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MOLECULAR CLONING OF A PLANT rDNA FRAGMENT CONTAINING THE 5′-PORTION OF 18S rRNA GENE AND ITS USE AS A PROBE IN INDIRECT END LABELING EXPERIMENTS

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Summary Plasmid pBG 35 contains the whole rDNA unit from flax (*Linum usitatissimum* L.) (Goldsbrough and Cullis, 1981). We cloned four different Eco RI restriction rDNA fragments of pBG 35 in the plasmid vector pBluescript SK^+ (pBS). One of the subclones (p2.1) contained the most part of the 5´-end of 18S rRNA gene (1581 bp) and was used as a heterologous DNA probe in molecular hybridization experiments. By means of the "indirect end labeling" technique, the plasmid p2.1 was used to study the methylation pattern of the intergenic spacer (IGS) of rRNA genes in excised cotyledons of *Cucurbita pepo* L. (zucchini) after treatment with the cytokinin 6-benzyladenine (BA). Heavy methylation of cytosine residues in the sequence -CCGG- both in IGS and 18S rRNA gene were observed. The methylation pattern was not changed upon hormonal treatment with BA.

Key words: cloning (subcloning) of plant rDNA, methylation pattern of IGS of plant rRNA genes, *Cucurbita pepo* L. (zucchini), excised cotyledons, cytokinins.

Abbreviations: Amp – ampiciline, BA – 6-benzyladenine, bp – base pairs, EDTA – ethylene diamine tetraacetic acid, DMSO – dimethylsulphoxide, EtBr – ethidium bromide, IGS – intergenic spacer, LB – Luria-Bertani medium, pBS – plasmid Bluescript SK^+, SDS – sodium dodecyl sulphate, 1xSSC – 4.41 g sodium citrate.l^{-1}, 8.76 g. l^{-1} sodium chloride, pH 7.0, TB – transformation buffer (10 mM Pipes, pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 0.250 M KCl,), TE – 10 mM Tris-HCl, pH 7.5 , 1 mM EDTA, pH 8.0.

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Introduction

The nuclear ribosomal RNA (rRNA) genes in eukaryotic organisms constitute a multigene family organized in tandemlly repeated units localized at the sites of nucleolus organizers (Long and Dawid, 1980). Each repeat unit consists of a noncoding IGS region situated upstream of the genes coding for 18S, 5.8S and 28S (25S in plants) rRNAs. In spite of the similarity of the overall transcriptional organization in plants and animals, the plant rRNA genes are different by two main reasons: they are highly repeated and the length of the repeated rDNA unit is much shorter than in animals (Long and Dawid, 1980). The great number of rRNA genes in plants is in excess of that needed for the cytoplasmic ribosome pool even in case of increased protein synthesis (Flavel et al., 1988). So, it seems likely that the plant cell can control the number of active rRNA genes *via* a specific mechanism of discriminating between transcriptionally active and silent rDNA copies. A lot of data on eukaryotic gene expression showed that such a control could be exerted through a concerted regulation including regulation of transcription at chromosome level by alterations of chromatin conformation and changes in the content of methylated cytosine residues on DNA template (Bird, 1995; Razin, 1998). Now it is firmly established that plant genomes contain much more 5-methylcytosine (5-mC), (about one third of cytosine) than animal genomes (Gruenbaum et al., 1981). Ribosomal RNA genes in plants seem to be also heavily methylated (Delseny et al., 1984; Flavell et al., 1988; Torres-Ruis and Hemleben, 1994). However some specific sites in the repeated rDNA unit were found to be regularly hypomethylated (Flavell et al., 1988; Jupe and Zimmer, 1990). Special interest deserves the IGS of rRNA genes where the promotor region and transcription termination site (TTS) as well as other repetitive elements are localized and they are supposed to play an important role in regulation of transcription. Now it is well known the primary structure and the methylation pattern of IGS in a single rDNA clone of *C. pepo* (zucchini) (King et al., 1993), but little is known about the functional regulation of transcription of these genes under different physiological conditions. The main objective of this work was to study the methylation pattern of IGS in zucchini after hormonal treatment *in vivo* of excised cotyledons with the cytokinin 6-benzyladenine (BA) when a 2-4-fold stimulation of the endogenous nuclear RNA polymerase I activity was previously observed (Ananiev at al., 1987).

