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TECHNICAL MANUSCRIPT 362

ABSCISSION: ROLE OF RNA SYNTHESIS

Robert E. Holm
Frederick B. Abeles

MAY · 1967

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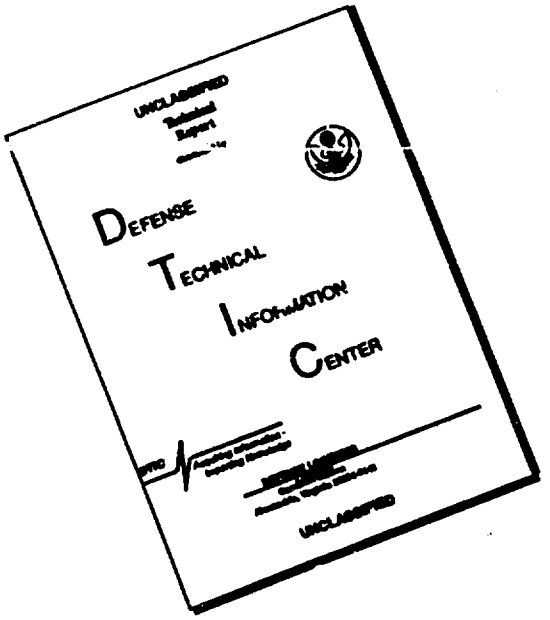

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TECHNICAL MANUSCRIPT 362

ABSCISSION: ROLE OF RNA SYNTHESIS

Robert E. Holm

Frederick B. Abeles

Crops Division
BIOLOGICAL SCIENCES LABORATORY

Project 1L013001A91A

May 1967

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ABSTRACT

Ethylene stimulated the incorporation of P^{32} into ribonucleic acid (RNA) in the abscission zone of bean explants (Phaseolus vulgaris L. var. Red Kidney). The enhancement was observed in all four peaks (soluble RNA, deoxyribonucleic acid, ribosomal RNA, and messenger RNA) separated by methylated albumin kieselguhr column chromatography, although the increase was not the same for each fraction. Differential extraction of the nucleic acids indicated that the ethylene stimulation was confined to the fraction extracted with sodium lauryl sulfate, with the increase mainly in the ribosomal and messenger RNA. Actinomycin D, which blocks ethylene-stimulated abscission, inhibited P^{32} incorporation into all column fractions. 5-Fluorouracil, which blocked 50% of the ethylene-enhanced P^{32} incorporation, did not inhibit ethylene-enhanced abscission. The results indicate that ethylene may regulate abscission through control of specific RNA.

CONTENTS

FIGURES

TABLES

I. INTRODUCTION

In an earlier paper,[1] we presented evidence that ribonucleic acid (RNA) synthesis was necessary for the cell separation process during abscission. However, Valdovinos and Muir[2] do not hold this viewpoint. With the increasing knowledge of the functions of different classes of RNA, it was of interest to study RNA synthesis with P^{32} during control and ethylene-stimulated abscission.

In this paper, we will show that ethylene enhanced all fractions of radioactive RNA separated on methylated albumin kieselguhr (MAK) columns, but only messenger and possibly ribosomal RNA appear critical for abscission. Other plant hormonal systems appear to exert their influence through nucleic acid regulation. The results of this study allow further comparisons of control mechanisms in abscission with other hormonally controlled plant processes.

II. MATERIALS AND METHODS

Methods used to grow and prepare explants and to measure ethylene in the surrounding gas phase have been described earlier.[1,3-5]

