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HORMONAL REGULATION OF RIBOSOMAL RNA GENE EXPRESSION IN EXCISED COTYLEDONS OF *CUCURBITA PEPO* L. (ZUCCHINI)

E. D. Ananiev[1], G. Abdulova[1], P. Grozdanov[2], L. Karagyozov[2]

[1]Institute of Plant Physiology "Acad. M. Popov", block 21, Sofia 1113, Bulgaria
[2]Institute of Molecular Biology, Bulg. Acad. Sci., block 21, Sofia 1113, Bulgaria

Summary: In the present work we have studied the effects of exogenously applied methyl ester of jasmonic acid (MeJA) and benzyladenine (BA) on rRNA gene expression in the model system of excised marrow cotyledons (*Cucurbita pepo* L. zucchini). Treatment of cotyledons with BA or MeJA resulted in a 2-fold increase and a 25–30% decrease in the endogenous nuclear RNA polymerase I activity, respectively. The methylation pattern of the intergenic spacer (IGS) of rRNA genes was studied as a measure of their activity using the couple of the restriction enzymes Msp I and Hpa II and the method of "indirect end labeling". A cloned fragment containing the 5′ portion of 18S rRNA gene from flax was used as DNA probe. Results showed heavy methylation of the rRNA genes. As judged from the almost total lack of digestion with Hpa II, in the repeating rDNA units there were either no methylation free regions or just a few were observed. A hypomethylated Hpa II site near the promoter region was detected in a very small number of rDNA repeats. Digestion with Msp I affected nearly 50% of the repeating units. The Msp I digestion products were few in number and large in size. This suggested that in addition to -CpG- sequences, methylation in -CpNpG- might not be random. The methylation pattern of IGS was not changed upon treatment of the cotyledons *in vivo* with BA and MeJA. Thus, the hormone-mediated alterations in rRNA gene activity were not accompanied by any significant DNA methylation changes.

Key words: methylation pattern of IGS of rRNA genes in plants; cytokinins; methyl ester of jasmonic acid; excised cotyledons; *Cucurbita pepo* L. (zucchini)

* Corresponding author, e-mail: Ananiev@obzor.bio21.bas.bg

Introduction

It is well known that excised cotyledons of *Cucurbitaceae* can serve as useful tools for studying the effect of exogenous cytokinins (Longo et al., 1979; Kulaeva, 1981; Letham and Palni, 1983). On the other hand it has been shown that jasmonates – jasmonic acid (JA) and its methyl ester (MeJA) and cytokinins can counteract in different physiological processes like leaf senescence (Weidhase et al., 1987) or the growth responses of different plants (Ueda et al., 1981). Our previous results showed that treatment of excised cotyledons of *C. pepo* (zucchini) with BA in darkness resulted in an increase in fresh weight and decrease in dry mass in contrast to MeJA which did not alter significantly the growth of cotyledons (Ananieva and Ananiev, 1999). The effect of these phytohormones was accompanied by a 2-4-fold stimulation of rRNA synthesis in response to BA (Ananiev et al., 1987) and about 45–30% inhibition after treatment with abscisic acid (ABA) and MeJA, respectively (Ananieva and Ananiev, 1999). It is now firmly established that site-specific metnylation of eukaryotic DNA can play a crucial role in the regulation of gene expression (Bird, 1995; Razin, 1998). In this work we envisaged the possibility that hormone-mediated regulation of rRNA gene expression might account for changes in the content of 5-methylcytosine (5mC) in rRNA genes in *C. pepo.* In respect of methylation and its possible relations to regulation of transcription, of special interest is the intergenic spacer (IGS) of rRNA genes where the promoter region (transcription initiation site – TIS) and transcription termination site (TTS) as well as other regulatory elements are localized.