In the present work we mapped the methylation of C in the sequence -CCGG- within the IGS of *C. pepo* by the use of methylation sensitive restriction enzymes Msp I and Hpa II and the "indirect end labeling" technique. Based on the conservatism of 18S rRNA genes in eukaryotes we made use of the 5′-end portion of the structural gene of 18S rRNA from flax (*Linum usitatissimum* L.) as ^{32}P-labeled rDNA probe. The plasmid pBG 35 which contains the whole rDNA unit from flax served as a donor for cloning the appropriate rDNA probe (Goldsbrough and Cullis, 1981). Complete

digestion of pBG 35 with the restriction enzyme Eco RI results in generation of four rDNA fragments with different size. The smallest of them (2.1 kb) spans the 5′-end portion of 18S rRNA structural gene and was used as rDNA hybridization probe. In this work we describe the cloning procedure of Eco RI 2.1 kb fragment at the single Eco RI site in the polylinker of plasmid pBluescript and its use as rDNA hybridization probe.

Material and Methods

Plants and growth conditions

Seeds of *Cucurbita pepo* L. (zucchini), cv. Cocozelle, were germinated for 96 h in darkness at 28 ºC. After excision of the embryonic axes, cotyledons were transferred to Petri dishes with distilled water for another 24 h in order to reduce the endogenous cytokinins and abscisic acid content. Then the cotyledons were incubated on distilled water (control) for 12 h in darkness or on aqueous solutions of 45 µM BA for 6 h in darkness.

Isolation of nuclear DNA and purification

Preparation of nuclei

Crude nuclei preparation from excised cotyledons was obtained as previously described (Ananiev et al., 1987). Cotyledons were ground in liquid nitrogen or in a mortar with precooled bufer A (30 ml buffer per 5 g of plant material). Buffer A contained 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 20 mM KCl, 0.6 M sucrose, 30% glycerol, 0.5 mM EDTA and 10 mM 2-mercaptoethanol. 400 µg.ml^{-1} ethidium bromide (EtBr) was present in the grinding buffer as an inhibitor of endogenous DNase and RNase activities (Luthe and Quatrano, 1980). The homogenate was filtured through Miracloth and centrifuged at 3000 rpm at –5 ºC for 10 min.

Nuclear DNA extraction

The nuclear pellet was lysed in a buffer containing 100 mM Tris-HCl, pH 8.0, 1% SDS and 20 mM EDTA. DNA was extracted by gentle shaking in ice-cold water bath overnight, purified by subsequent deproteinization with phenol (2 times), phenol-chloroform and chloroform:isoamilyc alcohol (24:1), precipitated with 3 vol. ethanol and dissolved in TE buffer. Total cellular RNA was digested with RNase A (10 µg. ml^{-1}) according to Maniatis et al., 1982. DNA was purified by thorough deproteinization with phenol (at least 2 times), phenol-chloroform and chloroform:isoamilyc alcohol (24:1), precipitated with 3 vol. of ethanol and dissolved in TE buffer.

Molecular cloning of plant rDNA fragments

Preparation of competent cells of E. coli

All medias used throughout the experiments were prepared according to the procedure of Hanahan (1983). Competent for transformation cells of *E. coli* strain XL1-blue were prepared according to Okayama et al., 1990 with slight modifications. LB agar plates (containing 12.5 μg ml^{-1} tetracycline) were inoculated with thawed XL1-blue cells in Hogness medium (3.6 mM K_2HPO_4; 1.3 mM KH_2PO_4, 2 mM Na-acetate, 1 mM $MgSO_4.7H_2O$, 4.4% glycerol) and cultivated overnight at 37 ºC. Two or three colonies (2–3 mm in diameter) were used to inoculate 100 ml of SOB medium (Bacto Tryptone – 20 $g.l^{-1}$, yeast extract – 5 $g.l^{-1}$, NaCl – 0.5 $g.l^{-1}$, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl) in 500 ml flasks and grown to an absorbtion A_{600} of 0.6 at 18 ºC with vigorous shaking (200–500 rpm). The cell culture was centrifuged at 3000 rpm for 10 min at 4 ºC and the cell pellet was resuspended in 32 ml of precooled transformation buffer (TB). The cells were incubated in an ice bath for 10 min and spun down as above. The pellet was gently resuspended in 8 ml of TB, containing DMSO at final concentration of 7%. After incubation on ice for 10 min the cell suspension was divided to 1 ml into 1.5 ml Eppendorf tubes and kept at –70 ºC until used.

