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Gibberellic Acid Causes Increased Synthesis of RNA Which Contains Poly(A) in Barley Aleurone Tissue

[poly(U)-cellulose columns/polyacrylamide gel electrophoresis/base analyses]

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ABSTRACT **Incubation of isolated barley aleurone layers with gibberellic acid for 16 hr caused a 50% increase in the synthesis of RNA that contains poly(A) sequences [poly(A)-RNA], but had no measurable effect on the syntheses of the major RNA species. The syntheses of both the poly(A) and the heteropolymeric fractions of the poly(A)-RNA were increased.**

The poly(A) sequences were separated into two classes by size, one containing an average of 250 nucleotides and the other about 70 nucleotides. The two classes occurred in a molar ratio of about 1:1. Gibberellic acid increased the syntheses of both sequences to the same extent.

We interpret these results to mean that gibberellic acid increases specifically the synthesis of mRNA in this tissue.

Gibberellic acid (GA_3) causes the *de novo* synthesis of several hydrolytic enzymes in aleurone tissue of barley grain (1, 2). Since RNA synthesis is required for this hormone effect (3), the synthesis of the enzymes is dependent on synthesis of their mRNAs (4).

In animal and viral cells, most mRNA molecules contain poly(A) sequences (5–9), and recently there have been reports of similar RNA in plant cells (10–13). We have detected RNA that contains poly(A) [poly(A)-RNA] in barley aleurone, and on the assumption that it was at least in part mRNA, we have looked for changes in this RNA caused by GA_3.

MATERIALS AND METHODS

Preparation of RNA. Aleurone layers were isolated from barley (*Hordeum vulgare* L. cv Himalaya) grains and incubated as described by Chrispeels and Varner (14) in the presence of radioactive precursors of RNA with or without 1 μM GA_3. Incubation time was always 16 hr.

Where base compositions of RNA were to be determined, the layers were incubated with [^{32}P]orthophosphate (specific activity 20,000 Ci/g) in the presence or absence of GA_3. Each flask, containing 30 aleurone layers and 2 ml of incubation medium, received 270 μCi of ^{32}P. No unlabeled orthophosphate was added.

Radioactive RNA was prepared as follows. To several 30-ml Erlenmeyer flasks, each containing 20 aleurone layers and 1 μM GA_3, was added 40 μCi of [^{3}H]adenosine (specific activity 2 Ci/mmole) and to an equal number of flasks containing also 20 aleurone layers, but no GA_3, was added 10 μCi of [^{14}C]-adenosine (specific activity 450 mCi/mmole). After incubation, the layers were washed with 1 mM adenosine and mixed. RNA was isolated and purified from the layers by the method of Click and Hackett (15) except that the tissue was frozen in liquid nitrogen before homogenization in a Virtis homogenizer, and the phenol of the homogenization medium was replaced by an equal volume of phenol:chloroform (1:1) (16). Additional purification was achieved by precipitation of the RNA with cetyltrimethylammonium bromide (17).

Poly(A) Preparation. Poly(A)-RNA was isolated from the total labeled RNA by means of cellulose polyuridylic acid [poly(U)] columns which bind RNA molecules with poly(A) sequences and allow others to pass. These were prepared as described by Sheldon *et al.* (18). Binding of poly(A)-RNA to and elution from the columns were carried out as described by Kates (5) except that 0.3% sodium dodecyl sulfate was added to the binding and elution buffers and the binding temperature was 15°. The poly(A)-RNA was precipitated from the appropriate column fractions by addition of 200 μg of barley RNA added as carrier and 2.5 volumes of ethanol, and recovered by centrifugation. It was dissolved in 1 ml of 0.01 M Tris·HCl buffer at pH 7.5 containing 0.3 M KCl, 30 μg of bovine pancreas ribonuclease and 150 units (approximately 30 μg) of T_1 ribonuclease and then incubated at 37° for 1 hr. The solution was then made 1% with respect to sodium dodecyl sulfate (19) and extracted three times with equal volumes of phenol–chloroform (20). The total ribonuclease-resistant material, poly(A), was precipitated by the addition of 100 μg of carrier barley RNA and 2.5 volumes of ethanol. The precipitate was recovered by centrifugation, redissolved in 1 ml of 0.01 M Tris·HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.3% sodium dodecyl sulfate, and once more passed through a poly(U)-cellulose column. Both the run-off and bound RNA were collected. Carrier RNA and 2.5 volumes of ethanol were added to the appropriate eluate fractions and the precipitated material was once more recovered by centrifugation.

