Phytochemistry, Vol. 26, No. 1, pp. 31–35, 1987.
Printed in Great Britain.

0031-9422/87 $3.00 + 0.00
Pergamon Journals Ltd.

STIMULATION OF RNA SYNTHESIS IN COWPEA (*VIGNA SINENSIS*) SEEDLINGS BY GIBBERELLIC ACID AND ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE

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(*Revised received* 27 *May* 1986)

Key Word Index *Vigna sinensis*; Leguminosae; cowpea; RNA; GA_3; cyclic AMP; polydisperse RNA; protein synthesis.

Abstract—There is about 50% stimulation in the incorporation of [^{3}H]uridine into total RNA of cowpea following the application of gibberellic acid (GA_3) and adenosine 3′,5′-cyclic monophosphate (cyclic AMP). Cyclic AMP is very specific in its action. Co-fractionation of ^{3}H- and ^{14}C-labelled RNA on acrylamide-agarose gels reveal a control by GA_3 and cyclic AMP predominantly on its polydisperse fraction. Both GA_3 and cyclic AMP appear to act through a similar mechanism.

INTRODUCTION

Cyclic AMP has been recognized as an important regulatory agent in animals, micro-organisms, fungi and yeast [1–4]. These findings provoked the conjucture that it may have a similar function in higher plants, and there have indeed been many attempts to test this hypothesis [5, 6]. Recent mass spectrometric identification of this compound in plants [7 9] and the demonstration of the presence of cyclic AMP-binding proteins [10] in plant tissues further substantiate the view that a biochemical potential exists in plants for a regulatory role for cyclic AMP. It may have a secondary messenger role analogous to that in animals or a primary messenger role similar to that in certain bacteria.

Cyclic AMP has been reported to mediate the action of certain plant hormones in the control of RNA metabolism in oat coleoptile chromatin [11] and in seedlings of *Cicer arietinum* [12]. An immediate enhancement of RNA synthesis in cytoplasmic pre-ribosomal and heterogenous nuclear RNA by the application of cyclic AMP to gibberellic acid sensitive maize protoplasts has also been reported [13]. Earlier studies with cowpea seedlings have indicated that application of GA_3 or cyclic AMP resulted in an increase in ribonuclease activity and its isoenzymes, and this increase was accompanied by an increase in the incorporation of [^{3}H]uracil into RNA [14]. In the present study an attempt has been made to elucidate the possible role of GA_3 or cyclic AMP in RNA synthesis by co-purifying ^{3}H- and ^{14}C-labelled species of RNA and fractionating them on acrylamide gels.

RESULTS AND DISCUSSION

Application of GA_3 (10^{-5} M) stimulated the incorporation of [^{3}H]uridine into RNA to the extent of *ca* 50–60%. An almost similar stimulation was observed when cyclic AMP (10^{-5} M) was substituted for the hormone (Table 1). These results are in agreement with the data obtained in maize seedlings [13, 15]. Seedlings grown in the presence of optimum concentrations of GA_3 (10^{-5} M) + cyclic AMP (10^{-5} M) did not show any additive effect on [^{3}H]uridine incorporation into RNA. The values obtained were almost the same as those observed with GA_3 (10^{-5} M) and cyclic AMP (10^{-5} M) alone. However, when the hormone and the cyclic nucleotide were applied together in suboptimal concentrations, i.e. GA_3 (10^{-7} M) + cyclic AMP (10^{-6} M), the amount of [^{3}H]uridine incorporated into RNA was the same as that with GA_3 (10^{-5} M) or cyclic AMP (10^{-5} M) alone at their optimum concentrations. This suggested that both the hormone and cyclic nucleotide might be acting through a similar mechanism, probably at the same sites. Salomon and

Table 1. Effect of GA_3 and cyclic AMP on [^{3}H]uridine incorporation into RNA

Additions	RNA (% control)
None	100*
10^{-7} M GA_3	138
10^{-5} M GA_3	165
10^{-6} M cyclic AMP	130
10^{-5} M cyclic AMP	148
10^{-5} M GA_3 + 10^{-5} M cyclic AMP	153
10^{-7} M GA_3 + 10^{-6} M cyclic AMP	160

Seeds were germinated in the dark at 35 ± 2°. [^{3}H]uridine (2 μCi/ml; 2700 mCi/mmol) was added to the quartz sand germination medium after 96 hr of germination, while GA_3 and cyclic AMP were present throughout the germination period. Seedlings were harvested after 8 hr of incorporation. The results are the average of two experiments done in duplicate.