Transformation of competent cells

Frozen competent cells of *E. coli* (50 μl), strain XL-1 blue with genotype: F, proAB, lac I^q, lac ZDM15, Tn 10 (Tet^r), rec A1, gyr A96 (Na I^r), thi 1, sup E44, rel A1, were thawed in an ice bath. After that plasmid DNA was added in 1–5 μl (1 $ng.ml^{-1}$) to the cells and the mixture was swirled and incubated on ice for 30 min. The mixture was heat-pulsed without agitation at 42 ºC for 30 s and placed on ice for 10 min. Then 500 μl of LB medium were added and the tubes were incubated at 37 ºC for 1 h. LB agar plate (containing 100 $\mu g.ml^{-1}$ Amp) was inoculated with 100 μl of the cell culture and cultivated overnight at 37 ºC.

Plasmid DNA isolation ("maxi-prep")

70 ml of LB medium (Bacto Tryptone – 10 $g.l^{-1}$, yeast extract – 5 $g.l^{-1}$, NaCl – 10 g^{-1}), containing 100 $\mu g.ml^{-1}$ Amp, were inoculated with a single colony obtained after transformation of competent cells of *E. coli*. Cells were cultivated overnight at 37 ºC with vigorous shaking (200–250 rpm). The cell culture was pelleted at 5000 rpm for 10 min at 4 ºC. The cell pellet was resuspended in 4 ml solution I (50 mM glucose, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) and incubated in an ice bath for 10 min. 4 ml of solution II (0.2 N NaOH, 1% SDS) were added. After incubating in an ice bath for 10 min 4 ml of potassium acetate (pH 5.0) was added to the suspension and incubated on ice for another 10 min. After that 10–12 ml of chlorophorm/isoamyl alcohol (24:1, vol/vol) was added. Samples were centrifuged at 5000 rpm for 10 min and the supernatant was treated with RNase A (final concentration of 0.025 $mg.ml^{-1}$) for 1 h at 37 ºC. After that the supernatant was purified by several steps of deproteinization with phenol/chloro-

phorm (1:1, vol/vol) and chlorophorm/isoamyl alcohol (24:1 vol/vol) and then precipitated with equal volume of ispropanol. The precipitate was washed once with 70% ethanol, dried and finally dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Plasmid DNA was precipitated once more by equal volume of 15% PEG (Mw 8 000) and 0.8 M NaCl. After incubation in an ice bath for 1 h the samples were centrifuged in an Eppendorf microfuge (12000×g). The resulting precipitate was washed in 70% ethanol, dried and redissolved in TE buffer.

Molecular cloning of rDNA fragments from the repeated rDNA unit of flax and preparation of rDNA containing plasmids

Plasmid DNA pBG 35 containing the entire rDNA unit from flax (*Linum usitatissimum* L.) was digested to completion with the restriction enzyme Eco RI (1 µg DNA/5 units of enzyme) and the resulting four definitive DNA fragments were separated by a prolonged electrophoresis in 0.8% agarose gel. The appropriate gel strips, containing the four DNA fragments with lengths of 4.4, 3.05, 2.8 and 2.1 kb were cut with a scalpel from the gel, put into an Eppendorf tubes and frozen at –20 °C overnight. After thawing at room temperature the gel strips were homogenized with Vortex and an equal volume of Tris-HCl-saturated phenol, pH 7.6 was added to the slurry. The mixture was frozen at –55 °C for 2 h, thawed, vortexed and frozen once again at –20 °C for overnight. The water phase was collected after centrifugation of the thawed material and an equal volume of phenol was added. The appropriate DNA fragment was pelleted with 2 M ammonium acetate and 1 volume of isopropanol, washed in 70% of ethanol and redissolved in TE buffer. The extraction of DNA fragments from the gel was controlled by electopghoresis in an agarose gel.

