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 Endriß, 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-

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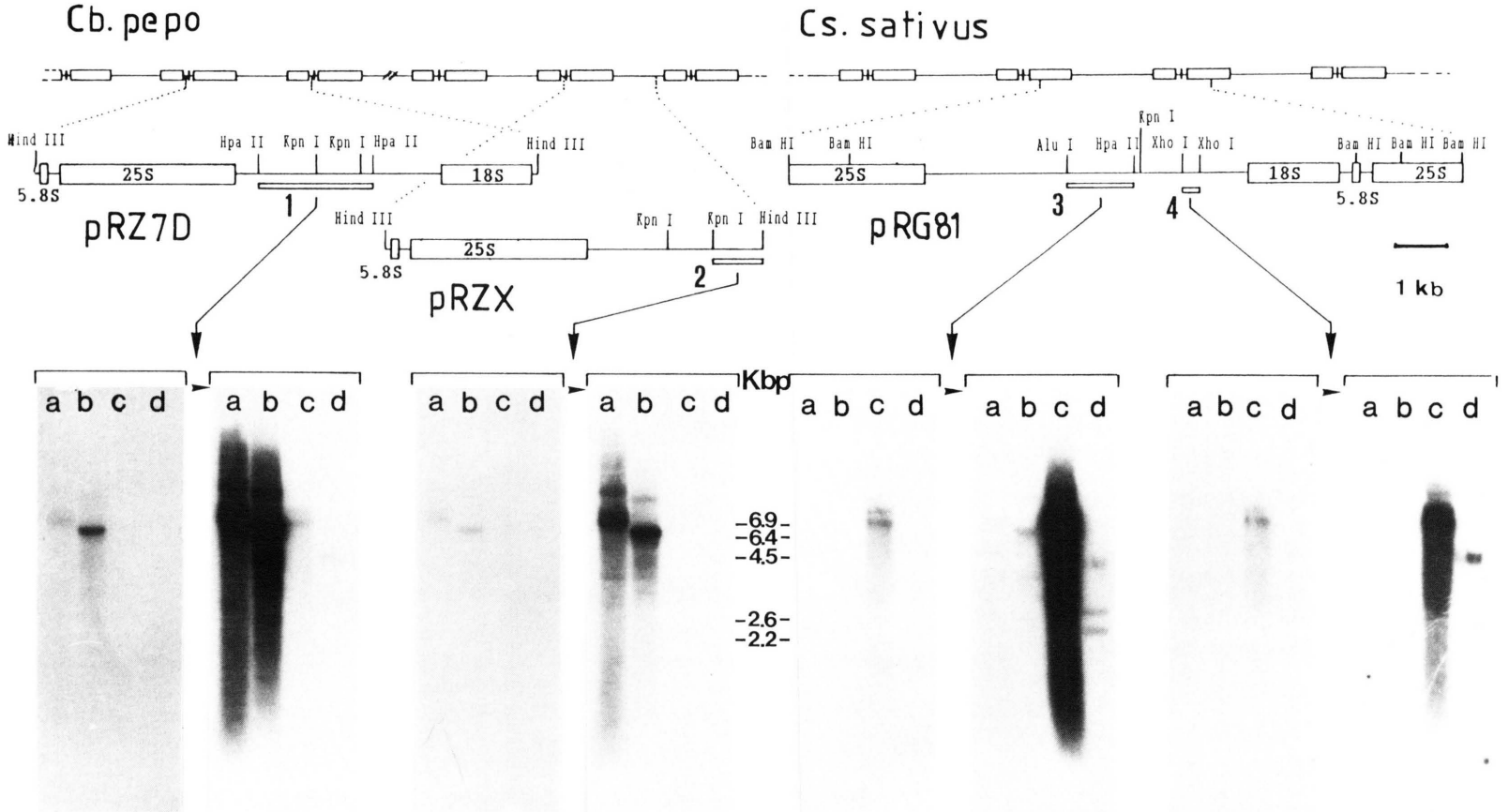

Fig. 1. Cross-hybridization of different ribosomal DNA intergenic spacer (IGS) fragments of zucchini (*Cb. pepo;* probes 1 and 2) and of cucumber (*Cs. sativus;* probes 3 and 4) to different species of the *Cucurbita* and the *Cucumis* genus. The tandemly arranged ribosomal DNA repeating units are schematically 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 pRG81 is indicated (middle; for description of the clones see Materials and Methods; restriction sites are given for *Hind*III, *Bam*HI, and *Xho*I; for *Alu*I and *Hpa*II 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 *Eco*RI, 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.

institut für Genetik und Kulturpflanzenforschung, Gatersleben, G.D.R. 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, electrophoresed 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).

