Organization of highly repetitive satellite DNA of two Cucurbitaceae species (Cucumis melo and Cucumis sativus)

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ABSTRACT

The prominent satellites of the Cucurbitaceae Cucumis melo (melon) and Cucumis sativus (cucumber) have been characterized. In actinomycin/CsCl gradients where the satellite sequences can be separated from ribosomal, organell, and main band DNA the location of the satellites is different indicating a different GC content. The purified satellite of C. melo is cut by HindIII into a repeat unit of 380 bp; AluI digestion gives rise to two bands (about 80 and 220 bp in size). The HindIII repeat unit if cloned into pBR325 exhibits new recognition sites for HpaII leaving two bands with 150 and 80 bp suggesting methylation of the C/CGG cutting site in the uncloned material. The restriction pattern indicates an internal sequence repeat within the 380 bp HindIII fragment. The C. sativus satellite is cut by AluI to a repeat unit of 180 bp showing no other recognition site for the restriction enzymes tested so far. About 10% sequence homology has been determined between the C. melo and C. sativus satellites by cross hybridization studies. A high methylation degree of cytosines has been measured for both satellites and the ribosomal DNA of C. sativus (about 30%). No transcription products of the C. melo satellite were found during seedling development.

INTRODUCTION

The occurrence of relatively short and simple DNA sequences which give rise to satellite fractions in buoyant density gradients is well established for animal and plant genomes (1). Localization of this DNA mostly in heterochromatic regions of the chromosome (centromer, telomer) suggests a function in meiosis and mitosis, and a possible role for recombination and crossing over (1, 2). A characteristic feature is the transcriptional inactivity of these genomic components. The amount of satellite DNA and the arrangement of the sequences vary enormously between different species of the same family indicating a function for the evolution of species (3).
Several plant families have been investigated with respect to their satellite components (4, 5). Here we report on the characterization and cloning of the prominent satellite of the Cucurbitaceae Cucumis melo (6 - 14) in comparison to the satellite DNA of Cucumis sativus (7). The sequence organization has been analyzed by restriction enzyme digestion of the satellites purified on actinomycin D/CsCl gradients and compared with the cloned satellite sequence of C. melo. The transcriptional activity of C. melo satellite and the methylation pattern of satellite, ribosomal, and main band DNA have been studied to find out whether a high methylation degree may be a tool for regulation of transcription.

MATERIALS AND METHODS

Plant material. Seeds of the sugar melon Cucumis melo (11) were cultivated under sterile conditions on sand for 5 - 7 days in the dark at 27°C. Seeds of Cucumis sativus (variety: Vorgebirgstraube) were grown in soil at 20°C.

Cell nuclei were isolated as described (15). The nuclear pellet was lysed in a proteinase K solution (100 µg/ml, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM NaCl, 0.5% SDS) for 4 h at 37°C. The lysate was centrifuged for 10 min at 5,000 rpm and dialyzed against 25 mM sodium tetraborate buffer pH 9.2. To 6.65 ml lysate 8.3 g CsCl was added and the solution was centrifuged for 48 h at 32,500 rpm, rotor Ti 50, Spinco, Beckman. The gradients were fractionated with an Isco density gradient fractionator and the DNA containing fractions were combined. The volume was raised to 7.5 ml and the mean density was adjusted to 1.580 g/cm³ by dissolving additional CsCl. 0.25 ml actinomycin D (1 mg/ml stock solution) was added to this solution in the cold. The gradients were centrifuged for 80 h at 3°C and 30,000 rpm, rotor Ti 50, Spinco. Satellite I containing fractions were combined, actinomycin D was removed by chloroform:isoamylalcohol (24:1) extraction, and the last aqueous phase was dialyzed against 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA. DNA was precipitated after addition of Na-acetate (0.15 M final conc.) with 2 Vol. of ethanol at -20°C.
RNA isolation, iodination, and RNA/DNA hybridization. Total RNA was isolated and purified from 6-day-old *C. melo* seedlings - roots, hypocotyls, and cotyledos, separately (16). Iodination with $^{125}$Iodine (Amersham-Buchler) was performed according to the reported method (17, 18). For hybridization to blotted Sat.I DNA the RNA fractions were separated into high and low molecular weight components on Sephadex G 25. Hybridization conditions (19, 20): 25% formamide, 6 x SSC, 2 µg $^{125}$I - 1m or hm RNA (specific radioactivity: 2 - 5 x $10^5$ cpm/µg) for 18 h at 60°C.