Alkaline phosphatase treatment of plasmid vector DNA and ligation of DNA fragments

Briefly, 100 ng of Eco RI-digested pBS was treated with alkaline phosphatase (2 units) in dephosphorilation buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ at 37 °C for 40 min. Alkaline phosphatase was inactivated by heating for 15 min at 65 °C. Dephosphorilated and linearized pBS-Eco RI plasmid was electrophoretically purified in 0.8% agarose gel and extracted from the gel as described above. After that 100 ng of dephosphorilated Eco RI-digested pBS was mixed with 100 ng Eco RI 2.1 kb fragment isolated from the gel. DNA fragments were ligated using 5 units of T4 DNA ligase. Ligation reaction was carried out in 10 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP for 16 h at 16 °C. After incubation the ligation mixture was used to transform competent cells of *E. coli*, strain XL1-blue. Transformants were screened visually by α-complementation for blue/white colour selection of recombinants in medium containing 100 µg.ml^{-1} ampiciline. The structure of recombinant plasmids was then verified by restriction analysis of minipreparations of plasmid DNA.

Restriction enzyme analysis and molecular hybridization

Single or double digests of purified DNA were carried out with restriction enzymes Eco RI, Hind III, Msp I or Hpa II (1 μg DNA with 2–5 units of enzyme) according to the supplier·s instructions. All restriction enzymes used in this work and the DNA markers (1 kb DNA ladder and λ-phage DNA digested with Eco RI and Hind III) were purchased from FERMENTAS, Riga, Lithuania. Restriction enzyme digests of nuclear DNA were electrophoreticaly separated in 0.8% agarose gel and transferred to nylon membranes (Hybond N+) according to standard methods as described by Maniatis et al., 1982 As a hybridization probes was used the cloned 2.1 kb Eco RI fragment of pBG35 spanning the 18S rRNA gene in flax (*Linum usitatissimum* L.). The rDNA probe was labeled with α[^{32}P]dCTP by the method of "random priming" using hexanucleotides as primers. Hybridizations were carried out at 42 ºC in 5xSSC, 50 mM phosphate buffer pH 6.8, 5% SDS and 50% formamide. Nylon membranes were washed 3 times each in 2xSSC, 50 mM phosphate buffer pH 6.8, 1%SDS; in 1xSSC, 50 mM phosphate buffer pH 6.8 and finally in 0.1xSSC, 50 mM phosphate buffer pH 6.8, 1% SDS at room temperature. Membranes were then dried and exposed to AGWA films using intensifying screens (Du Pont) for 6–24 h at –70 ºC.

Results and discussion

In order to subclone the most part from the 5′-end of 18S rRNA structural gene we used as a donor the plasmid pBG 35 which contained the whole rDNA unit from flax inserted into the plasmid pAT 153 at its single Bam HI site (Goldsbrough and Cullis, 1981). Complete digestion of pBG 35 with Eco RI results in generation of four DNA fragments with sizes of 4.4, 3.05, 2.8 and 2.1 kb pairs, respectively. Fig 1. shows the behaviour of the donor plasmid pBG 35 in the agarose gel (lane 1), the migration of the four Eco RI–generated restriction fragments of pBG (lane 2) and the case when each of them was extracted from the gel and put to electrophoresis (lanes 3–6). It must be noted the purity of the bands corresponding to the fragments with 4.4 and 2.1 kb, respectively, (Fig. 1, lanes 3 and 6) as well as the cross-contamination of the fragments with similar size (3.05 and 2.8 kb), (lane 4). The electrophoretic pattern of the recipient plasmid pBS in its three forms (supercoiled, circular and relaxed) is shown in lane 7 while its linearized form after Eco RI digestion is presented in lane 8. The electrophoretic pattern of plasmid p2.1 containing the inserted 2.1 kb fragment in pBS is shown in lane 9. Complete digestion with Eco RI of p2.1 resulted in the appearance of two fragments with size of 2.1 and 2.96 kb (the linearized size of pBS) (lane 10). This experimental approach was applied also for the checking of plasmids p2.8 and p4.4 (lanes 11–12 and 13–14, respectively). We do not show the proper orientation of the inserted Eco RI fragments in the recipient plasmid pBS which is not of critical importance for