A. PREPARATION OF TOTAL RNA

Modification of the perchloric acid method of RNA extraction of Key and Shannon[6] is described fully in an earlier paper.[1] The phenol method of nucleic acid extraction was modified after Ingle et al.[7] Sixty 6-gram explants were homogenized in 1 ml of bentonite solution (100 mg per ml), 3.5 ml of 10% sodium lauryl sulfate (SLS), 7 ml of 0.01 M tris buffer [tris(hydroxymethyl)aminomethane] at pH 7.4, and 17.5 ml of phenol washed and saturated with the tris buffer at high speed for 2 minutes with a VirTis 23 homogenizer, then stirred at low speed for 3 minutes. After centrifugation at 10,000 x g for 10 minutes, the aqueous phase was removed and further deproteinized by two 2-minute extractions with equal volumes of phenol. The final aqueous phase was made 0.15 M with respect to potassium acetate; the nucleic acid was precipitated by adding ethanol to a final concentration of 70%. After standing at -15 C for 2 or more hours, the nucleic acid was recovered by centrifugation at 20,000 x g for 10 minutes and dissolved in 3 ml of 0.05 M sodium phosphate buffer, pH 6.7. This solution was dialyzed against 10 liters (two changes of 5 liters each) of 0.05 M phosphate buffer, pH 6.7, for 3 days. The nucleic acids were fractionated on MAK columns

prepared as described by Mandell and Hershey.[8] The sample of nucleic acid, usually 0.3 mg, was added to 1 mg of carrier RNA that was extracted from petiole tissue. The combined sample was added to the MAK column in 50 ml of 0.4 M NaCl and washed with an additional 50 ml of NaCl. The nucleic acids were eluted with a linear gradient of NaCl from 0.4 M to 1.2 M (400 ml each) (Figs. 1-3). The column was run under a pressure of 3 psi to give a flow rate of 2.5 ml per minute. The optical density (OD) of each 6-ml fraction was determined at 260 mμ and the P^{32} incorporation was checked by plating a 1-ml sample from each tube. All solutions and operations prior to MAK chromatography were maintained at 0 to 4 C.

B. PREPARATION OF DEOXYRIBONUCLEIC ACID

Deoxyribonucleic acid (DNA) from explants labeled with thymidine-C^{14} was extracted by the perchloric acid method. After hydrolysis in 0.3 N KOH for 18 hours at 37 C, subsequent acidification to 0.3 N $HClO_4$, and centrifugation to remove the RNA nucleotides, the DNA in the pellet was removed by the method of Tuan and Bonner.[9] The DNA was extracted from the $KClO_4$ precipitate by hydrolysis at 70 C for 40 minutes in 0.5 N perchloric acid. The resultant hydrolyzate was then cooled and neutralized with KOH, the potassium perchlorate was precipitated by centrifugation, and the DNA content of the supernatant fraction was determined by measuring the OD at 260 mμ. A sample was plated to determine isotope incorporation. The DNA was also extracted by the phenol method described above and separated by MAK column chromatography.

C. INCUBATION OF EXPLANTS

After excision, the explants (4.5 mm of pulvinal tissue and 9.5 mm of petiole tissue) were aged by placing them in petri plates, petiole end down, in 3 mm of 1.5% agar for 22 or 24 hours. The plates were vented after 7 hours to reduce the accumulation of ethylene. We have shown earlier[1] that only aged or senescent explants respond to ethylene. In all experiments except one (Table 1), the top 2 mm of pulvinal tissue were cut from senescent explants before placing the agar block containing radioactive isotopes on the pulvinal stump. When the top 2 mm were cut from the explant there was no effect on subsequent control or ethylene-stimulated abscission. Biggs[10] has also shown that shortening the pulvinus does not alter abscission rates. All tissue samples were frozen immediately after the conclusion of the experiment and thawed just before homogenization.

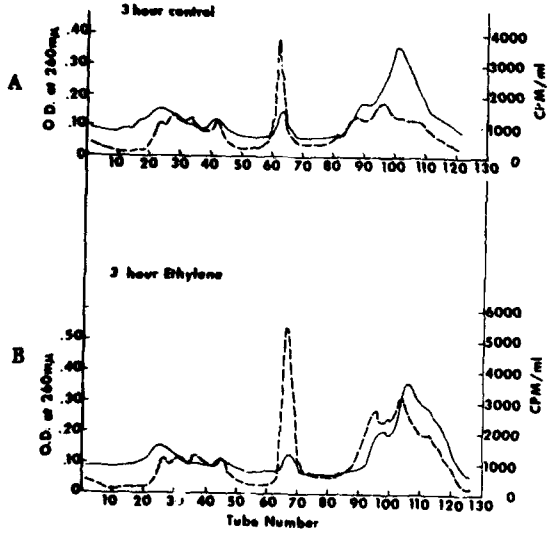

Figure 1. Distribution of Radioactivity in Various Nucleic Acid Fractions after 3 Hours' Incubation of Aged Explants. A. In air. B. In 2 ppm ethylene. In this and succeeding figures the solid line represents the OD at 260 mμ and the dashed line represents counts per minute.

