Physical mapping of 45S rRNA gene loci in the cucumber (*Cucumis sativus* L.) using fluorescence *in situ* hybridization

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Abstract — The fluorescence *in situ* hybridization (FISH) using two different rDNA probes (18S and 45S) was applied to cucumber (*Cucumis sativus* L.) chromosomes. After counterstaining with 4’-6-diamidino-2-phenylindole (DAPI), all chromosomes showed visible bands similar to C-banding pattern and could be identified. The 18S and 45S rDNA signals appeared at the pericentromeric regions of chromosomes 1, 2 and 5. After silver staining to identify the nucleolar organizer region (NOR), the mitotic cells had two to four dots in nucleoli at interphase, two bands at the pericentromeric regions of two larger chromosomes at mid-metaphase. Although no NOR-bands appeared clearly at the prometaphase stage, certain silver-stained chromosomes were identified. DAPI staining made it possible to identify the silver-stained chromosomes at mid-metaphase and suggested that chromosome 1 or 2 had a silver-stained band at mid-metaphase.

Key words: cucumber, physical mapping, NORs, rDNA, FISH

INTRODUCTION

A chromosomal study in cucumber (*Cucumis sativus* L.) has been rarely studied in the last decade. Not only small size and poor stainability with usual staining procedures (TRIVEDI and ROY 1970) but also difficulty with chromosome identification, since the haploid complements show seven submedian chromosomes (TRIVEDI and ROY 1970) or six median- to submedian chromosomes and one subterminal chromosome (RAMACHANDRAN and SESHADRI 1986), make cucumber a very difficult subject for cyto-logical study. There are only a few reports of chromosome identification of cucumber using C-banding method (RAMACHANDRAN and SESHADRI 1986; HOSHI et al. 1998). HOSHI et al. (1998) reported that chromosome 2 showed a remarkable tendency of elongation at the primary constriction at the pro- and early-metaphase. The phenomena suggested that these sites might include nucleolar organizer regions (NORs). Previous papers on cucumber chromosome studies described that ten chromosomes (AYYANGAR 1967) or six chromosomes (TRIVEDI and ROY 1970; RAMACHANDRAN and SESHADRI 1986) had secondary constrictions in diploid plants, suggesting all or some of them were NORs.

As a first step in cytomolecular work, the molecular characteristic of NORs using rDNA probes for *in situ* hybridization was performed (e.g. SHI et al. 1996; HANSON et al. 1996; XU and EARLE 1996). The use of rDNA gene as probe in *in situ* hybridization contribute to give un-equivocal information about both active and inactive NORs.

Recently, the identification of cucumber chromosomes using fluorescent dyes were reported by PLADER et al. (1998) and offered an advantage for the further physical mapping. In this paper, we report a chromosome study of *Cucumis sativus* L. var. Borszczagowski, analyzed by morphological, cytological and molecular methods. We discovered the localization of the rDNA genes on chromosomes counterstained with DAPI and investigated active
rDNA gene in the mitosis using silver and DAPI staining on the same cells.

MATERIALS AND METHODS

Plant materials

The *Cucumis sativus* L. variety Borszczagowski (2n=2x=14), a highly inbred line, was used in this study. The lines are maintained at the Department of Plant Genetics, Breeding and Biotechnology, Warsaw Agricultural University.

Chromosome preparation

Chromosome slides were prepared with the squash method and enzymatic digestion method. In the squash method, root tips for chromosome preparation were collected from three day-old seedlings, pretreated in 0.05% colchicine for 1.5 h at 18°C and fixed in 45% acetic acid. After fixation, they were hydrolyzed in a 2:1 mixture of HCl and 45% acetic acid for 15 sec at 60°C. Then, they were squashed in 45% acetic acid and air-dried for 18 h at room temperature after removal of the coverslips by freezing. In the enzymatic digestion method, the root tips were fixed in 3:1 ethanol-acetic acid after the pretreatment described above, and washed using an ethanol series (50%, 30%, 10% and distilled water two times, 10 min each). After washing with distilled water, the root tips were treated with 4% Cellulase 'Onozuka' RIO (Serva) and 2% Pectolyase Y-23 (Sigma) at pH 4.8 at 37°C for 1 h. Then, they were washed three times with distilled water for 10 min each, transferred and chopped on a glass slide. Suspended cells were mixed with a few drops of the fixative, and then their chromosomes were spread and air-dried for 18 h at room temperature.