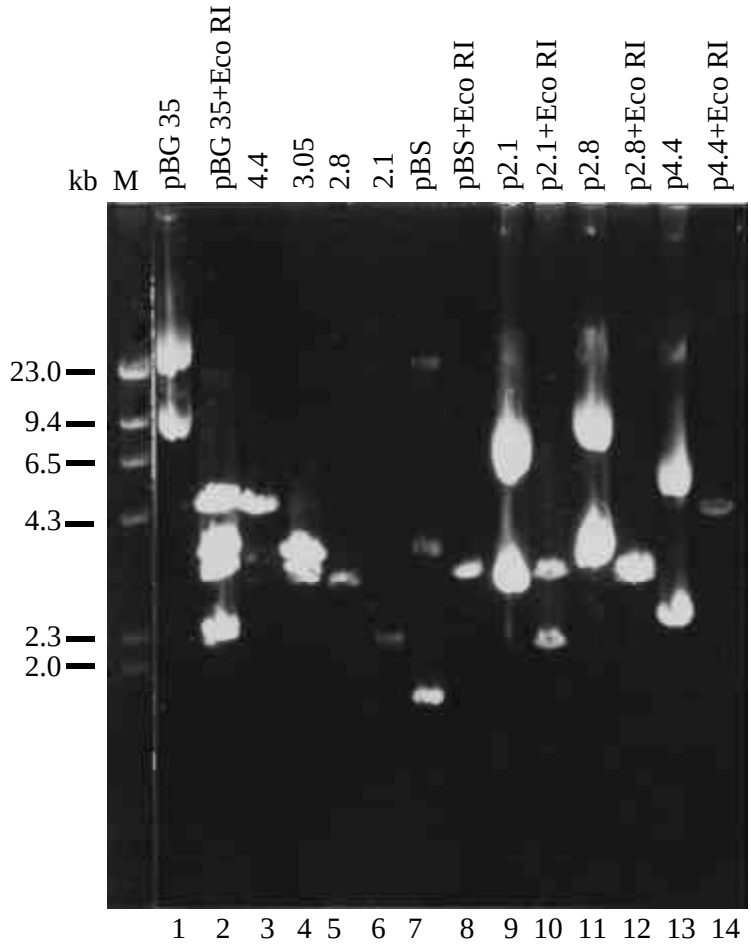

Fig. 1. Cloning of different fragments from the rDNA unit of *L. usitatissimum* L. in the plasmid pBluescript SK+. Plasmid pBG 35 harbouring the whole rDNA unit of flax was digested with Eco RI and the resulting fragments were electrophoretically separated in 0.8% agarose gel, isolated from the gel and cloned at the single Eco RI site in the polylinker of pBluescript SK^+ as described in "Material and methods". M – marker DNA (λ-phage DNA digested with Hind III). Electrophoretic behaviour of nondigested (Lane 1) and Eco RI-digested pBG 35 (Lane 2). Lanes (3-6) – electrophoresis of the four Eco RI-generated fragments of pBG 35 isolated from the gel (4.4, 3.04, 2.8 and 2.1 kb, respectively). Electrophoretic pattern of the circular (Lane 7) and linearized form of the recipient plasmid pBS after Eco RI digest (Lane 8). Electrophoretic pattern of subclones p2.1 (Lane 9), p2.8 (Lane 11) and p 4.4 (Lane 13). Lanes 10,12, and 14 represent the electrophoretic pattern of p2.1, p2.8 and p4.4 sublones after a complete Eco RI digestion.

our objectives. So, as a result of these cloning experiments we dispose of different specific rDNA probes for molecular hybridization.

Further on we made use of the plasmid p2.1 to investigate the methylation pattern in IGS of the rRNA genes in *C. pepo* (zucchini). The full nucleotide sequence of IGS

in a single rDNA clone of *C. pepo* is now well known (King et al., 1993). Based on the sequence data as well as on the conservatism in the primary structure of 18S rRNA genes in dicots, (GenBank AF223066, 14531 bp of of *Humulus lupulus* rDNA), we represented a combined physical restriction map of rDNA unit in *C. pepo* (Fig 2). *C. pepo* (zucchini) has two different types of repeated rDNA units with length of about 10 and 9.3 kb (Ganal and Hemleben, 1986). Except the length heterogeneity due to different repetitive elements in IGS, sequence heterogeneity of rDNA is also observed. Thus, the major repeat unit (~10 kb) consists of two subtypes that could be distinguished by the presence of an additional Hind III site in front of the 18S rRNA gene. Therefore, complete digestion of the major rDNA unit with Hind III results in two definite fragments of 10 kb and 2.2 kb, while digestion with Eco RI generates two main DNA fragments of 6.2 kb and 3.7 kb from the major unit and a slight 5.7 kb fragment from the minor repeat units (Ganal and Hemleben, 1986). The bigger Eco RI fragments contain the IGS itself and can be used to study the methylation of cytosine residues in -CpG- met in the regulatory regions of IGS using digestion with the methylation-sensitive restriction enzymes Msp I/Hpa II. In addition, double digestion of genomic DNA with Eco RI and Hind III will further facilitate this investigation**.** Methylation of the C residues in the short palindrome -CCGG- can be best studied by the pair of isoschizomers Msp I and Hpa II which have different specificity with respect to methylation. Both enzymes and only Hpa II can cut unmethylated cytosine sequences while Msp I cuts sites methylated on the second C but not those methylated on the first. If both Cs are methylated, neither enzyme is able to cut the sequence. Indicated in Fig. 2