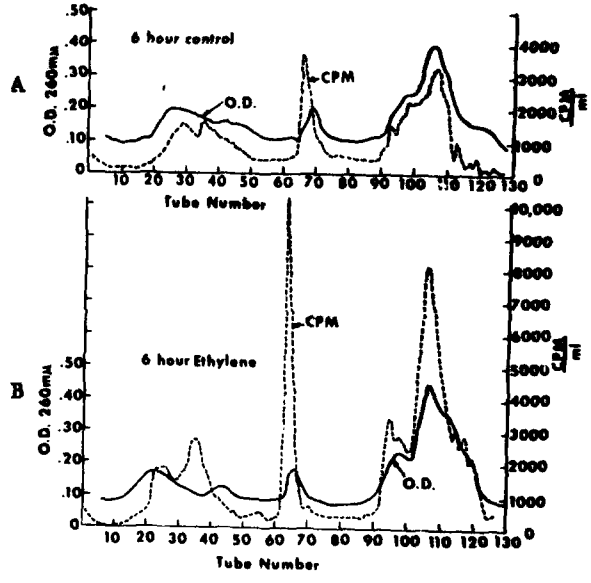

Figure 2. Distribution of Radioactivity in Various Nucleic Acid Fractions after Incubation of Aged Explants for 6 Hours. A. In air. B. In 2 ppm ethylene.

TABLE 1. ETHYLENE STIMULATION OF RNA SYNTHESIS IN THE ABSCISSION ZONE OF BEAN EXPLANTS

Section	RNA, µg		cpm[a]/ per mg RNA		%
	Control	Ethylene	Control	Ethylene	Change
Pulvinus (top 2 mm)	116±5	89±3	592,309	659,863	+11.4
Abscission zone (next 4 mm)	203±3	204±6	25,301	39,420	+55.8
Petiole (bottom 9 mm)	234±5	221±4	1,216,536	1,080,216	-11.2

a. Counts per minute.

D. APPLICATION OF INHIBITORS OF NUCLEIC ACID SYNTHESIS

Inhibitors of nucleic acid synthesis used in these experiments, 5-fluorouracil (5-FU), 5-fluorodeoxyuracil (5-FDU), phenethyl alcohol (PEA), mitomycin C, and methotrexate, were added to the explants by injection with a microliter syringe,[3] by a 1.5% agar droplet on the pulvinus, or by placing explants, pulvinal or petiole end down, in the agar containing the inhibitors. In the experiment illustrated in Figure 4, the explants were cut and stored in plain agar in petri dishes. At the various times indicated after excision, the explants were placed pulvinal or petiole end down in 10^{-2} M 5-FU. At 24 hours all explants were taken out of the 5-FU and placed petiole end down in gas collection bottles containing plain agar with or without 2 ppm ethylene. Abscission was measured 6 hours later, or 30 hours after the start of the experiment. 5-FU had no effect on the endogenous ethylene production of the tissue.

E. TREATMENT OF BANANAS

Bananas (Musa acuminata) were purchased from a local wholesaler before they were gassed with ethylene to induce ripening and were either kept untreated or induced to ripen by exposure to 25 to 50 ppm ethylene. Green (untreated) or yellow (ethylene-treated) peels (7 g of 1-cm squares) were incubated for 7 hours in sealed 125-ml Erlenmeyer flasks containing 10 ml of 5×10^{-3} M NaH_2PO_4 at pH 6, 0.08 M sucrose, 20 µg of streptomycin per ml, and 300 µc P^{32}, with or without 30 ppm ethylene. The RNA was extracted by the perchloric acid method.