Fluorescent staining.

Chromomycin A₃ (CMA) and 4'-6-diamidino-2-phenylindole (DAPI) were used to distinguish the chromosomes. The slides were preincubated in McIlvaine's buffer (pH 7.0) supplemented with 5 mM MgSO₄, stained with 0.1 mg/ml CMA in McIlvaine's buffer supplemented with 5 mM MgSO₄ for 10 min. Some slides were stained with 0.1 mg/ml distamycin A (DMA) before CMA treatment. Then, they were mounted in glycerol and stored at 4°C for at least 10 h to prevent fading. After CMA staining, the slides were destained in 45% acetic acid for 30 min, 3:1 ethanol-acetic acid for 30 min and rinsed in distilled water for 5 min. Then slides were stained with 0.25 mg/ml actinomycin D (AMD) before 0.1 µg/ml DAPI treatment. DAPI staining after high-temperature heating were applied to compare the banding pattern of counterstained chromosomes after FISH.

The slides were incubated in 50% formamide solution for 15 min at 80°C, rinsed in McIlvaine's buffer for 10 min and then stained with 0.1 µg/ml DAPI for 30 min. Chromosomes stained with CMA and DAPI were observed using V and U filter cassettes, respectively.

Silver staining

The silver staining technique of Hizume et al. (1980) was used with some modifications. Before covering the preparations with the coverslip, a drop of 50% AgNO₃ in distilled water was added. The preparations were then sealed with a rubber solution and incubated in a moisture chamber for 7 days at 55°C. Some slides were mounted in 50% glycerol in 1 µg/ml DAPI.

Probe and probe preparation

The ribosomal probes prGSl and pRZlS for *in situ* hybridization were supplied by Prof. Dr. V. Hemleben, Tubingen University, Germany. The prGSl contains a 13.5 kb BamHI fragment of rDNA isolated from *Cucumis sativus* L. (Ganal et al. 1988), cloned in pBR322. It contained the coding sequence for the 18S, 5.8S and 25S genes and the non-transcribed spacer sequences. The pRZlS contains a 2.4 kb Hind III fragment of 18S rDNA isolated from *Cucurbita pepo*. These probes were labeled by random priming with digoxigenin-dUTP (Boehringer Manheim DIG DNA Labeling and Detection Kit) according to the manufacturer's instructions.

FISH

FISH followed mainly Fuchs and Schubert (1995) with some modifications. Slides were pretreated in 0.1 mg/ml DNAase-free RNAase at 37°C for 1 h, then washed two times in 2xSSC for 15 min each. After postfixation in 4% paraformaldehyde for 5 min, slides were washed again in 2xSSC for 10 minutes and kept in 2xSSC at 0°C for 10 min. On each slide, 20 ng of labeled probe DNA was mixed with 5 ng of herring sperm DNA and denatured in a hybridization buffer (50% formamide, 10% dextran sulphate in 2xSSC) for 5 min at 100°C. Chromosomes were also denatured in 70% formamide for 2.5 min at 80°C and quickly immersed in cold 50% formamide. The probe was dropped onto slides and chromosomes and probes were denatured together for 10 min at 80°C. They were slowly cooled to 37°C overnight. Following hybridization, the slides were washed at high stringency in 2xSSC at room temperature, 50% formamide in 2xSSC at 42°C, 2xSSC at 42°C and 2xSSC at room temperature, for 10 min each. According to Schwarzacher et al. (1989), these conditions allow hybridization between DNA-DNA duplexes sharing approximately.
80% sequence homology. Signals were detected with anti-dig fluorescein isothiocyanate (FITC) (Boehringer Mannheim, Germany). Chromosomes were counterstained with DAPI. Photographs were taken by Fuji super G ISO 400 film with the Olympus PM-20 camera system, using an Olympus microscope BX60. The superimpose images were created by adobe photoshop.

