signaux à la fin des chromosomes associés aux satellites.

Des séquences d'ADN répété détectées directement sur des chromosomomes somatique en métaphase ont été localisées dans les régions hétérochromatiques des satellites.