* 100% = 3.3×10^3 cpm/mg RNA.

Mascarenhas [11] reported that IAA (10^{-6} M) and cyclic AMP (10^{-7} M) both stimulated RNA synthesis in oat coleoptiles. They interpret the results by suggesting that both the hormone and cyclic nucleotide act through a similar mechanism. Stimulation of RNA synthesis by IAA and cyclic AMP was also reported in seedlings of *C. arietinum* and this enhancement was not found to be additive in nature. It was suggested that the stimulatory effect of IAA on RNA synthesis is mediated via cyclic AMP [12].

As is evident from Table 2, the response to cyclic AMP was found to be highly specific, since various related compounds failed to enhance RNA synthesis. To ascertain that the observed stimulation in RNA synthesis by GA_3 or cyclic AMP was not a result of degradation products entering the cells, which do eventually provide an additional source of nitrogen or phosphorus, the effects of inorganic nitrogen (KNO_3 10 mM), inorganic phosphorus (KH_2PO_4, 5 mM), and a few amino acids (5 mM) were also tested on [^{3}H]uridine incorporation into RNA. None of these compounds could mimic the action of cyclic AMP, indicating that cyclic nucleotide acted specifically in invoking such a response, and the question of non-permeability of plant cells to this nucleotide does not arise. Some workers have already shown that cyclic AMP does enter the plant cells [16, 17].

The stimulation of RNA synthesis elicited by GA_3 or cyclic AMP was extremely sensitive to the action of inhibitors of RNA synthesis (actinomycin D and cordycepin). 5-Fluorouracil (5-FU), however, was less effective (Table 3). This indicated a control of GA_3 and cyclic AMP on the synthesis of all species of RNA, preferentially the minor species. These results were further substantiated when RNA was isolated, purified and fractionated.

In preliminary experiments (data not given), when RNA prepared from seedlings incubated in the presence of [^{3}H]uridine was fractionated on gels, very little GA_3 or cyclic AMP-promoted differences were observed. In order for the differences in minor species present in the region of low counts to be detected, the sensitive labelling method used by Chandra and Duynstee [18] and later adopted by Zwar and Jacobson [19] was used. Figure 1 shows the absorbance ($A_{265\,nm}$) of different fractions of RNA and the ^{3}H:^{14}C ratio obtained when seedlings were grown in the presence or absence of either GA_3 (10^{-5} M) or cyclic AMP (10^{-5} M). The nucleic acid peaks from left to right correspond to DNA, 25S RNA, 18S RNA and 4S RNA. As is evident from the figure, the overall ^{3}H:^{14}C ratio was

Table 2. Effect of cyclic AMP and related compounds on the incorporation of [^{3}H]uridine into RNA

Additions	RNA (% control)
None	100*
10^{-5} M cyclic AMP	152
10^{-5} M adenine	101
10^{-5} M 5'-AMP	111
10^{-5} M ADP	108
10^{-5} M ATP	124

Seeds were germinated in the dark at 35 ± 2°. Adenine, 5'-AMP, ADP, ATP and cyclic AMP were present throughout the period of germination. Seedlings raised for 96 hr were used for incorporation of [^{3}H]uridine (2 μCi/ml; 2700 mCi/mmol) for another 8 hr. The results are the average of three experiments done in duplicate.

*100% = 3.16×10^3 cpm/mg RNA.