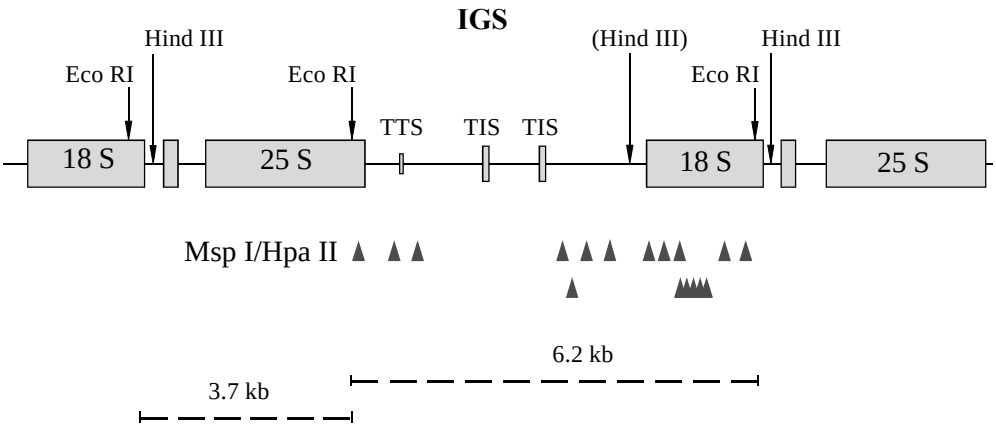

Fig. 2. Restriction map of rDNA unit in excised cotyledons of *Cucurbita pepo* L. (zucchini). The major length variant of rRNA genes (~10 kb) determined after digestion with Hind III is presented. In brackets is presented the minor Hind III site in IGS. IGS – intergenic spacer; TIS – transcription initiation site; TTS – transcription termination site. Nuclear DNA was subsequently digested with Eco RI, Hind III and afterwards with Msp I or Hpa II, respectively. The length of Eco RI-generated restriction fragments and the positions of Msp I/Hpa II sites in IGS and the structural gene for 18S rRNA are indicated.

are the positions of all six putative Msp I/Hpa II sites in the IGS as revealed from the primary structure of one single rDNA clone (King et al., 1993). Based on the conservatism of the structural gene for 18S rRNA the positions of all possible 10 Msp I/Hpa II sites in the 18S rRNA gene are also shown. Hind III and Eco RI single and double digests of bulk DNA isolated from control and BA-treated cotyledons are shown in Fig. 3A, lanes 2,3,4 and 8,10. Further on, the Eco RI and Hind III-digestion fragments and the position of the active Msp I and Hpa II sites within IGS were precisely located, following Southern transfer to Hybond^{+} membrane and hybridization with the ^{32}P-labelled 2.1 kb Eco RI fragment subcloned in p2.1 (Fig. 3B). As expected the complete digestion of genomic DNA with Hind III resulted in two fragments hybridizing with the 18S rDNA probe (Fig. 3B, lanes 2, 8). The bigger one (10 kb) represents the whole length of rDNA unit in marrow and is generated by the single conservative (present in all rDNA units) Hind III site localized in the internal transcribed spacer 1 (ITS1), 128 bp after the 3′-OH end of the 18S rRNA gene (Jobst et al., 1998). The smaller Hind III fragment (2.2 kb) is defined by the presence of an additional Hind III site in some of the repeats at position 264, upstream from the 5′-end of 18S rRNA gene. In the case of Eco RI digestion only the bigger Eco RI fragments from the major and minor rDNA units were detected in autoradiographs as 6.2 kb and 5.7 kb, respectively (Fig. 3B, lanes 3,9). Double digestion with Eco RI and Hind III preserved the bigger Eco RI fragments but provoked also the appearance of a new fragment of 1.845 kb (Fig. 3B, lanes 4,10). The latter fragment was generated when additional Hind III site in IGS appeared as a result of substitution of the outer C in the sequence -AAGCTC- with T, thus generating the proper site for Hind III (-AAGCTT-) in front of the 5′-end of 18S rRNA gene. Digestion with Msp I resulted in generation of fragments with smaller size (between 0.5 and 2.5 kb), but they were not completely digested with Msp I (Fig. 3B, lanes 5,11). This suggests that in addition with -CpG- sequences, methylation in -CpNpG- might not be random throughout the IGS and 18S rRNA gene in marrow. Among the Msp I fragments three of them hybridized most intensively with this probe thus suggesting that they were generated from the structural 18S rRNA gene itself. Their relative length was estimated to be about 450–500 bp for the smallest one, about 750 bp for the middle-sized and about 1100–1200 bp for the greatest of them (Fig. 3B, lanes 5,11). Special interest deserved the highest Msp I fragment in the electrophoretic pattern since it was also detected after Hpa II digestion. This Hpa II/Msp I major fragment was 2.4–2.5 kb long (Fig. 3B, lanes 5,11). By calculating the length of all possible Msp I/Hpa II fragments generated from 6.2 kb Eco RI fragment and by overlapping their lengths it was possible to suggest that these Hpa II fragments could reside from the group of hypomethylated -CCGG- sites near the second TIS in the IGS. The major Hpa II-generated fragment could arise from a hypomethylated site located 313 bp downstream from the “spacer” promotor and the Hpa II site at position 1118 in the 18S rRNA gene. So, it appears that a hypomethylated -CCGG- site could exist also in the 18S rRNA gene.

