Species and Genus Specificity of the Intergenic Spacer (IGS) in the Ribosomal RNA Genes of Cucurbitaceae

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The use of intergenic spacer (IGS) fragments of plant ribosomal DNA (rDNA) for the differentiation between genera and species is tested by cross-hybridization experiments with different IGS probes of two Cucurbitaceae, Cucurbita pepo (zucchini) and Cucumis sativus (cucumber). Hybridization with cloned fragments of different parts of the IGS of ribosomal DNA exhibit a different degree of conservation within and between the Cucurbitaceae genera. In general, Cucurbita species seem to be closer related to each other than the Cucumis species. A repetitive element of the external transcribed spacer (ETS) shows a more genus-specific pattern, reacting only with the respective genera; the region preceding the ETS is conserved between the Cucurbita species but also cross-hybridizes weakly with the Cucumis species. A GC-rich element of the Cucumis sativus IGS (“Cfo-cluster”) is present in small amounts in Cucumis melo (melon) and even less represented in other genera of the Cucurbitaceae.

Introduction

One characteristic feature of higher plants is the high amount of nuclear ribosomal DNA (rDNA: [1–4]). Each of the tandemly arranged rDNA repeating units contains the 18S, 5.8S, and 25S rRNA coding region which is separated from the next one by an often length heterogeneous intergenic spacer (IGS; [3, 4]). The RNA components are transcribed as a large precursor which is processed to the mature RNAs. Therefore, parts of the IGS are transcribed, named external transcribed spacer (ETS); the 5.8S rRNA coding region is flanked by internal transcribed spacers (ITS 1 and ITS 2; [1]).

The organization of the ribosomal DNA repeating unit and the regulation of transcription have been analyzed in more detail in animals, especially in Xenopus and mouse [5, 6]. Different elements are involved in the control of transcription by RNA polymerase I; transcription initiation sites (TIS), promoter elements, enhancers, and terminators have been identified in the IGS [6, 7]. Recent data show that the ribosomal RNA genes of higher plants exhibit a comparable structural architecture of the IGS [3, 4] although an enormous nucleotide sequence variability is found even between closely related species. In contrast to this sequence diversity of the IGS the coding regions are highly conserved. However, within a given species the IGS of this multigene family is rather homogeneous regarding the nucleotide sequence and only heterogeneous with respect to the spacer length [3, 4]. This mode of evolution has been termed “concerted evolution” [8].

Different representatives of the Cucurbitaceae, especially of the genera Cucurbita and Cucumis, with often several thousands of rDNA repeats per nucleus [2, 9] were chosen for this cross-hybridization study to provide data on the evolution of the IGS of higher plants. In addition, the use of spacer fragments for the identification and differentiation between genera and species of a family should be tested. Only for cucumber (Cucumis sativus) the nucleotide sequence of the IGS is known yet ([10]; U. Zentgraf, M. Ganal, and V. Hemleben, in preparation); the other species are mainly characterized by restriction enzyme mapping [9, 11].

Materials and Methods

Plant material and DNA isolation

Seeds of the following Cucurbitaceae were purchased commercially (Hild, Marbach a. N., or Endrilli, Tübingen, F.R.G.): Cucurbita pepo (zucchini), Cucurbita maxima (squash), Cucumis sativus (cucumber), Cucumis melo (melon), Lagenaria leucantha, Citrullus lanatus. Seeds of Cucurbita mixta, Cucurbita ficifolia, Cucurbita lundelliana, and Cucurbita moschata were obtained from the Zentral-
Fig. 1. Cross-hybridization of different ribosomal DNA intergenic spacer (IGS) fragments of zucchini (Cb. pepo; probes 1 and 2) and of cucumber (Cs. sativus; probes 3 and 4) to different species of the Cucurbita and the Cucumis genus. The tandemly arranged ribosomal DNA repeating units are schematically drawn (above). The localization of the hybridization probes 1 and 2 of the zucchini clones pRZ7D and pRZX and of probes 3 and 4 of the cucumber clone pRGS1 is indicated (middle; for description of the clones see Materials and Methods; restriction sites are given for HindIII, BamHI, and XhoI; for AluI and HpaII they are only marked, if there were used to generate the probes). The 18S, 5.8S, and 25S rRNA coding regions are indicated.

Total nuclear DNA (approximately 10 µg each) of Cucurbita maxima (a), Cucurbita pepo (b), Cucumis sativus (c), and Cucumis melo (d) was digested with EcoRI, separated on 1% agarose gels, blotted on nitrocellulose filters, hybridized to the respective 32-P-nick-translated probes, and exposed to X-ray films. For each hybridization and autoradiogram of a short (left) and a longer (right) exposure is shown (see arrows). 1 kb = 1000 bp.
Seeds were cultivated under sterile conditions at 25 °C under continuous light. Seedlings were harvested after 7–14 days. Nuclei were isolated and DNA was purified as described by Hemleben et al. [12]. DNA of *Matthiola incana* R. Br. (Brassicaceae) was used as control.