Restriction enzyme analysis. *C. melo*, *C. sativus* or pSat 107 DNA were digested with various restriction endonucleases: HindIII, EcoRI, BamHI, SmalI, Alul, BglII, HpaI, HpaII, HhaI (all Boehringer, Mannheim), and PstI (BRL) under the appropriate enzyme conditions. DNA fragments were separated on 1 or 2% agarose gels (15) together with HindIII ·λDNA fragments or HaeIII · φ X174 DNA fragments (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp), stained with ethidium bromide, and if indicated blotted for hybridization (19, 20).

Cloning of satellite I DNA. The purified and HindIII digested satellite I DNA of *C. melo* was ligated to the HindIII digested *E. coli* plasmid pBR325 conferring the ampicillin, tetracycline, and chloramphenicol resistance markers. *E. coli* K12, RRI was transformed (21), recombinant clones selected, and screened by a miniscreen procedure (22) for inserts of satellite I DNA. Clone pSat 107 containing three repeat units of the satellite DNA was amplified, and the DNA was isolated by ethidium bromide-CsCl density gradient centrifugation (23). The satellite insert DNA was cut out of the plasmid by HindIII digestion and separated from the plasmid DNA by centrifugation on linear sucrose gradients (5 -30% sucrose in 10 mM Tris-HCl pH 8, 1 mM EDTA; centrifugation conditions: rotor SW 40, Spinco, Beckman, 40,000 rpm, 6 h at 4°C).

$^{32}$P-nick translation of pSat 107 DNA and DNA/DNA hybridization. pSat 107 DNA was linearized with EcoRI and nick translated with *E. coli* DNA polymerase I according to Maniatis et al. (24). Hybridization of denatured satellite I DNA blotted on nitrocellulose filters (Ø 13 mm) was performed after preincubation
of the filters at 37°C in 50% formamide, 5 x SSC, 0.01 M EDTA, 0.02 M HEPES pH 7.4, 5 x Denhardt's solution (1 x Denhardt's: 0.02% Ficoll, polyvinylpyrrolidon, bovine serum albumin, each) for 5 h. The preincubated filters were hybridized to 32P-nick translated pSat107 DNA (5 x 10^6 cpm; specific radioactivity: 4 x 10^7 cpm/μg) in 10 ml preincubation medium for 18 h at 40°C. Washing procedure: 3 times in 5 x SSC + 0.5% SDS for 1 h each at 60°C; 3 times in 0.5 x SSC + 0.05% SDS for 15 min each at room temperature. The filters were dried and counted in a liquid scintillation counter (Packard).

Determination of methylcytosine. Methyl group determination was performed by a modified gas chromatography procedure as described (25). An OV-101 glass capillary was used as the column.

RESULTS

Isolation and purification of satellite I DNA. High molecular weight DNA preparations are necessary to purify satellite DNA components. Therefore, isolated nuclei of seedlings of C. melo were lyzed with proteinase K, dialyzed, and the DNA was separated on CsCl gradients (Fig.1A). All DNA containing fractions were combined and separated on an actinomycin D/CsCl gradient (Fig.1B). The DNA sequences coding for rRNA were localized in these gradients by hybridization of the denatured fractions on filters with 3H-labelled 18S, 25S and 5S rRNA (11). After actinomycin D/CsCl gradient centrifugation of total DNA at least four different DNA components can be distinguished (Fig.1B): The ribosomal DNA is clearly separated from satellite I (10 - 12); satellite II mainly consists of organell DNA as judged by the restriction enzyme pattern (10). Ribosomal DNA and satellite I DNA fractions purified from actinomycin D/CsCl gradients were further analyzed by restriction enzyme digestion with HindIII and separation of the fragments on 1% agarose gels (Fig.2). The rDNA shows the known 7.6 x 10^6 molecular weight HindIII fragment containing the total gene repeat unit coding for 18S and 25S rRNA plus spacer regions (26) and slight contaminations with satellite I DNA. HindIII digested satellite incubated for 1, 2, and 4 h gives a typical fragment ladder with a basic repeat unit of about 380 bp (10).
Figure 1. Fractionation of Cucumis melo DNA on buoyant density gradients. A. DNA from isolated nuclei of seedlings was fractionated on CsCl gradients. Fractions 10-50 were combined and separated on an actinomycin D/CsCl gradient (B). From parallel gradients the DNA of every fraction was denatured and loaded onto nitrocellulose filters. Filters were cut in halves and hybridized to $^3$H-5S rRNA (O---O) or $^3$H-18S and 25S rRNA (●●●●), respectively; (■■■■) absorbance at 260 nm.