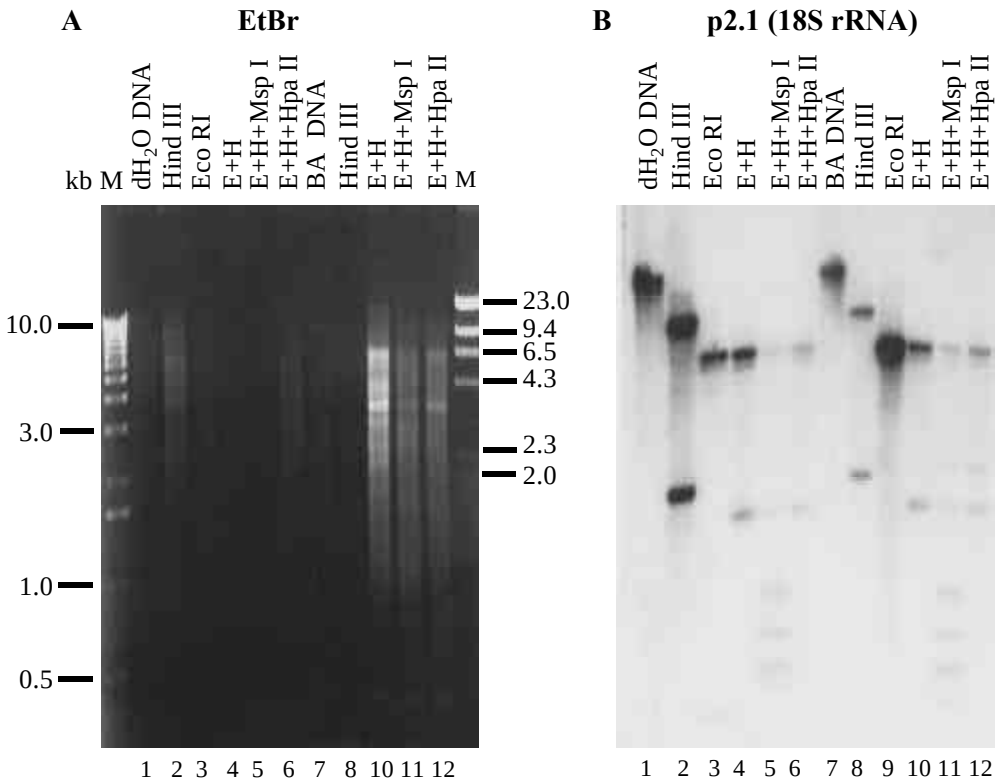

Fig. 3. Methylation pattern of rDNA in excised *C. pepo* cotyledons. **A** – Restriction enzyme analysis of genomic DNA with indicated enzymes and fluorescence of EtBr-stained DNA fragments in UV-light. **B** – Hybridization with cloned ^{32}P-labelled 2.1 kB Eco RI fragment (p2.1) spanning the 18S rRNA gene from flax (*Linum usitatissimum* L.). Lanes (1–6) – genomic DNA from control cotyledons (dH_2O-treated for 12 h in darkness). Lanes (7–12) – genomic DNA from BA-treated cotyledons for 6 h in darkness.