Materials and methods

Seeds of *Cucurbita pepo* L. (zucchini), cv. *Cocozelle* were germinated for 96 h in darkness at 28 °C. After excision of embryonic axes, cotyledons were kept in darkness for a further 24 h in distilled water in order to decrease the endogenous cytokinin and abscisic acid (ABA) levels. The cotyledons were then incubated for different periods of time on distilled water or aqueous solutions of 45 μM BA or 45 μM MeJA. In some experiments a 45 μM solution of ABA was also used. Nuclei were isolated and endogenous nuclear RNA polymerase activity was determined as described previously by Ananiev et al., (1987). High molecular genomic DNA was isolated and purified after adsorption on a Celite column as described by Abdulova et al. (2002). Single Eco RI and double Eco RI-Msp I or Eco RI-Hpa II digestion products were precisely located within the IGS of rRNA genes, following Southern transfer to Hybond^{+} membranes and hybridization to cloned ^{32}P-labeled 2,1 kb Eco RI fragment of pBG 35 (Goldsbrough and Cullis, 1981). This DNA fragment spans the most part of the 18S rRNA gene in flax from its 5′-end just to the Eco RI site at position 1581.

Results and discussion

Fig.1 represents the kinetic analysis of the activity of endogenous nuclear RNA polymerase I in cotyledons after different hormonal treatments. BA provoked a very rapid stimulation of this activity with a maximum (about 2.5-fold) observed after 3–6 h of cytokinin treatment. Later on, the stimulatory effect of BA sharply decreased and by 24 h the values were approximately the same as at the start of cytokinin action. Treatment of cotyledons with ABA and MeJA resulted in a reduction of RNA polymerase I activity (about 45 and 25% inhibition, respectively). These results showed that a different quantity of ^{3}H-labeled RNA product was measured in all tested variants for the same time (duration of RNA assay was 10 min, Ananiev et al., 1987). The effect of phytohormones on rRNA synthesis might be accounted for by many reasons, but two of them are of critical importance for the regulation of rRNA gene transcription.

1. Increased (decreased) rate of transcription (elongation of the pre-formed nascent rRNA chains)
2. Increased (decreased) number of template bound molecules of RNA polymerase I.

Our previous results revealed that as regards the stimulatory effect of BA no significant changes in the number of transcribing RNA polymerase I molecules were observed. At the same time the effect of cytokinins on RNA polymerase I activity coincided with a 2-fold increase in the elongation rate of transcription (Ananiev et al., 1987). The main goal of this work was to check the possibility that the increased rate of transcription of rRNA genes might be due to or accompanied by an altered methyla-

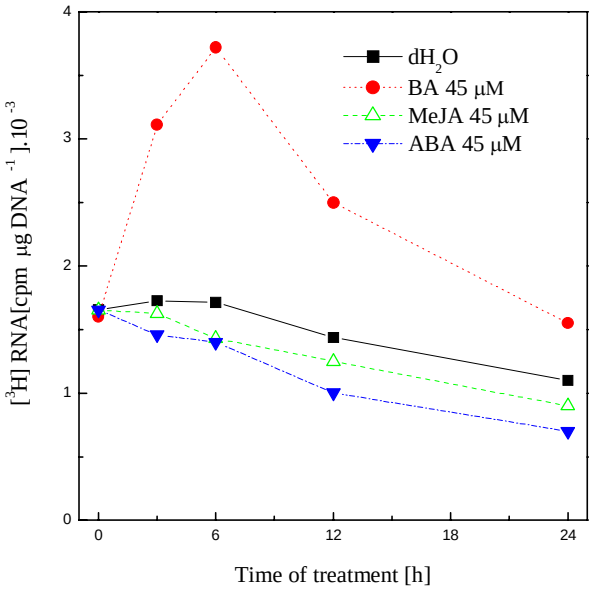

Fig. 1. Effect of BA, MeJa and ABA on the activity of nuclear RNA polymerase I in excised cotyledons of *C. pepo* L. (zucchini) in darkness. Nuclei were isolated from cotyledons treated with 45µM solutions of BA, MeJA and ABA for the indicated periods of time. Endogenous nuclear RNA polymerase I activity was estimated by the incorporation of ^{3}HUMP in RNA synthesized in vitro in the presence of 5 µg.ml^{-1} α-amanitin.