Polyacrylamide Gel Electrophoresis. Acrylamide gels, containing 0.5% agarose (21), were prepared and run as described by Loening (22). Total RNA was fractionated on 2.4% acrylamide gels and poly(A) on gels containing 5% acrylamide. After electrophoresis the gels were cut into 1 mm segments and ^{3}H and ^{14}C in each segment was counted. The ratios of ^{3}H dpm to ^{14}C dpm were then calculated.

Purification of Major RNA Species for Base Analyses. [^{32}P]RNA which did not bind to poly(U)-cellulose [presumably poly(A)-RNA free] was fractionated on 2.4% and 7.5% acrylamide gels. Sections of the 2.4% gels containing heavy

Abbreviations: poly(A)-RNA, RNA that contains sequences of poly(A); GA_3, gibberellic acid.

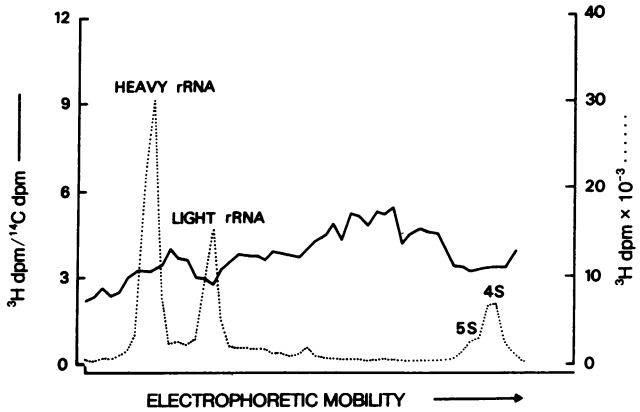

FIG. 1. Acrylamide gel electrophoresis of total RNA. Total labeled RNA was fractionated by electrophoresis on 2.4% acrylamide gels. The gels were then sectioned and the radioactivity in each section counted as described in *Materials and Methods*.

and light rRNA (detected by scanning the gels at 260 nm) were cut out. The tRNA and 5S RNA were resolved on the 7.5% gels and sections containing these species were also cut out. The pieces of gel were sealed in glass tubes with piperidine and hydrolyzed as described below. The hydrolyzate was eluted from the gel pieces in water.

Base Analyses. ^{32}P-labeled RNA was hydrolyzed in 10% piperidine at 100° for 90 min (23) and the nucleotides were separated by paper electrophoresis using the method of Sebring and Salzman (24) except that the separations took place on Whatman 3 MM paper and electrophoresis was continued for 90 min at 59 V/cm. The labeled nucleotides were located by passing the chromatogram strips through a radiochromatogram scanner, and radioactivity was quantitated by placing pieces of chromatogram directly into scintillation fluid and counting in a scintillation counter. The size of the piece of paper had no effect on the counting efficiency.

RESULTS

The effect of GA_3 on RNA synthesis was measured by labeling aleurone layers with [^{3}H]adenosine in the presence of GA_3 and [^{14}C]adenosine in the absence of GA_3, mixing the tissues, and extracting RNA. A typical fractionation of such labeled RNA on an acrylamide gel and the ^{3}H dpm to ^{14}C dpm ratios associated with each gel segment are shown in Fig. 1. The ratios for total RNA, heavy rRNA, light rRNA and 4S and 5S RNAs were very similar (between 3.0 and 3.3), indicating that GA_3 had no differential effect on the synthesis of the major RNA species. In fact, GA_3 seems to have no effect at all on synthesis of the major RNA species (25, 26). However, since the ratios of ^{3}H to ^{14}C not associated with major RNA species were elevated (see also ref. 25), GA_3 caused increased synthesis of some minor RNA component.

The total RNA was passed through a poly(U)-cellulose column to extract the poly(A)-RNA. The run-off RNA (major species) had a radioactivity ratio of 3.1, as expected, but since the poly(A)-RNA had a ratio of 4.6, a 50% increase of poly-(A)-RNA synthesis was caused by GA_3.