Fractionating the DNA of lyzed nuclei of C. sativus seedlings in the same way as described for C. melo results in a similar separation pattern (Fig.3) although the DNA components were localized in different regions of the actinomycin D/CsCl gradient (Fig. 3). Satellite I is located between rDNA and satellite II plus main band DNA. Table 1 summarizes the buoyant densities of the different DNA components of C. melo and C. sativus in actinomycin D/CsCl gradients. The fact that C. sativus satellite I bands at a higher density than the C. melo satellite I demonstrates its lower GC content.

Sequence arrangement of satellite I DNA. The combined and purified fractions of satellite I from actinomycin D/CsCl gradients (Fig.1B and Fig.3 ) were digested with various restriction endonucleases and the fragments were separated on agarose gels (Fig.4 and Tab.2). The basic repeat unit of C. melo satellite I DNA cut with HindIII is about 380 bp (10). AluI digestion results in two bands for C. melo (220 and 80 bp) and in a single band for C. sativus of 180 bp. Obviously the two satellites do not contain
Figure 2. HindIII restriction enzyme analysis of purified ribosomal DNA and satellite I DNA of Cucumis melo. The respective DNA fractions purified from actinomycin D/CsCl gradients were digested with HindIII and the fragments were separated on 1% agarose gels with HindIII · λ DNA (lane D) as marker. A-C: C. melo satellite I digested for 1, 2, and 4 h; lane E: ribosomal DNA (1 h digest).

Figure 3. Fractionation of Cucumis sativus hypocotyl DNA on an actinomycin D/CsCl gradient. Separation and hybridization conditions as described for Fig.1. (■■■) absorbance at 260 nm; (○——○) hybridization with 3H-5S rRNA; (●●●) hybridization with 3H-18S and 25S rRNA.
Table 1: Buoyant densities of the DNA components of C. melo and C. sativus in actinomycin D/CsCl density gradients

| DNA component    | C. melo (g/cm³) | difference to main band DNA (g/cm³) | C. sativus (g/cm³) | difference to main band DNA (g/cm³) |
|------------------|------------------|-------------------------------------|--------------------|-------------------------------------|
| main band DNA    | 1.583            | 0                                   | 1.583              | 0                                   |
| ribosomal DNA    | 1.536            | 0.047                               | 1.526              | 0.057                               |
| satellite I      | 1.521            | 0.062                               | 1.561              | 0.022                               |

...any cutting sites for all the other enzymes tested so far (Tab.2). To clarify whether more restriction endonuclease cleavage sites may be found in unmethylated satellite sequences and to have a hybridization probe available, the HindIII C. melo satellite DNA fragment, 380 bp in length, was ligated to pBR325 and cloned. After transformation of E. coli most of the recombinant plasmids

Figure 4. Restriction enzyme analysis of satellite I DNA of C. melo, the cloned C. melo satellite (insert of pSat 107) and of C. sativus. Satellite I DNA was purified from actinomycin D/CsCl gradients or from the recombinant plasmid pSat 107, digested with the indicated restriction endonucleases, and the fragments were separated on 1.5% agarose gels together with HaeIII·ΦX174 DNA (lane I, Q) and HindIII·λ DNA (lane H) as markers. Lane A-G: C. melo satellite I; lane K-P: Insert of pSat 107; lane R-X: C. sativus satellite I. Restriction enzymes used: HindIII (A,R); SmaI (B; K; S); AluI (C, L, T); BglII (D, M, U); HpaII (E, N, V); HpaI (F, O, W); PstI (G, P, X).
Table 2: Restriction enzyme pattern of satellite DNA of *C. melo*, the cloned *C. melo* satellite (insert of pSat 107), and *C. sativus*.

