Phytochemistry, 1975, Vol. 14, pp. 403–407. Pergamon Press. Printed in England.

CHROMATIN RNA POLYMERASE ACTIVITY FROM SOYBEAN HYPOCOTYLS TREATED WITH GIBBERELLIC ACID AND AMO-1618*

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(*Received* 23 *June* 1974)

Key Word Index—*Glycine max*; Leguminosae; soybean; chromatin; RNA polymerase; AMO-1618; gibberellic acid; growth retardant.

Abstract—Chromatin isolated from control, AMO-1618 [2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenyl piperidine carboxylate] and gibberellic acid (GA) treated soybean hypocotyl tissue incorporates labeled nucleoside triphosphates into acid-insoluble RNA. Gibberellic acid, sprayed on intact soybean hypocotyls, is shown to have enhanced the level of chromatin RNA polymerase activity while chromatin isolated from hypocotyls pretreated with AMO-1618 exhibits a lower polymerase activity relative to the control. Chromatin extracted from the treated or untreated seedlings are all sensitive to the inhibition (in varying degrees) by the presence of actinomycin D, pyrophosphate, or ribonuclease. Thus enhanced (or decreased) RNA-synthesizing capacity of chromatin in response to chemical treatments may be due to enhanced (or decreased) synthesis of RNA polymerase.

INTRODUCTION

Current literature on plant growth retardants AMO-1618, CCC (2-chloroethyltrimethylammonium chloride), phosfon-D (tributyl-2,4-dichlorobenzyl-phosphonium chloride), B-995 (*N*-dimethylaminosuccinamic acid) and AMAB (alkyltrimethyl ammonium bromide) deals mainly with their morphological and physiological effects especially in their use as a tool in the study of gibberellin physiology in various plant systems. However, studies on the effects of growth retardants on nucleic acid metabolism are rare.

Previous work of Soteros and Pillay [1] in this laboratory was concerned in correlating the actions of the plant growth hormone GA and the growth retardant AMO-1618 at the level of nucleic acid metabolism. Plant and animal hormones are reported to affect nucleic acid metabolism in a wide variety of systems [2,3]. Evidence indicates that plant hormones generally enhance RNA synthesis in hormone-responsive tissues. Nevertheless, it is still not clear whether the rate of RNA synthesis or the rate of degradation is affected in different tissues. In order to gain additional information on the significance of the hormonal effect on RNA metabolism, various studies have been undertaken to investigate the effect of hormones on RNA synthesis by isolated, cell-free, nuclei and chromatin.

Isolated chromatin of higher organisms possess several properties characteristic of the same chromatin *in vivo* [4]. Among the properties are the presence of histone bound to DNA, the state of repression of the genetic material, and the ability to serve as template for RNA synthesis and for the readout of the derepressed portion of the genome by RNA polymerase. This report is an attempt to determine the effect of AMO-1618 and GA on the RNA-synthesizing ability in the nucleus by studying the *in vitro* chromatin-directed RNA synthesis by isolated chromatin and its bound RNA polymerase of treated and untreated tissues. The chromatin-DNA template availability was also studied. Lastly, it was of interest to understand the relationship between changes induced by these chemicals at the genetic level with particular reference to the morphological and/or physiological changes in the treated plant system.

* This work was supported by a Grant A-1984 from the National Research Council of Canada.

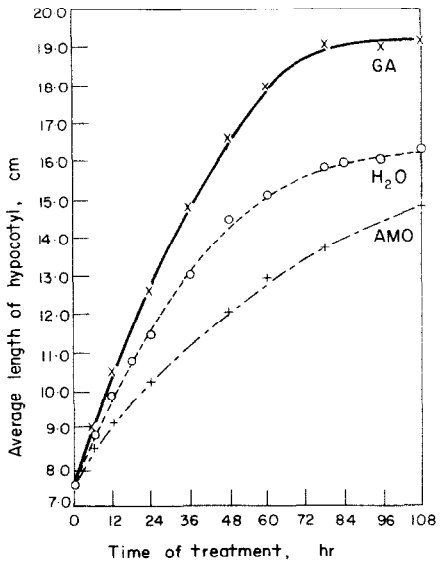

Fig. 1. Elongation of hypocotyl of intact soybean seedlings. Each point is average of 15 hypocotyls. Curves labelled with GA, AMO, and H_2O represent seedlings sprayed with gibberellic acid (10^{-3} M), AMO-1618 (10^{-3} M), and water respectively 72 hr after sowing the seeds.