It must be noted that the methylation patterns of IGS and 18S rRNA gene were not altered in the case of 2-4 fold stimulated transcription of rRNA genes after *in vivo* treatment with BA (Fig. 3B, lanes 11,12).

Conclusions

1. The plasmid pBG 35 which harbors the whole rDNA unit of flax (*Linum usitatissimum* L.) was used as a donor for cloning different Eco RI-generated fragments in the plasmid pBluescipt SK$^+$. One of these clones (p2.1) contained a 2.1 kb insert which spans the most part of the 5′-end portion of the structural gene for 18S rRNA.

2. Digestion of genomic DNA with methylation sensitive restriction enzymes Msp I and Hpa II showed heavy methylation of rRNA genes in *C. pepo* L. As judged from the almost total lack of digestion with Hpa II, in rDNA units there are no methylation free regions or little if any were observed. A hypomethylated Hpa II site was detected in IGS near the promoter region of some of the repeats.
3. The methylation pattern in the IGS and 18S rRNA structural gene was not changed upon *in vivo* treatment of the cotyledons with cytokinin. This suggested that the alterations in rRNA gene activity were not due to or accompanied by significant DNA methylation changes.

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References

Ananiev, E. D., L. K. Karagyozov, E. N. Karanov, 1987. Effect of cytokinins on ribosomal RNA gene expression in excised cotyledons of *Cucurbita pepo* L. Planta, 170, 370–378.

Bird, A., 1995. Gene number, noise reduction and biological complexity. Trends Genet., 11, 94–100.

Delseny M., M. Laroche, P. Penon, 1984. Methylation pattern of radish (*Raphanus sativus*) nuclear ribosomal RNA genes. Plant Physiol., 76, 627–632.

Flavel, R. B., M. O·Dell, W. F. Thompson, 1988. Cytosine methylation of ribosomal RNA genes and nucleolus organizer activity in wheat. J. Mol. Biol., 204, 523–534.

Ganal, M., V. Hemleben, 1986. Comparison of ribosomal RNA genes in four closely related *Cucurbitaceae*. Plant Syst. Evol., 154, 63–77.

Goldsbrough, P. B., C. A. Cullis, 1981. Characterization of the genes for ribosomal RNA in flax. Nucl. Acids Res., 9, 1301–1309.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol., 166, 557–580.

Inoue, H., H. Nojima, H. Okayama, 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene, 96, 23–28.

Jobst, J., K. King, V. Hemleben, 1998. Molecular evolution of the internal transcribed spacer (ITS1 and ITS2) and phylogenetic relationships among species of the family *Cucurbitaceae*. Mol. Phylogenet. Evol., 9, 204–219.

Jupe, E. R., E. A. Zimmer, 1990. Unmethylated regions in the intergenic spacer of maize and teosinte ribosomal RNA genes, Plant Mol. Biol., 14, 333–347.

King, K., R. A. Torres, U. Zentgraf, V. Hemleben, 1993. Molecular evolution of the intergenic spacer in the nuclear ribosomal RNA genes of *Cucurbitaceae*. J. Mol. Evol., 36, 144–152.

Long, E. D., I. B. Dawid, 1980. Repeated genes in eukaryotes. Ann. Rev. Biochem., 49, 727–764.

Luthe, D. S., R. Quatrano, 1980. Transcription in isolated wheat nuclei. I. Isolation of nuclei and elimination of endogenous ribonuclease activity. Plant Physiol., 65, 305–308.

Maniatis, T., E. F. Fritsch, J. Sambrook, 1982. Molecular cloning, A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Razin A., 1998. Cytidine methylation, chromatin structure and gene silencing – a three way connection. The EMBO J., 17, 4905–4908

Torres-Ruis R. A., V. Hemleben, 1994. Pattern and degree of methylation in ribosomal RNA genes in *Cucurbita pepo* L. Plant Mol. Biol., 26, 1167–1179.