| Restriction enzyme | *C. melo* satellite I* | Insert of pSat 107* | *C. sativus* satellite I* |
|--------------------|------------------------|---------------------|--------------------------|
| HindIII            | 380                    | 380                 | -                        |
| AluI               | 220 and 80             | 220 and 80          | 180                      |
| HpaII              | -                      | 150 and 80          | -                        |
| HpaI               | -                      | -                   | -                        |
| HhaI               | -                      | -                   | -                        |
| Smal               | -                      | -                   | -                        |
| BgII               | -                      | -                   | -                        |
| PstI               | -                      | -                   | -                        |
| BamHI              | -                      | -                   | -                        |
| EcoRI              | -                      | -                   | -                        |

* Indicated are fragment sizes in base pairs.

contained the basic satellite repeat unit of 380 bp or multimers (Fig.5). Plasmid pSat 107 conferring three HindIII satellite repeat units was amplified and used for hybridization studies or, after isolation of the insert, for restriction enzyme analysis and methyl group determination. The HindIII-380 bp insert was further analyzed and digested with the same restriction endonucleases. New cutting sites are only detected for HpaII which produces two fragments about 150 and 80 bp in size (Tab.2 and Fig.4) suggesting that in vivo these sequences are methylated and therefore protected against cleavage. (The weak bands in the gel result from slight contamination with plasmid pBR325 DNA).

Determination of sequence homology between satellite I DNA sequences of *C. melo* and *C. sativus*. Since the restriction endonuclease patterns of *C. melo* and *C. sativus* satellite DNA seem to be different the percentage of homology between these sequences has been determined. Equal amounts of *C. melo* and *C. sativus* satellite I DNA purified by actinomycin D/CsCl gradients were denatured, fixed onto nitrocellulose filters, preincubated and hybridized to the $^{32}$P-labelled insert of pSat 107. The degree
Figure 5. Characterization of the recombinant plasmids. 
*C. melo* satellite I DNA was purified from actinomycin D/CsCl gradients, digested with HindIII, ligated to pBR325, and *E. coli* K12, RRI was transformed. DNA from the recombinant clones was digested with HindIII and the fragments separated on 1% agarose gels with HindIII · λ DNA as marker (lane E); clone pSat 103 (A), pSat 106 (B), pSat 107 (C), pSat 114 (D).

of homology between both satellites was estimated to be about 10% by setting the amount of radioactivity on the filters from the homologous hybridization as 100% and calculating the percentage from the radioactivity fixed on the *C. sativus* DNA filters after heterologous hybridization. Hybridization was also performed along an actinomycin D/CsCl gradient of *C. sativus* hypocotyl DNA (Fig.6). Only fractions in the region of satellite I hybridized to the cloned *C. melo* satellite probe.

Test for transcriptional activity and 5-methylcytosine content of satellite I. One characteristic feature of highly repetitive satellite DNA is the transcriptional inactivity except in the stage of oocyte development in certain amphibians (27, 28). To see whether satellite I sequences are transcribed during seedling development total RNA isolated separately from roots, hypocotyls, and cotyledos of *C. melo* seedlings were iodinated in vitro and hybridized to the cloned satellite DNA blot-
Figure 6. Cross hybridization between C. melo satellite sequences and C. sativus DNA. C. sativus DNA from hypocotyl cells was separated on an actinomycin D/CsCl gradient. The DNA of each fraction was denatured, fixed onto nitrocellulose filters (Ø 13 mm) and hybridized after preincubation to the cloned and $^{32}$P-labelled C. melo satellite II (insert of pSat 107). (---) absorbance at 260 nm; (O---O) $^{32}$P-radioactivity.

ted onto nitrocellulose filters. No RNA fraction from any developmental stage hybridized to this DNA (data not shown), indicating that no transcription of these sequences occurs during seedling development as expected for a typical satellite DNA.

Methylation of DNA sequences especially by modification of cytosine residues to 5-methylcytosine may be a tool to regulate transcriptional activity. The methylation degree of the satellite I DNA was therefore measured by a modified gas chromatography procedure (25) and compared to the methylation of purified ribosomal DNA and mainband DNA. As standard either herring sperm DNA or the unmethylated cloned satellite sequence (insert of pSat 107) were used. Table 3 shows the percentage of methylated cytosines for different fractions of C.melo and C.sativus DNA. Both satellite I fractions show a similar amount of methylated cytosines. Most striking is the fact that the ribosomal DNA sequences of C.sativus exhibit a similar methylation degree as the inactive satellite sequences.