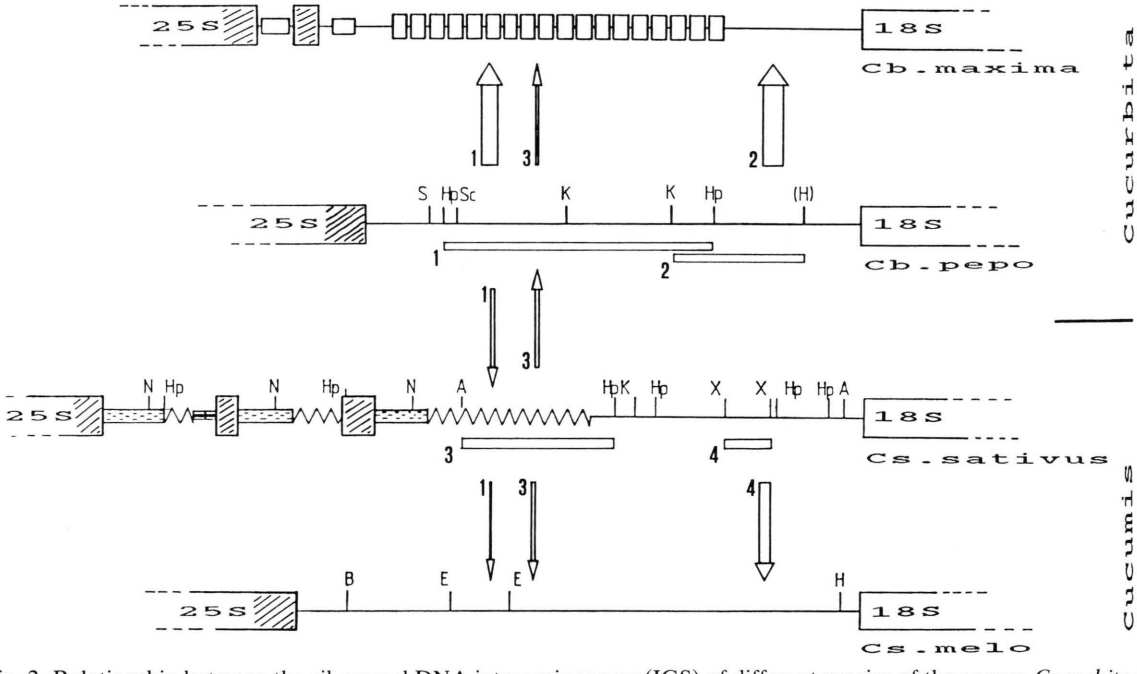

Fig. 2. Relationship between the ribosomal DNA intergenic spacer (IGS) of different species of the genera *Cucurbita* and *Cucumis* as deduced by the strength of cross-hybridization with different IGS probes of *Cucurbita pepo* and *Cucumis sativus* (see Fig. 1). The IGS of the four Cucurbitaceae investigated 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.

Comparison between the IGS of Cucurbita and Cucumis species

From restriction mapping experiments the *Eco*RI 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 *Eco*RI, 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 *Hpa*II fragment of the *Cb. pepo* clone pRZ7D; probe 2 (an 1.1-kbp fragment) resulted from a *Kpn*I/*Hind*III digest of pRZX and represents part of the ETS region (R. A. Torres and V. Hemleben, unpublished results). The *Cs. sativus* 1.4-kbp *Alu*I/*Hpa*II 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 *Xho* I 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-

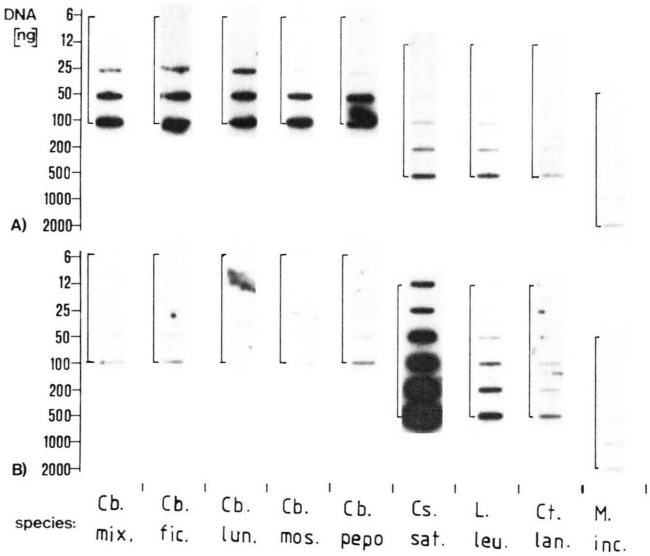

Fig. 3. Autoradiogram of "slot-blot" hybridizations with 32-P-labelled intergenic spacer fragments to DNA of other Cucurbitaceae. A) Hybridization with probe 1 (see Fig. 1 and 2) of *Cb. pepo*. B) Hybridization with probe 3 (see Fig. 1 and 2) of *Cs. sativus*. Increasing amounts of denatured total nuclear DNA of the *Cucurbita* species *Cb. mixta* (Cb. mix), *Cb. ficifolia* (Cb. fic.), *Cb. lundelliana* (Cb. lun.), *Cb. moschata* (Cb. mos.), and *Cb. pepo*, and of *Cucumis sativus* (Cs. sat.), *Lagenaria leucantha* (L. leu.), and *Citrullus lanatus* (Ct. lan.) were fixed onto nitrocellulose filters, hybridized to the respective 32-P-labelled probes, and exposed to X-ray films. Nuclear DNA of *Matthiola incana* (Brassicaceae) was used as control.

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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