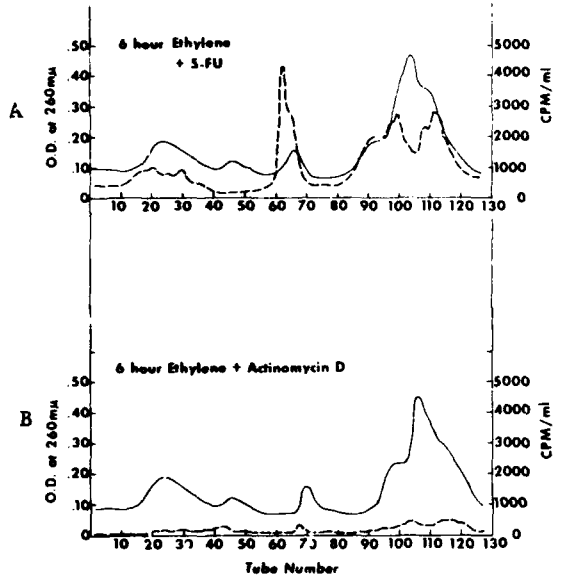

Figure 3. Distribution of Radioactivity in Various Nucleic Acid Fractions after Incubation of Aged Explants. A. Treated with 5-FU in 2 ppm ethylene for 6 hours. B. Treated with actinomycin D in 2 ppm ethylene for 6 hours.

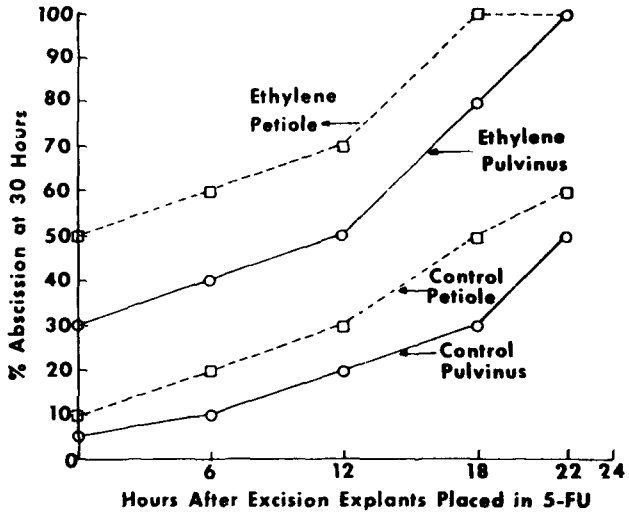

Figure 4. Effect of Time and Site of 5-FU Application on Abscission.

F. INORGANIC PHOSPHATE DETERMINATION

Ten abscission zones (2-mm pulvinal and 3-mm petiole tissues) were homogenized in 10 ml of ice-cold deionized water for 2 minutes. The homogenate was filtered through Miracloth and an 8-ml portion was added to 2 ml of 50% trichloroacetic acid (TCA). After shaking, the tubes were centrifuged at 2,000 x g for 10 minutes at 0 to 4 C. Three ml of the supernatant were analyzed for inorganic phosphate by the method of Fiske and Subbarow.[11]

G. DIFFERENTIAL EXTRACTION OF NUCLEIC ACID

The tissue was homogenized as described for total nucleic acid except that the SLS was omitted. After centrifugation and removal of the aqueous layer, which contained 76% of the tissue nucleic acid and was designated tris-NA, the phenol residue was re-extracted by shaking for 3 minutes with 16 ml of 0.01 M tris buffer at pH 7.4 containing 4 ml of 10% SLS. The aqueous layer after centrifugation contained the fraction of nucleic acid designated SLS-NA. Both fractions of nucleic acid were deproteinized and purified as described above for total RNA.