The poly(A) regions were obtained by T_1 and pancreatic ribonuclease digestion of the labeled poly(A)-RNA, and were separated into two fractions on 5% acrylamide gels (Fig. 2). One fraction migrated as a broad band with a mean mobility corresponding to a length of 250 nucleotides and the other as a narrow band with a mobility corresponding to about 70 nucleotides. Since the proportions of radioactivity ([^{3}H]-adenosine) in the bands were 77% (250 nucleotides) and 23% (70 nucleotides) and the ^{3}H dpm to ^{14}C dpm ratios were 4.9 and 4.5, respectively, GA_3 increased the synthesis of both sizes of poly(A) to about the same extent.

If the total RNase-resistant material was loaded onto a second poly(U)-cellulose column, to free it from contaminating nonpoly(A) oligonucleotides, an unexpectedly large proportion of the radioactivity (49%) did not bind to the column although both the bound and unbound fractions were largely adenylic acid (see below). Since the ratios of ^{3}H dpm to ^{14}C dpm for the run-off and bound poly(A) fractions were 4.6 and 4.9, the two fractions were not differentially affected by GA_3. We do not yet know why so much of the poly(A) did not bind to the column but it could be an artifact associated with residual RNase in the poly(A) preparation which removed poly-(U) and its associated poly(A) from the poly(U)-cellulose. Recently we have found that a much larger proportion of the poly(A) (85%) would bind to poly(dT)-cellulose.

Using ^{32}P-labeled RNA, we found that the poly(A)-RNA constituted about 2% of the total labeled RNA. The RNase resistant fraction constituted 16.7% of the poly(A)-RNA synthesized in the absence of GA_3, and 13.5% of the poly(A)-RNA synthesized in its presence. When this material was ap-

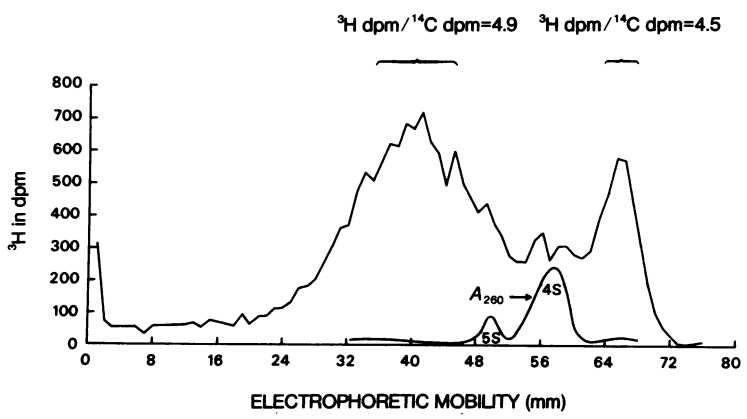

FIG. 2. Acrylamide gel electrophoresis of poly(A). Labeled poly(A)-RNA was prepared and then digested with pancreatic and T_1-ribonuclease as described in *Materials and Methods*. The total RNase resistant material, poly(A), was recovered and subjected to electrophoresis on a 5% acrylamide gel. The gel was sectioned and the radioactivity in the sections was counted.

TABLE 1. *Base compositions of RNA fractions*

Fraction	Moles percent C	A	G	U
(Without GA_3)				
Total poly(A)-RNA	20.9	31.9	26.2	21.1
RNase sensitive poly(A)-RNA	21.4	26.3	30.4	22.0
RNase resistant poly(A)-RNA				
(1) bound to poly(U)-cellulose	1.7	94.9	1.4	2.0
(2) not bound to poly(U)-cellulose	1.1	93.8	3.0	2.1
Heavy rRNA	22.1	23.5	36.0	18.4
Light rRNA	20.5	24.6	32.5	22.5
5S RNA	21.3	24.8	32.3	21.6
Transfer RNA	30.0	18.4	32.8	18.8
(With GA_3)				
Total poly(A)-RNA	20.4	32.8	27.1	19.8
RNase sensitive poly(A)-RNA	25.5	23.4	30.4	20.7
RNase resistant poly(A)-RNA				
(1) bound to poly(U)-cellulose	1.4	91.7	3.9	3.1
(2) not bound to poly(U)-cellulose	1.1	92.4	4.5	2.0
Heavy rRNA	22.1	24.3	34.3	19.4
Light rRNA	20.4	24.6	31.9	22.9
5S RNA	21.9	23.6	32.7	21.8
Transfer RNA	30.4	18.6	31.8	19.2