RESULTS AND DISCUSSION

Effect of AMO-1618 and GA on growth of soybean hypocotyls

The growth retardant AMO-1618 at concentrations of 10^{-5} M or below appeared to enhance the elongation of soybean hypocotyls but caused retardation at 10^{-3} M. Gibberellic acid concentrations below 10^{-3} M appear to promote the elongation of hypocotyls in dark-grown soybean seedlings. Figure 1 indicates expansion of dark-grown soybean hypocotyls treated with GA (10^{-3} M), AMO-1618 (10^{-5} M) and water respectively. Each point represents average of 15 hypocotyls from treated and untreated plants. Zero time is taken to be the 72nd hr after sowing of seeds. At the concentrations used, GA caused elongation of hypocotyls over a period of 108 hr after treatment while AMO-1618 retarded their growth during the same period. Similar results were obtained when measurements were based on average fr. wt. of hypocotyl. It should be pointed out that the action of AMO-1618 is highly specific in inhibiting shoot elongation, but it does not affect the number of nodes and the weight of leaves [5]; therefore any reduction in weight of the treated plants appears to be a primary result of decrease in stem length. Previous work in this laboratory has shown that AMO-1618 retards the growth of soybean hypocotyls [1] and also the growth of pea seedlings [6]. It is clear that GA promoted growth of etiolated soybean seedlings while AMO-1618 (10^{-3} M) retarded it. The curves level off beyond the 96th hr; that is, when the dark-grown hypocotyls were fully elongated.

Effect of GA and AMO-1618 on chromatin-directed RNA synthesis over a 48-hr period

Chromatin RNA polymerase activity was assayed by measuring the incorporation of $\mu\mu$mol of ^{3}H-UMP per 100 μg DNA in GA and AMO-1618 treated seedlings over a period of 48 hr. Results (Table 1) indicate that RNA polymerase activity was enhanced in GA-treated hypocotyls but reduced in AMO-1618 treated samples. GA-induced increase in RNA synthesis occurred 18 hr after treatment but declined after 36 hr. Similarly,

Table 1. Induction of chromatin-RNA polymerase activity by treatments of GA and AMO-1618

	Incorporation of UMP				
	Chromatin from H_2O-treated seedlings	Chromatin from AMO-1618 treated seedlings		Chromatin from GA-treated seedlings	
Time (hr)	$\mu\mu$mol UMP*	$\mu\mu$mol UMP*	% of control	$\mu\mu$mol UMP*	% of control
0	15·36	—	100	—	100
6	15·70	14·60	93	20·40	129·9
18	18·56	11·05	59·5	36·92	198·9
24	17·32	11·08	64	32·90	190
36	16·70	14·10	84	24·70	148
48	13·76	13·62	98	17·88	130

* Per 100 μg DNA.

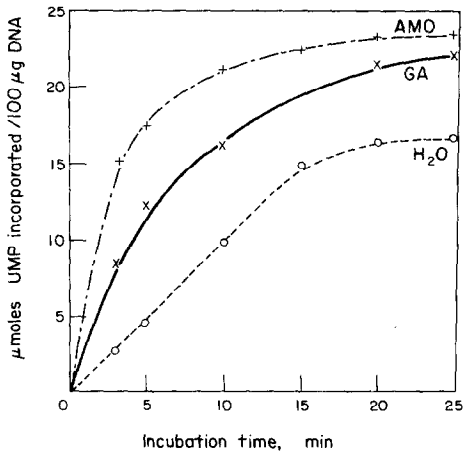

Fig. 2. Reaction kinetics of chromatin–RNA polymerase activity. The curves represent RNA synthesis as a function of time by chromatin-bound RNA polymerase isolated from AMO-treated (upper curve), from GA-treated (middle curve), and from untreated hypocotyls (lower curve).

RNA synthesis by AMO-1618 was reduced during the first 18 hr and thereafter increased up to 48 hr.