Table 3. Effect of actinomycin D, cordycepin and 5-fluorouracil (5-FU) on the [^{3}H]uridine incorporation into RNA

Additions	RNA (% control)
None	100*
10^{-5} M GA_3	162
10^{-5} M cyclic AMP	150
10 μg/ml actinomycin D	35
5×10^{-4} M cordycepin	25
1.0 mM 5-FU	62
10^{-5} M GA_3 + 10 μg/ml actionomycin D	40
10^{-5} M cyclic AMP + 10 μg/ml actionomycin D	45
10^{-5} M GA_3 + 5×10^{-4} M cordycepin	30
10^{-5} M cyclic AMP + 5×10^{-4} M cordycepin	28
10^{-5} M GA_3 + 1.0 mM 5-FU	77
10^{-5} M cyclic AMP + 1.0 mM 5-FU	71

For experimental conditions, see legend of Table 1.
*100% = 3.4×10^3 cpm/mg RNA.

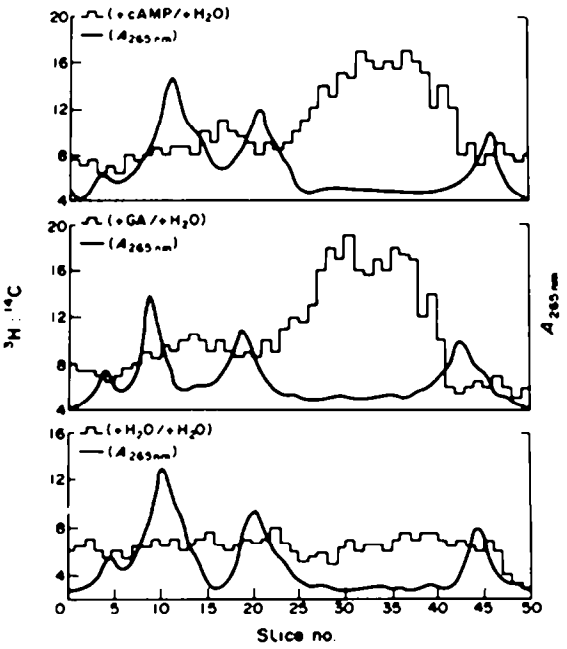

Fig. 1. Effect of GA_3 (10^{-5} M) and cyclic AMP (10^{-5} M) on the incorporation of [^{3}H]uridine and [^{3}H]adenosine into RNA fractions separated by PAGE. The RNA was prepared from cowpea seedlings incubated for 8 hr: in water (+H_2O/+H_2O), neither ^{3}H- nor ^{14}C-labelled seedlings contained added GA_3 or cyclic AMP; in +GA_3/+H_2O and in +cyclic AMP/+H_2O, only the ^{3}H-labelled seedlings received GA_3 or cyclic AMP.

much higher in GA_3-treated seedlings than that obtained in the water-grown seedlings. This effect was more pronounced (2- to 3-fold) in the 5S–14S region of the gel scan. An almost similar pattern of incorporation was observed when seedlings were raised in the presence of cyclic AMP (10^{-5} M). If GA_3 or cyclic AMP is absent from ^3H and ^{14}C treatments, the ^3H:^{14}C ratio would be expected to be constant over the length of gel and if the synthesis of any particular species of RNA were promoted by GA_3 or cyclic AMP, this species would contain more ^3H label than others. Consequently the ^3H:^{14}C ratio would be raised in the region of the gel in which it is localized. Thus a rise in observed ratio in any particular region of gel indicates stimulation of incorporation into the species of RNA which run in that region. To ensure that labelling of RNA was due to plant RNA rather than to bacterial RNA, all the experiments were done under bacteria-free sterile conditions in the presence of chloramphenicol (20 μg/ml). The results indicated that both GA_3 and cyclic AMP stimulated ^3H-label incorporation into the RNA species but the effect was not the same for all fractions of RNA. A high ^3H:^{14}C ratio observed in the 5S–14S region indicated the presence of GA_3-stimulated RNA or cyclic AMP-stimulated RNA in this region. The overall counts obtained in the region containing ribosomal RNAs were very large compared to those in the 5S–14S region. The small increase in ratio suggests the presence of GA_3-stimulated RNA or cyclic AMP-stimulated RNA in this region too. Thus GA_3 or cyclic AMP enhanced considerably polydisperse RNA synthesis besides a comparatively lower increase of pre-ribosomal RNA. Comparison of RNA synthesis patterns induced by cyclic AMP or GA_3 resulted in very impressive similarities.