H. BASE COMPOSITION OF THE RNA

The tubes from the peak on the MAK column to be analyzed were pooled and 1 to 2 mg of carrier RNA were added. The RNA was precipitated by adding $HClO_4$ and centrifuging at 4,000 x g for 10 minutes in the cold. The supernatant was poured off and the resultant pellet was hydrolyzed in 0.3 N KOH for 18 hours at 37 C. After chilling, 2.4 N $HClO_4$ was added to give a pH of 1 and the DNA and $KClO_4$ were precipitated by centrifugation at 2,000 x g for 10 minutes. The supernatant fraction was adjusted to pH 6.0 with 1 N KOH and recentrifuged. The nucleotides were separated on a Dowex 1 by 8 formate column by stepwise elution with formic acid [100 ml of 0.15 N cytosine monophosphate (CMP), 100 ml of 1.0 N adenosine monophosphate (AMP), 180 ml of 3.0 N guanosine monophosphate (GMP), and 100 ml of 4.0 N uridine monophosphate (UMP) containing 0.1 N ammonium formate]. The peaks were combined and mixed, and samples were plated. Composition is expressed as P^{32} distributed among the four nucleotide peaks. The ratio of GMP to AMP has been used as a convenient parameter for comparison of RNA composition.[7]

III. RESULTS

A. ENHANCEMENT OF RNA SYNTHESIS IN THE ABSCISSION ZONE BY ETHYLENE

Abeles and Holm[3] had shown earlier that ethylene stimulated L-leucine-C^{14} incorporation into protein in the abscission zones of beans and cotton (Gossypium hirsutum L. var. Acal 4-42), but not in the surrounding tissues. Experiments were performed to see whether ethylene had the same effect on RNA synthesis.

In this experiment, bean explants were aged for 24 hours before being placed on their sides. Agar blocks containing 2.5 μc P^{32} were placed on each (pulvinal and petiole) end. The explants were incubated for 6 hours in the presence or absence of 2 ppm ethylene. Then the agar blocks were removed and the explants were divided into three sections (the top 2 mm, the next 4 mm that contained the abscission zone, and the basal 9 mm); RNA was extracted by the perchloric acid method. Abscission after 30 hours was 24% for controls, 91% for ethylene-treated explants. Table 1 shows that the ethylene enhancement occurred mainly in the abscission zone (56%) and to a lesser extent in the top section. The lower rate of synthesis of RNA-P^{32} in the abscission zone appears to result from less P^{32} diffusing into that area rather than from a slower rate of synthesis. Subsequent experiments, in which the top 2 mm of tissue were removed, showed that the abscission zone incorporates P^{32} at rates similar to those in the other two sections. The rest of the experiments reported in this paper were performed using explants with the top 2 mm removed after aging about 22 hours. The diffusion path for P^{32} was shortened by removal of the top 2 mm of pulvinus before placing the P^{32} agar block. This increased the rate of P^{32} incorporation into abscission zone RNA.

Other possible explanations for the enhancement of RNA synthesis are that ethylene either caused increased uptake of P^{32} from the agar blocks or altered the inorganic phosphate pool in the tissue, resulting in more isotope incorporation. Table 2 indicates that neither of these explanations can account for the ethylene-stimulated increase in P^{32}. Ethylene had no effect on the uptake of P^{32} from the agar blocks or on the amount of the isotope in the tissue. The soluble inorganic phosphate pool size did not seem to be altered by ethylene.

TABLE 2. EFFECT OF ETHYLENE ON P^{32} UPTAKE AND INORGANIC PHOSPHATE POOL SIZE IN EXPLANT TISSUES[a]

Time, hr	Treatment	cpm[b] 10 Agar Blocks	cpm 0.1 ml Homo.	iP x 10^{-4}, moles[c] 10 Absc. Zones	% Absc.
3	Control	745,710	19,159	2.62	0
	2 ppm C_2H_4	751,979	18,063	2.65	0
6	Control	603,387	35,987	2.46	10
	2 ppm C_2H_4	704,656	35,017	2.71	60

a. Explants aged for 22 hours, agar blocks contained 5 μc P^{32}. Agar blocks were spread and dried on planchets. Samples of the homogenate were taken prior to precipitation with $HClO_4$.
b. Counts per minute.
c. Initial inorganic phosphate content at time 0 was 3.05×10^{-4} M.