Reaction kinetics of chromatin–RNA polymerase

Recently McComb *et al.* [7] indicated that GA markedly enhances RNA-synthesizing ability of chromatin–RNA polymerase of treated pea seedlings over that of control. They also provided evidence that the maximum time required for enzyme to complete synthesis of RNA *in vitro* was *ca.* 15 min. Results of a time-course experiment in Fig. 2 indicate that the maximum incorporation of ^{3}H-UMP was achieved at about 20 min of incubation; the three curves (GA, AMO-1618, and water) show a similar pattern as they all level off after 15 min of incubation. Chromatin isolated from GA and AMO-1618 treated soybean seedlings 18 hr after treatment showed a marked increase in RNA synthesis over control. AMO-1618 induced enhancement of RNA polymerase activity in this experiment may be due to the insufficient amounts of the chemical available to check the growth of hypocotyls in a single spray of 10 ml (10^{-3} M) per tray of size 36 × 30 cm, whereas previous results [1, 6] have shown that AMO-1618 treatments caused considerable decrease in RNA synthesis in soybean and pea seedlings. This observation could be due to the fact that plants grown in their experiments were subjected to longer and continuous treatment of retarding chemical.

Incorporation of label from UTP into a trichloroacetic acid precipitable product was dependent on the presence of a divalent cation, and the presence of the other nucleoside triphosphates. Incorporation was inhibited by pyrophosphate, but not phosphate, by actinomycin D, and by RNase. It was clear from these observations that the product is RNA. In the presence of Mn^{2+} and Mg^{2+}, either alone or in combination, maximum incorporation was obtained, at pH 8·0. (Data not reported here.)

In vitro *effect of GA and AMO-1618 on chromatin-bound RNA polymerase*

It was reported that growth retardants, CCC and phosfon-D each stimulated up to 50% *in vitro* synthesis of polynucleotides by polynucleotide phosphorylase isolated from wheat roots [8]. However, the chromatin-bound RNA polymerase activity was reported to be neither enhanced nor inhibited *in vitro* by plant growth regulators, such as auxins [9–11] and gibberellins [7, 12]. Experiments were designed to determine whether or not AMO-1618® and GA have any promotive or inhibitory effect on the *in vitro* RNA polymerase activity. AMO-1618 (10^{-5} M) and GA (10^{-5} M) were tested separately, and the results given in Table 2 indicate that these plant regulators had no effect on the enzyme activity *in vitro*.

Effect of different concentrations of GA and AMO-1618 on chromatin–RNA polymerase activity

Table 3 shows the *in vivo* effect of various concentrations of AMO-1618 and GA, either applied singly or in combination, on the induction of chromatin-directed RNA synthesis. When applied singly to soybean seedlings, GA at various concentrations appeared to promote chromatin–RNA

Table 2. The *in vitro* effect of GA and AMO-1618 on chromatin and its associated RNA polymerase

Additives	Incorporation of UMP $\mu\mu$mol UMP per 100 μg DNA	% of control
Complete	26·89	100
+AMO 10^{-5} M	27·65	102·8
+GA 10^{-5} M	28·96	107·7

GA and AMO-1618 were added *in vitro* to control chromatin in the assay mixture.

Table 3. Effect of GA, AMO-1618 and AMO-1618 + GA treatments on chromatin-bound RNA polymerase activity

Treatment*	% Incorporation of UMP Chromatin–RNA polymerase activity GA	AMO-1618	AMO-1618 + GA‡
0	100†	100†	100†
10^{-7} M	393·9	151·6	174·6
10^{-5} M	195·4	89·6	86·6
10^{-3} M	198·9	59·8	156·2

* Seedlings harvested 18 hr after treatment with various concentrations as indicated in the table.

† 100% represents incorporation of 18·56 μμmol per 100 μg DNA.