Earlier studies [20] on the same plant material had shown that both GA_3 and cyclic AMP treatment resulted in the synthesis *de novo* of ribonuclease. The increase in ribonuclease activity was quite sensitive to inhibitors of RNA synthesis. This is probably a good indication that the synthesis of most of the hydrolases may be regulated by GA_3 or cyclic AMP in this plant material. To equate the production of these enzymes with GA_3-stimulated RNA or cyclic AMP-stimulated RNA, inhibitors of RNA synthesis, i.e. actinomycin D, cordycepin and 5-fluorouracil, were tried to see the labelling pattern of RNA on gels in their presence. When actinomycin D was supplied with GA_3 or cyclic AMP, it reduced the incorporation of radioactive label to slightly less than half the original value in the ribosomal region while there was almost a 70–80% inhibition in the incorporation in the polydisperse region. This showed that both GA_3- and cyclic AMP-induced synthesis of all the species of RNA, especially polydisperse fraction, were equally sensitive to actionomycin D inhibition (Fig. 2). Cordycepin, a well-known inhibitor of *m*RNA synthesis, when tried together with GA_3 or cyclic AMP resulted in inhibition of incorporation of ^3H-label in the 5S 14S region specifically (Fig. 3). When 5-fluorouracil was included together with the different treatments the incorporation in the ribosomal regions was sensitive to a larger extent with no or very little effect on the incorporation into the polydisperse fraction (Fig. 4). These results corresponded well with the inhibition of ribonucleases with respective inhibitors [20].

The GA_3- or cyclic AMP-induced RNA occurred over most of the gel scan but the increase in ratio was more predominant in the polydisperse region, indicative of a

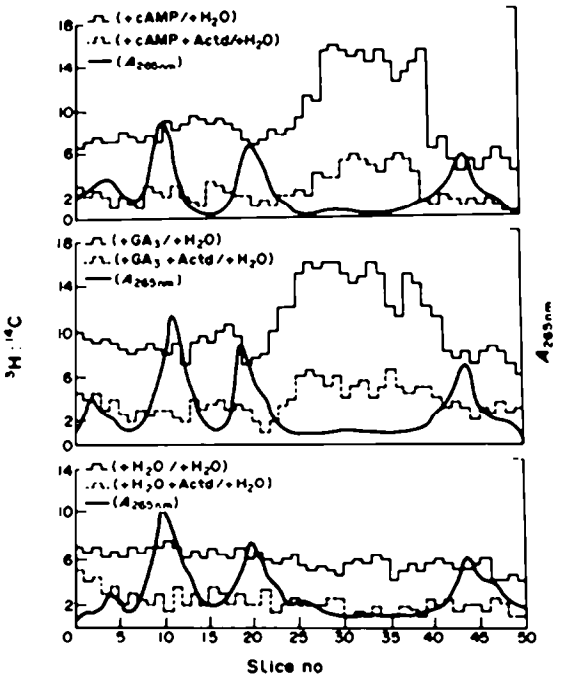

Fig. 2. Inhibitory effects of actinomycin D (Act. D) on the GA_3- and cyclic AMP-dependent incorporation of [^{3}H]uridine and [^{3}H]adenosine into RNA fractions separated by PAGE. Conditions were the same as for Fig. 1, except that one of the treatments contained actinomycin D (10 μg/ml) in addition to the normal treatments.