**Cloning**

Standard methods for cloning were followed as described by Maniatis et al. [13]. The zucchini clone pRZ7D contains a complete 10-kbp rDNA repeating unit, cloned in the *Hind*III site of pBR 322, pRZX has a 7.5-kbp *Hind*III insert originating of a minor represented 10-kbp repeat of zucchini (*Cucurbita pepo*) rDNA (see Fig. 1; [9, 11]). pRG81 containing more than one 12.5-kbp rDNA repeat of cucumber (*Cucumis sativus*; Fig. 1) was a gift of Dr. M. Ganal [10]. The fragments used for hybridization (probe 1–4; Fig. 1 and 2) were cut out of the respective plasmids by restriction endonucleases, electroforeosed on agarose gels and electroeluted out of the gels.

**Hybridization**

Nick translation with 32-P-dCTP and DNA-DNA hybridization (stringent conditions: 67 °C) were carried out as described [13]. For “slot-blot” hybridizations the Minifold II chamber (Schleicher and Schuell) was used following the procedure of Imamura et al. [14].

**Results and Discussion**

Different DNA probes of the IGS of zucchini (*Cb. pepo*) and cucumber (*Cs. sativus*) were selected for hybridization experiments in order to follow the evolutionary relationship between various genera and species of the family of Cucurbitaceae (Fig. 1 and 2; for the description of the clones see Materials and Methods).

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![Fig. 2. Relationship between the ribosomal DNA intergenic spacer (IGS) of different species of the genera *Cucurbita* and *Cucumis* as deduced by the strength of cross-hybridization with different IGS probes of *Cucurbita pepo* and *Cucumis sativus* (see Fig. 1). The IGS of the four Cucurbitaceae investigated is drawn flanked by the 25S and 18S rRNA coding regions. Number and width of the vertical arrows indicate the strength of the cross-hybridization of the respective probes 1–4 with the DNA of another species. The data for the IGS organization are taken from R. Kelly and A. Siegel, cited in [4] for *Cb. maxima*, from [9] and R. A. Torres and V. Hemleben (unpublished) for *Cb. pepo*, from [10] for *Cs. sativus*, and from [9] for *Cs. melo*. Hatched boxes indicate the duplications of the 3’end of the 25S rRNA coding regions and subsequent sequences [4, 10], the zigzag lines represent the GC-rich “Cfo-clusters” in the IGS of *Cs. sativus* [10]. Abbreviations for the restriction enzyme sites indicated: A = *Alu*I, B = *Bam*HI, E = *Eco*RI, H = *Hind*III, Hp = *Hpa*II, K = *Kpn*I, N = *Nsi*I, S = *Sma*I, Sc = *Sac*I, X = *Xho*I.](image-url)
Comparison between the IGS of Cucurbita and Cucumis species

From restriction mapping experiments the EcoRI sites in the rDNA of the four investigated Cucurbitaceae are known [9]. Therefore, equal amounts of total nuclear DNA of Cucurbita maxima and Cucurbita pepo and of Cucumis sativus and Cucumis melo was digested with EcoRI, separated on agarose gels, blotted on nitrocellulose filters and hybridized to the probes 1–4, respectively (Fig. 1). The strength of the hybridization signals obtained indicated the similarity between the different IGS regions (see Fig. 2).

The following probes were used for cross-hybridization experiments: Probe 1 is a 2.3-kbp HpaII fragment of the Cb. pepo clone pRZ7D; probe 2 (an 1.1-kbp fragment) resulted from a KpnI/HindIII digest of pRZX and represents part of the ETS region (R. A. Torres and V. Hemleben, unpublished results). The Cs. sativus 1.4-kbp Alul/HpaII fragment of the clone pRG81, probe 3, is mainly composed of a GC-rich “Cfo-cluster” [10] localized at different parts of the IGS and surrounding the duplications of the 3’ end of the 25 S rRNA coding region plus adjacent sequences occurring in the Cs. sativus spacer (see Fig. 2). Probe 4 is a 350-bp XhoI fragment of the ETS of Cs. sativus (Fig. 1 and 2).

Probe 1 strongly reacts with Cb. pepo and Cb. maxima DNA and shows only a weak signal with the Cucumis species. Hybridization with the Cb. pepo ETS probe 2 results in equally strong signals with both Cucurbita species and shows no reaction with the Cucumis species. Interestingly, the Cs. sativus ETS probe 4 reacts with a comparably weak hybridization signal with Cs. melo; no cross-hybridization occurs with the Cucurbita species. The GC-rich element (probe 3) of Cs. sativus, however, hybridizes to both the Cucurbita and the other Cucumis DNA to a very low extent, the weakest signal arises with Cb. maxima.