RESULTS

Chromomycin A₃ and DAPI staining

Each chromosome stained with DMA-CMA and CMA alone could be identified unequivocally (Fig. la, b). The chromosomes stained with DMA-CMA displayed higher contrast bands than those of CMA alone. All early- to mid-metaphase stained with DAPI alone did not show any band (figure not shown). The early-metaphase complement stained with AMD-DAPI after DMA-CMA staining is shown in Fig. 1c. The result of DAPI alone staining is the same as that of AMD-DAPI staining. The sequential staining method using CMA and DAPI made it possible to show clearly CMA-positive and DAPI-negative (CMA⁺DAPI⁻) bands at pericentromeric regions on early- to mid-metaphase chromosomes 1, 2, 3, 5 and 7 and slightly CMA⁺DAPI⁻ bands at the pericentromeric region on chromosome 6. In contrast to DAPI staining, post-heated DAPI staining showed quite similar banding patterns to that of CMA staining (Fig. 1d). All CMA⁺ and telomeric bands were well enhanced with post-heated DAPI staining. The long and short arms of chromosome 2 were always widely separated at prometaphase (figure not shown) and well-separated in many early-metaphase cells (Fig. 1b arrows).

Fig. 1. — Early-metaphase chromosome complements stained with DMA-CMA (a) CMA alone (b), AMD-DAPI (c) and post-heated DAPI staining (d). The identified chromosomes were indicated. Bar = 5µm
Fig. 2. — Early-metaphase chromosome complements were counterstained with DAPI (a and c) after 18S (b) and 45S (d) rDNA FISH. The identified chromosomes were indicated (a and c). Bar = 5 µm.

Fig. 3. — Superimposed image helped to see the localization of the 45S rDNA signals. The major signals were located at the proximal regions of the long arms of chromosomes 1, 2 and 5, and short arms of chromosomes 1 and 2. Bar = 5 µm.
**Fluorescent in situ hybridization**

The results of rDNA fluorescent *in situ* hybridization (FISH) are shown in Figures 2-4. Certain chromosomes and some segments were identified at the prophase stage (figure not shown). From pro- to mid-metaphase, each chromosome counterstained with DAPI could be identified and showed the same banding pattern to that of post-heated DAPI. The proximal regions of six chromosomes had the strong hybridization signals of 18S and 45S rDNA probe in the diploid complement (Fig. 2). Tiny 45S signals were detected at the proximal regions of four chromosomes (Fig. 2d). Superimposed images of chromosomes counterstained with DAPI and the hybridization signals of the same cells made it possible to localize each of the gene loci to a chromosome region (Fig. 3). All strong signals of 18S and 45S rDNA were located on the same regions. These signals were in CMA-DAPI heterochromatic regions. Larger strong signals were located at the proximal regions of the long and short arms of chromosomes 1 and 2; a smaller strong signal was located at the proximal region of the long arm of chromosome 5 (Fig. 2). Tiny signals were detected at the proximal regions of chromosomes 3 and 7 (Figs. 2 and 3). The haploid complement showed a 6% ratio of total strong signals to total chromosome length. The hybridization signal on chromosome 1 is the same size as that of chromosome 2 and two times larger than that of chromosome 5. Signals of chromosome 5 comprised approximately 6% of the total length of the chromosome hybridized with the rDNA gene. Chromosomes 1 and 2 showed 20% relative ratios of the signals to the full length. Major hybridizing sites are shown in the cytological map (Fig. 4).

![Fig. 4. — Cucumber cytological map representing major 45S rDNA sites.](image-url)
Silver staining