DISCUSSION

Cucurbitaceae are characterized by a large percentage of satellite DNA (5, 7, 14). A satellite is defined as consisting
Table 3: Percentage of 5-methylcytosine and cytosine in different DNA fractions of *C. melo* and *C. sativus.*

| DNA fraction               | Cytosine (%) | Methylcytosine (%) |
|----------------------------|--------------|--------------------|
| Insert pSat 107            | 93.5         | 6.5                |
| **C. melo:**               |              |                    |
| Satellite I                | 67.7         | 32.3               |
| rDNA                       | 77.3         | 22.7               |
| mainband DNA               | 81.5         | 18.5               |
| **C. sativus:**            |              |                    |
| Satellite I (hypocotyls)   | 70.0         | 30.0               |
| rDNA (" )                 | 71.5         | 28.5               |
| Satellite I (cotyledos)    | 68.8         | 31.2               |
| rDNA (" )                 | 70.5         | 29.5               |
| herring sperm DNA          | 93.0         | 7.0                |

of highly repetitive, tandemly arranged and clustered simple DNA sequences (30). This satellite DNA can be separated from the middle repetitive DNA coding for 25 S and 18 S, and 5 S ribosomal RNA, and from mainband plus organell DNA on actinomycin D/CsCl (11, 12) or Ag⁺-Cs₂SO₄ (10) gradients if the DNA is of high molecular weight. Satellite I sequences of *C. melo* and *C. sativus* differ clearly in their position in these gradients and thus in the GC content, since actinomycin D binds specifically to GC rich DNA sequences. The separation of satellite I DNA on actinomycin D/CsCl gradients allows only a rough estimate of its percentage of total DNA and can be determined as 10 - 20% for *C. melo* and 20 - 30% for *C. sativus* depending on the tissue investigated (see 7).

Cross hybridization between the cloned *C. melo* satellite and *C. sativus* revealed about 10% homologous sequences in the *C. sativus* satellite I sequences. No similarities in the restriction enzyme pattern of both species could be found. Further sequencing data will identify the exact location and arrangement of similar sequence families. The restriction enzyme pattern of the *C. melo* satellite I allows a preliminary suggestion on the internal sequence arrangement within in the 380 bp HindIII
repeat unit: The AluI and HpaII bands add up only to 300 bp and 230 bp, respectively. Therefore, it can be assumed that an internal repeat is present forming a palindrome-like structure. A model of the AluI restriction map is presented based on the fact that the 4 bp-enzyme cleaves the inner tetranucleotide sequence of the 6 bp-HindIII recognition site (Fig.7). A palindromic structure was also found for Scilla satellites and may be a common feature for specific satellite sequences (31).

The comparison of the methylation degree of cytosine residues in different DNA components of both Cucumis species investigated showed that the satellite I DNA sequences are highly methylated but the methylation is still lower than the reported percentage of methylated cytosines in the prominent satellite of Scilla species (31). Purified ribosomal DNA of the Cucumis species revealed a similarly high methylation degree especially for the C.sativus rDNA supporting the suggestion that the transcriptional activity of the abundant ribosomal RNA genes in plants may be regulated by methylation (29, 32). This mechanism is suggested to inactivate supernumeral ribosomal RNA cistrons in animal cells (33). Mainband DNA cytosines are methylated up to 18%. This is in the lower range of the methylation degree found for other higher plants (34).

Satellite I sequences of C.melo were shown to be transcriptional inactive since no hybridization to in vitro labelled RNA from different developmental stages of seedlings was detected. This result does not exclude, however, the transcription of these sequences during a defined stage of development as it occurs in the oocytes of Triturus (27, 28)

The actual functions of the highly repetitive DNA sequences are still unknown but certainly the arrangement of sequence families play a role in the evolution and separation of new species. The family of Cucurbitaceae seems to be very appropriate

![Figure 7. Arrangement of the AluI fragments within the HindIII satellite repeat unit (insert of pSat 107) of C. melo.](image-url)
to study the possible functions of satellite DNA.

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