‡ Equimolar concentrations of AMO-1618 and GA were used.

polymerase activity; AMO-1618 concentrations at 10^{-5} M and above tended to inhibit the enzyme activity but at lower concentration (10^{-7} M) enhanced its activity. When applied in combination (AMO-1618 and GA both of 10^{-5} M and 10^{-7} M) the results appear as though they were treated with AMO-1618 of 10^{-5} M and 10^{-7} M respectively. However, their combined concentration of 10^{-3} M somewhat mimicked the effect of GA concentration 10^{-3} M. It appears that at lower combined concentrations (10^{-5} M and 10^{-7} M) AMO-1618 rather than GA is the controlling factor whereas at 10^{-3} M the reverse is true for GA. This finding is consistent with the data of Soteros and Pillay [1]. They also demonstrated that a combined concentration of AMO-1618 (10^{-4} M) and GA (10^{-4} M) promoted an increased synthesis of nucleic acid in soybean hypocotyl. The high concentration of GA might possibly compete effectively and reduce greatly the inhibitory action of AMO-1618, thus increasing RNA polymerase activity.

Determination of chromatin–DNA template availability

To determine the DNA template availability, the chromatin–DNA from treated and untreated hypocotyls were separately saturated at the same levels of exogenous *E. coli* RNA polymerase. The assay method was the same as that for endogenous chromatin–RNA polymerase, except that the amount of chromatin–DNA used per reaction tube was reduced to *ca* 0·4–0·6 μg. The results showed that there was neither an increase nor a decrease in template availability due to treatments of GA or AMO-1618.

Relation between RNA polymerase activity and growth rate

Figure 3 shows the relation between RNA polymerase activity induced by AMO-1618 and GA and changes in growth rate. This graph was derived from data of Fig. 1 and Table 1. The percentage RNA polymerase activity for GA and AMO-1618 was calculated for the respective increase (or decrease) over control at various time intervals (Table 1); percentage growth rate was obtained by calculating growth rate (cm/hr) for different points along the curves in Fig. 1 and expressed in terms of control. Thus the chemical-induced RNA polymerase activity and the growth rate can be related. Maximum increase or decrease in RNA synthesis induced by GA or AMO-1618 respectively precedes the maximum increase or decrease in growth rate.

The maximum increase in chromatin–RNA polymerase activity induced by GA treatment occurred 18 hr after treatment, and gradually decreased with increasing time. This finding is in agreement with published data [2, 13]. Chrispeels

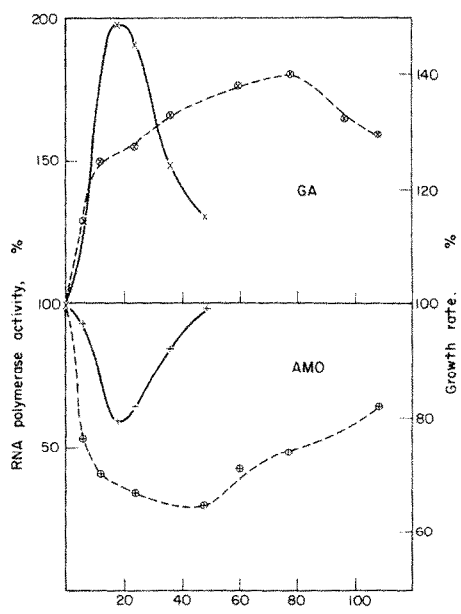

Fig. 3. The comparison between the % increase in RNA polymerase activity induced by GA (×—×) and AMO-1618 (+—+) respectively, and changes in growth rate for GA (⊗—⊗) and for AMO-1618 (⊕—⊕).

and Varner [14] related the decrease in RNA synthesis after the initial 24 hr treatment to GA-regulated increase in RNAase activity in intact plants. It seems also logical to assume that one spray of GA solution applied to hypocotyls could only induce a certain amount of RNA synthesis, which was not sufficient enough to maintain RNA synthesis for continued growth. It is also clear that the maximum decrease in RNA polymerase activity brought about by AMO-1618 was also at the 18th hr. The enzyme activity gradually approached the control value after 24 hr of treatment. It may be concluded that one spray of AMO-1618 was not enough to check RNA synthesis, thus allowing the growth of the hypocotyls.

EXPERIMENTAL

Plant material. Soybean seeds (*Glycine max.* L. var Wayne) sown in vermiculite at 26° in the dark for 3 days, were sprayed with AMO-1618 (10^{-3} M) and/or GA (10^{-3} M). Hypocotyl sections (taken 1 cm below the cotyledonary hook to the first lateral root) were excised at various times after treatment and kept in a beaker on ice.