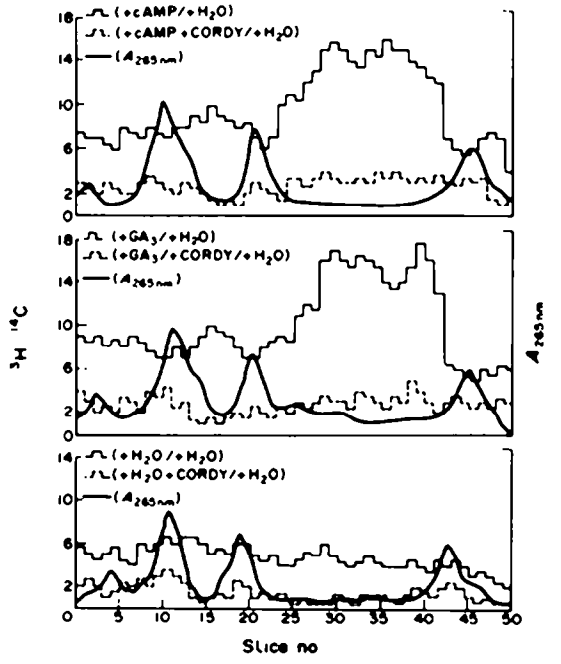

Fig. 3. Inhibitory effect of cordycepin (CORDY) on the GA_3- and cyclic AMP-dependent incorporation of [^{3}H]uridine and [^{3}H]adenosine into RNA fractions separated by PAGE. The conditions were the same as for Fig. 1, except that one of the treatments contained cordycepin (5×10^{-4} M) in addition to normal treatments.

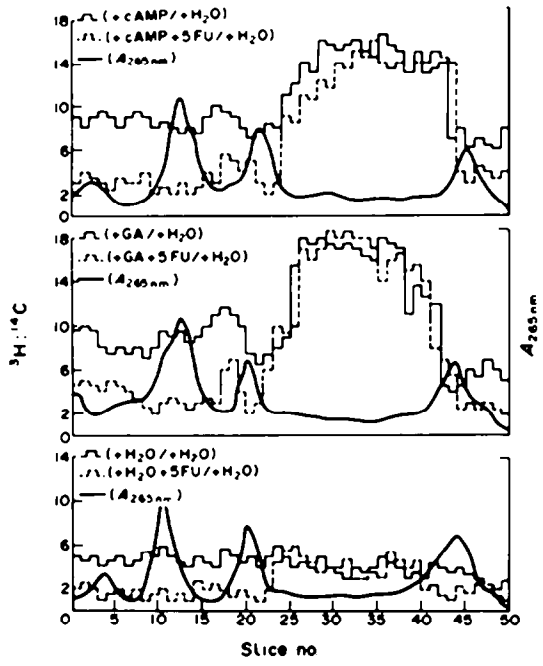

Fig. 4. Inhibitory effect of 5-fluorouracil (5-FU) on the GA_3- and cyclic AMP-dependent incorporation of [^{3}H]uridine and [^{3}H]adenosine into RNA fractions separated by PAGE. Conditions were the same as for Fig. 1, except that in addition to normal treatments, one treatment contained 5-fluorouracil (1.0 mM).

control by GA_3 and cyclic AMP on transcription. The results obtained, when discussed in the light of earlier data on ribonuclease [14, 20], suggest that in cowpea seedlings both GA_3 and cyclic AMP appear to control the various processes by synthesizing new *m*RNAs specific for different hydrolysing enzymes. These *m*RNAs, having different M_rs, together constitute the polydisperse fraction of RNA.

Comparison of RNA patterns induced by GA_3 or cyclic AMP show that both the hormone and cyclic nucleotide are acting through a common mechanism. It is possible that phytohormones effect processes which regulate the cyclic AMP levels within the cell and that the cyclic nucleotide in turn regulates gene expression.

EXPERIMENTAL

The seeds of cowpea (*Vigna sinensis* L.) were surface sterilized with a 0.1% soln of $HgCl_2$ for 5 min, rinsed thoroughly with sterile distilled water (SDW) and then presoaked in SDW for 4 hr in the cold. They were allowed to germinate at 35 ± 2° in Petri plates containing acid-washed quartz sand. Chloroamphenicol (20 μg/ml) was added to prevent bacterial contamination. This complete step was performed on a Laminer flow bench to help maintain sterile conditions.