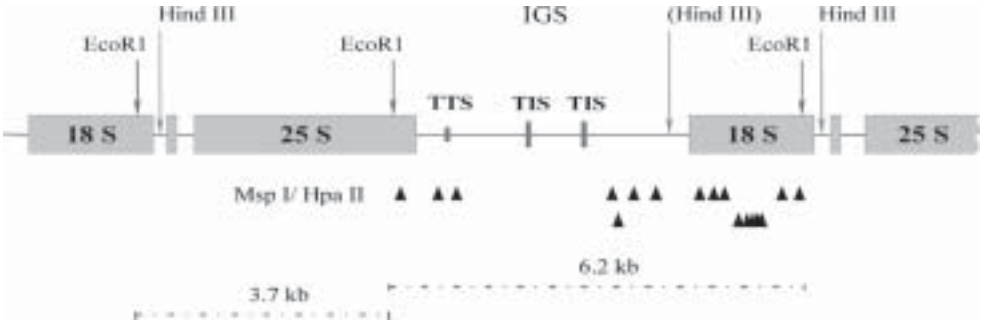

Fig. 2. Restriction map of of rDNA unit in excised cotyledons of *Cucurbita pepo* (zucchini). The major length variant of rRNA genes (~10 kb), determined after digestion with Hind III is presented. In brackets is presented the minor Hind III site in IGS. IGS – intergenic spacer; TIS – transcription initiation site; TTS – transcription termination site. Nuclear DNA was completely digested with Eco RI 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.

tion pattern of this class of genes. There is evidence that in plants some specific sites in the IGS of rRNA genes are regularly hypomethylated and the pattern of methylation correlates with the rate of transcription (Flavell et al., 1988; Jupe and Zimmer, 1990; Torres-Ruis and Hemleben, 1994). Methylation of the C residues in the sequence -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. In Fig. 2 is represented the restriction map of the rDNA unit in *C. pepo*. Indicated in Fig 2 are the positions of all six Msp I/Hpa II sites in 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 18S rRNA gene are also shown. The complete digestion of the major genomic rDNA unit (~10 kb) with Eco RI results in two distinct DNA fragments of 6.2 and 3.7 kb, respectively (Ganal and Hemleben, 1986). The bigger one contains IGS and is very convenient to study the methylation of Msp I/Hpa II sites in IGS by the method of "indirect end labeling". Further on, the single Eco RI-digested and double Eco RI-Msp I/Hpa II-digested products in IGS were precisely located, following Southern transfer to Hybond$^+$ membrane and hybridization to ^{32}P-labelled 2.1 kb Eco RI fragment of pBG35 spanning the 18S rRNA gene in flax (Fig. 3B). Eco RI-digested DNA preparations from both control and BA-treated cotyledons hybridized with 18S rDNA probe and only the bigger Eco RI fragments from the major (~10 kb) and minor (~9.3 kb) rDNA repeat unit were detected in autoradiographs (Fig. 3B, lanes 1,4). Surprisingly, the 6.2 kb Eco RI fragment spanning the IGS sequences was not completely digested with Msp I. Digestion with Msp I affected

nearly 50% of the repeated units and resulted in generation of fragments with smaller size (between 0.5 and 2.5 kb). This suggests that in addition with -CpG- sequences, methylation in -CpNpG- might not be random throughout the IGS in marrow. Three Msp I fragments 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 2,5). Special interest is deserved for 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 and was surrounded by two other minor fragments with similar size (Fig. 3B, lanes 3,6). 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 IGS. The major Hpa II-generated