Interpreting these results it is obvious that in contrast to the highly conserved ribosomal RNA coding regions [9] the IGS is more divergent between the genera investigated (Fig. 1 and 2). However, the two Cucurbita species seem to be closer related to each other with respect to IGS sequences than the two Cucumis species.

Remarkably, different regions of the IGS diverge specifically: The selected region of the putative ETS of Cb. pepo (probe 2) shows a clear genus-specific reaction; however, within the Cucumis species this part of the spacer seems to be more variable. Sequencing studies on Cs. sativus and Cb. pepo demonstrate that this part of the IGS is build up by repetitive elements which are organized in a structurally related but sequence independent form (U. Zentgraf, R. A. Torres, and V. Hemleben, in preparation). The strength of hybridization indicates that the two Cucurbita species are similar in this region, whereas in the two Cucumis species these sequences diverge to a greater extent. Other dicotyledonous rDNAs exhibit a similar repetitive organization but no sequence similarity in the ETS region (e.g. mung bean [15] and carrot [16]). In rye, wheat and maize the ETS is also divergent across the genus barrier [17–19].

In the region further upstream (probe 1) of the ETS there are at least some sequences also strongly conserved between the Cucurbita species (Fig. 1 and 2); slight cross-reaction with the Cucumis species suggests the presence of a distantly related element in the IGS of this genus. A repetitive element is described for Cb. maxima (R. Kelly and A. Siegel, cited in [4]), however, there is no indication for a corresponding repeated sequence in Cb. pepo rDNA. Further sequencing will reveal this similarity.

The sequence of the 1.3-kbp probe 3 of Cs. sativus is known [10]; it is mostly composed of repetitively organized GC-rich 30-bp elements (called “Cfo-cluster”, according to the alternating GCs). Interestingly, with respect to these sequences Cs. sativus seems to be equally distantly related to Cs. melo and to the Cucurbita species. The observed weak cross-reaction of probe 3 with the three other plants, therefore, is possibly due to the existence of an altered “Cfo-cluster” in these plants. The “Cfo-clusters” in the Cs. sativus IGS probably stimulated unequal crossing-over processes resulting in duplications of the 3’ end of the 25 S rRNA coding region plus some adjacent sequences observed in the IGS [10]. Such a duplication is also reported for Cb. maxima (Fig. 2; R. Kelly and A. Siegel, cited in [4]) but is not detected in the Cb. pepo rDNA (R. A. Torres and V. Hemleben, unpublished results).

Cross-hybridization with other Cucurbitaceae

Extending our studies to other species and other genera of the Cucurbitaceae using the method of “slot-blot” hybridization (Fig. 3) it can be demon-
strated that the IGS region represented by the Cb. pepo probe 1 is strongly conserved within the genus Cucurbita; comparably weak signals are observed with Cs. sativus, Lagenaria leucantha, and Citrullus lanatus. Hybridization with the Cs. sativus probe 2 shows the same weak hybridization reaction with all the Cucurbita species and gives a slightly stronger answer with the Lagenaria and Citrullus species. DNA of Matthiola incana (Brassicaceae) is used background control.

Interspecific crossings, sometimes with the support of bridging species like Cb. lundelliana, demonstrated that Cucurbita species are closely related [20, 21]. The common occurrence of related highly repetitive satellite sequences in the genome of Cb. pepo and Cb. maxima [22] and other Cucurbita species [23] has been described. In contrast, Cs. sativus and Cs. melo seem to be more distantly related shown by cross-hybridization with rDNA intergenic spacer sequences. This observation is confirmed by isozyme analysis [24], number of chromosomes [25], meiotic analysis of different Cucumis hybrids [26], and characterization of species-specific highly repetitive satellite DNA [27, 28].

For Raphanus sativus rDNA it was shown that the short subrepeats within the region upstream of the transcription initiation site are highly specific for the genus Raphanus and do not cross-hybridize to other genera of the Brassicaceae e.g. Brassica [29]. However, within species of a given genus a certain sequence variability of the IGS is found. In plant breeding differences even within varieties of a species can be used for e.g. RFLP (restriction length polymorphism) mapping as shown for potato cultivars (Solanum tuberosum; [30]). The evolutionary mechanisms generating divergency between and homogenization within the intergenic spacer as demonstrated here for the rDNA of various Cucurbitaceae species obviously act differently at the respective regions of the intergenic spacer (see Fig. 2). The question whether this correlates with the functional significance of these regions is under further investigation.

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