Chromatin extraction and purification. 150 g Lots of chilled hypocotyls from AMO-1618 and GA treated and untreated plants were used for chromatin extraction, using the method of Huang and Bonner [15], with minor modifications. All steps of extraction were performed in the cold 0–4°. The tissue was homogenized in an equal vol. (w:v) of (sucrose, 0·25 M; Tris–HCl, 0·05 M, pH 8·0; $MgCl_2$, 0·01 M; 2-mercaptoethanol, 0·01 M), and the homogenate filtered through 4 layers of cheese cloth and finally through a layer of miracloth. The filtrate was centrifuged in a Sorvall RC2-B centrifuge for 30 min at 5000 *g*. The supernatant containing mitochondria and other smaller particles was discarded. The crude chromatin which formed a brownish, gelatinous layer over the underlying, firm starch layer was suspended in a dounce homogenizer containing 20 ml wash buffer (Tris–HCl, 0·01 M, pH 8·0; sucrose, 0·25 M; 2-mercaptoethanol, 0·01 M). The homogenate was centrifuged for 10 min at 10000 *g*. The chromatin layer was again scraped from the pellet and suspended in wash buffer. The chromatin obtained thus far contains *ca* 95% DNA but is contaminated by nonchromosomal protein. To remove the latter, the chromatin suspension was layered over 10 ml of 2 M sucrose (containing Tris–HCl, 0·01 M, pH 8·0; 2-mercaptoethanol, 0·01 M) in a 12·5 ml centrifuge tube and the top portion gently stirred and centrifuged for 3 hr at 20000 rpm. The supernatant was discarded and the purified chromatin pellet finally suspended in a dounce tissue homogenizer (containing Tris–HCl, 0·01 M, pH 8·0; 2-mercaptoethanol, 0·01 M). Aliquots of this chromatin suspension were used in assays for RNA polymerase activity.

Chromatin-bound RNA polymerase assay. The incorporation of labeled ribonucleoside-5′-triphosphate into trichloroacetic acid-insoluble material was used. Aliquots of chromatin extract each of 0·1 ml (equivalent to 15 μg DNA) were added to 0·3 ml of cold assay reaction mixture containing: Tris–HCl, pH 8·0, 10 μmol; $MgCl_2$, 1·0 μmol; 2-mercaptoethanol, 0·125 μmol; ATP, 0·2 μmol; CTP, 0·2 μmol; GTP, 0·2 μmol; unlabelled UTP, 0·005 μmol; labelled ^{3}H-UTP, 10 μCi (sp. act. 13 μCi/μmol). A zero-time reaction was done by adding 4 ml of cold 10% TCA containing 0·04 ml tetrasodium pyrophosphate to the reaction mixture immediately after 0·1 ml chromatin had been added. The reaction was carried out at 37° for 20 min and stopped by the addition of 4 ml of ice-cold TCA containing 0·04 M pyrophosphate. The resultant ppt. was transferred to a Whatman GF/A glass fiber filter disc and washed 4× each with 5 ml cold 5% TCA containing 0·02 M pyrophosphate. The discs were dried under IR lamps and put in 10 ml scintillation liquid, and counted in a Nuclear–Chicago liquid scintillation system Mark II.

Determination of DNA template availability. The chromatin–DNA template availability was determined by using excess RNA polymerase and small amounts of chromatin as template to synthesize RNA. Assay methods and reaction mixtures used are the same as those employed in RNA polymerase activity assay, the only exception being the amount of chromatin reduced to 0·3–0·6 μg DNA. *Escherichia coli* RNA polymerase of various amounts of 1–10 units (units of enzyme are as described by Sigma Co., St. Louis, Mo.) was added in excess. The reaction was carried out at 37° for 20 min and stopped by adding 4 ml of cold 10% TCA. The RNA ppt. was processed in the same way as in RNA polymerase activity assay.

DNA determination. Chromatin–DNA was determined by the method of Giles and Myers [16] which is a modified method of Burton [17] after hydrolysis in 10% perchloric acid at 70° for 45 min. This was followed by adding 4% diphenylamine reagent and incubating the DNA sol. at 30° for 18 hr. The absorbance was measured using Calf thymus DNA as standard.

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