plied to a poly(U)-cellulose column, some was bound and some was not (as described above) and the adenylic acid contents of all these fractions (bound and unbound plus or minus GA_3) were in excess of 90% adenylic acid (Table 1). The base compositions for the two total poly(A)-RNA preparations were also the same and contained high amounts (31.9% and 32.8%) of adenylic acid. The RNase sensitive part of poly-(A)-RNA had a base composition which was similar to that of the total poly(A)-RNA preparations in that it was high in G. The C and A values obtained for this RNA synthesized in the presence of GA_3 were different, but the difference is of doubtful significance at this time because of the presence of salt in the hydrolyzed RNA solutions which lessened the resolution of C and A on paper electrophoresis. For the sake of comparison, base analyses of the major RNA species have been included in the table. It is noteworthy that GA_3 made no difference to the compositions of these species.

DISCUSSION

It is now established that plant cells, like animal cells, contain RNA with poly(A) segments since such RNA has been isolated from rice callus (10), corn roots (11), mung bean roots (12), soybean seedlings (13), and now barley aleurone. Rapidly labeled RNAs, rich in AMP, in pea and soybean have been known for some time (27, 28). In addition, poly(A) polymerase activity has been found associated with chromatin of maize (29) and wheat (30). Since poly(A)-RNA has been isolated from rice and mung bean polysomes (10, 12), it would appear that poly(A)-RNA accounts for at least some of the mRNA of plant cells as it does in animal cells.

O'Malley *et al.* (31) have shown that in the chick oviduct, progesterone induces the synthesis of the mRNA for the protein avidin; we show here that a plant hormone increases synthesis of poly(A)-RNA. The results are in harmony with the early suggestion by Varner (4) that the *de novo* synthesis of hydrolases by barley aleurone in response to GA_3 (1, 2) is dependent on the synthesis of hydrolase mRNA. It seems likely that we have measured such a GA_3-enhanced synthesis of mRNA.

Since the synthesis of total poly(A)-RNA is increased by GA_3, it is pertinent to inquire if both the poly(A) and the nonpoly(A) fractions, which are the informational part of the presumptive mRNA, are involved. We have shown that the ratios of ^3H dpm to ^{14}C dpm for the two poly(A) sequences isolated are both raised to the same extent and are about the same as the overall poly(A)-RNA ratio. This demonstrates that the enhancement of poly(A) synthesis is about the same as for the poly(A)-RNA. Therefore, the ratio of the non-poly(A) fraction must also be elevated.

The poly(A) sequences from rice callus and from mung bean roots both range in size from 100 to 150 nucleotides but that from barley aleurone is more disperse and has a bimodal distribution. The large fragments have an average mobility corresponding to about 250 nucleotides, like mammalian poly(A), and the small ones correspond to about 70 nucleotides. The significance of the difference between the two sizes of poly(A) is unknown. Short sequences have been reported in mitochondrial RNA (32) and from both nuclei and cytoplasm of HeLa cells (33, 34). Our preparation would contain RNA from nuclei, cytoplasm, and mitochondria.

The ratio of the numbers of nucleotides in the two poly(A) classes (250/70 = 3.6) is similar to the ratio of ^3H dpm in the two classes (77/23 = 3.4). This indicates that the two sizes of poly(A) are present in a molar ratio of about 1:1 and suggests that each poly(A)-RNA molecule might carry one of each of the two poly(A) sequences. In agreement with this, we have estimated from mobility measurements on acrylamide gels (26) that the average molecular weight of poly(A)-RNA is about 8×10^5; since about 15% of the poly(A)-RNA is poly(A), it follows that each poly(A)-RNA molecule should contain about 360 nucleotides as poly(A). This is approximately the number of nucleotides a poly(A)-RNA molecule would contain in the poly(A) sequences if it contained one short and one long poly(A) sequence, namely about 320. However, there is no further experimental support for this poly(A) distribution yet.

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