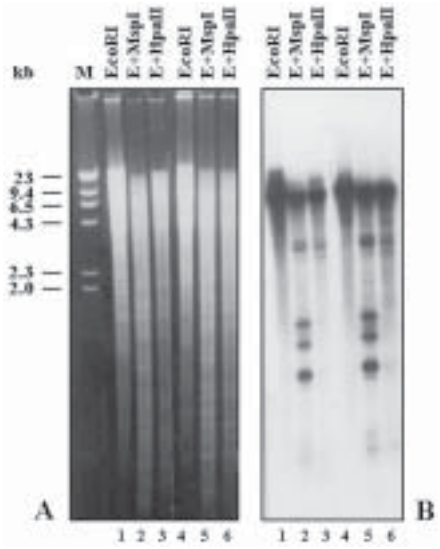

Fig. 3. Methylation pattern of rDNA in excised *C. pepo* cotyledons. A. Restriction enzyme analysis of genomic DNA with indicated enzymes and fluorescence of EtBr-stained DNA fragments in UV-light. B. Hybridization with gel purified ^{32}P-labelled 2.1 kB Eco RI fragment from pBG 35, spanning the 18S rRNA gene from flax (*Linum usitatissimum* L.). Lanes 1–3 contain genomic DNA from control and lanes 4–6 – from BA-treated cotyledons for 6 h in darkness.

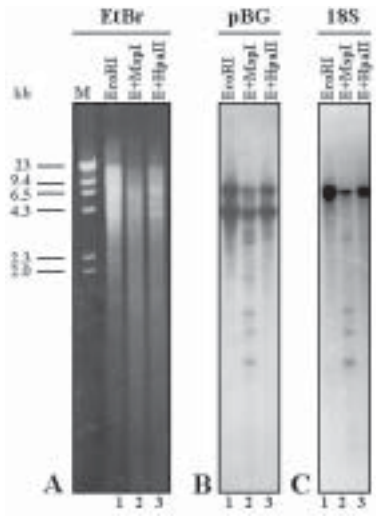

Fig. 4. Methylation pattern of rDNA in excised *C. pepo* cotyledons. A. Restriction enzyme analysis of genomic DNA with indicated enzymes and fluorescence of EtBr-stained DNA fragments in UV-light. B. Hybridization pattern with ^{32}P-labelled rDNA probe (pBG35) containing the whole rDNA unit from flax (*Linum usitatissimum* L.). C. Hybridization pattern with gel purified ^{32}P-labelled 2.1 kB Eco RI fragment from pBG 35, spanning the 18S rRNA gene from flax (*Linum usitatissimum* L.). Lanes 1-3 contain genomic DNA from MeJA-treated cotyledons for 24 h in darkness.

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 but this suggestion needs more precise experimental proofs.

It is worth mentioning that the methylation pattern of IGS was not altered neither in the case of two-four fold stimulated transcription of rRNA genes after *in vivo* treatment with BA (Fig. 3B, lanes 3,6) nor in the case of MeJA-treatment (Fig. 4B,C).

Conclusions

1. "Run-on" transcription experiments showed a 2-fold increase in RNA polymerase activity in nuclei isolated from BA-treated cotyledons, while treatment with MeJA decreased RNA polymerase I activity up to 35%.
2. The methylation pattern of rDNA in *C. pepo* was assayed by digestion with Msp I and Hpa II and by means of the "indirect end labeling" technique using a 18S rRNA gene as a ^{32}P-labeled DNA probe. Results showed heavy methylation of rRNA genes. As judged from the almost total lack of digestion with Hpa II, in rDNA repeated 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. Digestion with Msp I affected nearly 50% of the repeating units. The Msp I digestion products were few in number and large in size. This suggested that in addition with -CpG- sequences, methylation in -CpNpG- might not be random.
4. The methylation pattern in IGS of rRNA genes was not changed upon treatment of the cotyledons with cytokinin or jasmonate. This suggested that changes in rRNA gene activity were not due to or accompanied by significant DNA methylation changes.

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