To determine the activity of the rDNA sites, silver staining and counts of nucleoli of mitotic cells were performed on slides prepared with the squash or enzymatic digestion method. Slides prepared with the enzymatic digestion procedure obtained much better silver bands on the chromosomes than the squash method. Well-stained nucleoli were observed in inter-phase nuclei prepared by the standard squash and enzymatic digestion procedures. Generally, one or two silver-stained nucleoli were observed in interphase nuclei (Fig. 5a-c). Three silver-stained nucleoli were rarely observed (Fig. 5d) and four silver-stained nucleoli could not be found during our investigations. Superimposed images of chromosomes stained with DAPI and Ag-I are shown in Figures 6 and 7. In prometaphase, a banded chromosome, similar to that of CMA and DAPI, was obtained (Fig. 6). Silver-stained chromosomes with DAPI banding pattern did not appear at early- to mid-metaphase chromosomes (Fig. 7a, b). In contrast to prometaphase, two dark NOR-bands appeared in the diploid complements at mid-metaphase (Fig. 7a). DAPI staining after Ag-I made all chromosome easily identified without chromosomes 1 and 2 (Fig. 7b). Superimposed pictures of chromosomes stained with Ag-I and DAPI of the same cells made it possible to localize each of the active gene loci to a chromosome region (Fig. 7c). The band was located on the proximal region on the larger chromosome, suggesting chromosome 1 or 2.

Fig. 5. — Silver-stained interphase nuclei. Generally, one or two silver-stained nucleoli were observed (a-c). Rarely, three silver-stained nucleoli observed (d). Bar = 5 μm.
Fig. 6. — Superimposed image of prometaphase chromosomes stained with DAPI after silver staining. The banding pattern of silver-stained chromosomes is quite similar to that of DAPI. Bar = 5 µm.

Fig. 7. — Silver-stained (a) and DAPI-stained (b) mid-metaphase chromosomes. Two dark bands appeared in the diploid complements (a). Each chromosome was identified in DAPI staining (b). Superimposed picture of chromosomes stained with Ag-I and DAPI of the same cells made it possible to localize each of the active gene loci to a chromosome region (c). Bar = 5 µm.

DISCUSSION

Chromosome preparation using the squash method makes chromosome observation difficult in silver staining and FISH, because the cytoplasm of cucumber cells stained darkly or brightly. However, in chromosome preparation using the enzymatic digestion procedure, stainability of the cytoplasm was reduced, making it possible to obtain well-stained chromosomes with sharp bands. This allowed for an accurate determination of chromosome bands, especially
in silver staining. Chromosome preparation between squash and enzymatic digestion methods showed no differences in CMA, DAPI and post-heated DAPI staining. The results of our comparison between FISH and Ag-I nuclei count support the statement that, not all NORs are associated with active transcriptional machinery and therefore they will not form a visible nucleolus. The phenomenon that not all NORs are transcriptionally active during interphase is often seen in interspecific hybrids, and has been named nucleolar dominance (Reeder 1990). In this experiment, however, we used a highly inbred cucumber line. Thus, the results cannot be considered as a nuclear dominance. Another explanation could be that in cucumber may exist intraspecific NOR dominance. In this experiment, however, we used a highly inbred cucumber line. Thus, the results cannot be considered as a nuclear dominance.

FISH experiments and silver staining of cucumber chromosomes revealed that the NORs, localized on one of the largest chromosomes (1 or 2), are strongly stained with silver. Considering that the silver-reducing proteins are believed to belong to the transcriptional machinery (mainly C-23; Zurita et al. 1998) and to be associated with the NORs during mitosis, these sites have probably been active during the preceding interphase. On the other hand at met-aphase, the NORs of chromosome 3, as well as minor signals of chromosomes 5 and 7 do not show protein silver precipitation. The lack of silver staining not only suggests that these NORs do not possess an active transcriptional machinery, but also that they can remain inactive during a large part of the cell cycle. These results are similar to that described for Petunia hybrida (Montijn et al. 1998).

An appearance of the active NORs on chromosomes 1 or 2 which possess the strongest in situ hybridization signals, can be explained based on previous report describing the direct association between the amount of rDNA (FISH signal) and the level of expression of NORs (Ag-I signal) (Zurita et al. 1998). Level of transcriptional activity of NORs is directly related to the number of ribosomal cistrons, and then NORs with the largest size of rDNA are preferentially activated (Zurita et al. 1998). These results, together with previously described findings that on chromosomes 1 and 2 are located strong positive CMA bands (GC rich region; Plader et al. 1998); rDNA genes are GC rich (King et al. 1993) and the tendency of chromosome 2 to elongation in the centromeric region (Hosm et al. 1998; Plader et al. 1998), we would conclude that an active rDNA site is located at the pericentromeric region of chromosome 2.

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REFERENCES


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