The effects of GA_3, cyclic AMP, AMP, ADP, ATP, adenine, inorganic nitrogen (KNO_3), inorganic phosphorus (KH_2PO_4), amino acids (leucine, phenylalanine, tryptophan, tyrosine, methionine and proline) were also studied during germination, as were those of the inhibitors of RNA synthesis (actinomycin D, cordycepin and 5-fluorouracil).

For the incorporation of [^{3}H]uridine into RNA, the seedlings were grown in the dark in the continuous presence of respectively GA_3, cyclic AMP, AMP, ADP, ATP, adenine, KH_2PO_4, KNO_3, amino acids, actinomycin D, cordycepin and 5-fluorouracil containing chloroamphenicol (20 μg/ml). The seedlings were harvested after 96 hr and incubated in [^{3}H]uridine (2 μCi/ml; 2700 mCi/mmol) for a period of 8 hr in the presence of the respective treatments. The controls were processed in the same way except SDW was used instead of the treatment. RNA was extracted in 10 vols. of a soln containing 0.01 M Tris·HCl (pH 9.0), 0.05 M NaCl, 1% sodium tri-isopropyl naphthalene sulphonate and 6% butan-2-ol. NaCl was further added to give a final concn of 0.5 M. An equal vol. of PhOH·H_2O–8-hydroxyquinoline-*m*-cresol (50:10:0.05:7, V/V/W/V) was added and the mixture was shaken vigorously at 25°. The mixture was then centrifuged at 15000 *g* for 2 min and the aq. phase and interphase were re-extracted with $CHCl_3$ until the interphase disappeared (approx. 2–3 times). RNA was precipitated from the aq. phase with 2 vols. of 95% EtOH in the cold.

To maximize the chances of detecting GA_3- or cyclic AMP-induced changes in RNA, the fractionation of RNA on acrylamide gels was done by employing the labelling method of Zwar and Jacobson [19]. To each of the two Petri plates containing 15 seedlings (96-hr-old) [^{3}H]adenosine (2 μCi/ml; 2500 mCi/mmol) and [^{3}H]uridine (2 μCi/ml; 2700 mCi/mmol) were added. To a third Petri plate containing 15 seedlings [^{14}C]adenosine (0.5 μCi/ml; 500 mCi/mmol) and [^{14}C]uridine (0.5 μCi/ml; 450 mCi/mmol) were added. The seedlings were totally immersed in the incubation medium with constant shaking. The temp. of incubation was 25°. All the treatments contained chloramphenicol (20 μg/ml) to prevent bacterial contamination. The seedlings were harvested after 8 hr of incubation and washed with 1 mM uridine and adenosine. The 30 seedlings incubated with ^{3}H-label were mixed with 15 seedlings labelled with ^{14}C and their RNA extracted following the procedure described above. This treatment was designated as $+H_2O/+H_2O$. Different sets of labelled seedlings were prepared by following the same procedure except that ^{3}H-labelled medium contained the respective treatments. These were designated as $+Tr/+H_2O$.

RNA (30–40 μg) was subjected to electrophoresis on 2.4% acrylamide gels according to the method of Loening [21] but modified by the addition of 0.5% agarose [22]. The gels were scanned at 265 nm and frozen in liquid N_2 and sliced into 1 mm discs. Each slice was then transferred to a scintillation vial and solubilized in 0.5 ml 30% H_2O_2 at 60° for 12 hr [23]. The radioactivity contents of the vials were measured in a liquid scintillation counter using Bray's scintillation mixture [24]. The dpm values for both ^{3}H and ^{14}C were calculated by means of the set of equations described in ref. [25]. A rise or fall in the observed ratio in any particular region of gel, as a result of a particular treatment, will indicate stimulation or inhibition of incorporation into the species of RNA present in that region.

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