B. SEPARATION OF NUCLEIC ACIDS BY MAK CHROMATOGRAPHY

Figures 1 and 2 show the separation of P^{32}-labeled nucleic acids from bean explants after 3- and 6-hour exposures to atmospheres with or without 2 ppm ethylene. The total tissue nucleic acids (solid lines) separated as in other plant systems. The soluble RNA (s-RNA) eluted in the first two peaks (tubes 15 to 55), the DNA in one peak (tubes 60 to 70), the ribosomal RNA (r-RNA) in two peaks (tubes 80 to 105), and the messenger RNA (m-RNA) in tubes 106 to 125. The m-RNA peak was arbitrarily selected as starting five tubes past the heavy r-RNA OD peak because an OD shoulder usually occurred there. These separations were obtained by a single NaCl gradient from 0.4 to 1.2 M. The separations (Fig. 5 and 6) were obtained by two linear gradients (0.4 to 0.85 M and 0.85 to 1.2 M) that caused the various peaks to be shifted to the left.

At 3 hours, ethylene caused increases in all fractions of nucleic acids except the s-RNA (Fig. 1 and Table 3). The largest increase was in the ribosomal fraction, but the isotope peaks did not coincide with the OD peaks either in the control or in the ethylene-treated explants. After 6 hours (Fig. 2 and Table 3) ethylene-treated explants showed increases in all fractions, with more complete coincidence of label and optical density.

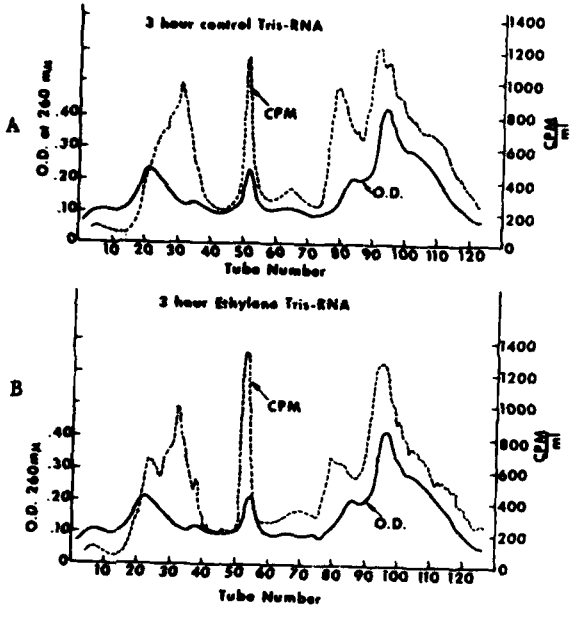

Figure 5. Distribution of Radioactivity in Various Nucleic Acid Fractions Extracted by the tris Buffer Method from Aged Explants. A. Incubated in air for 3 hours. B. Incubated in 2 ppm ethylene for 3 hours.

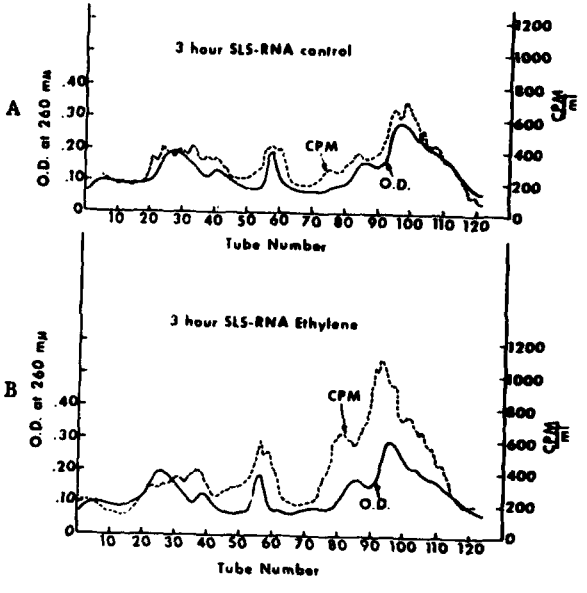

Figure 6. Distribution of Radioactivity in Various Nucleic Acid Fractions Extracted by the SLS Method from Aged Explants. A. Incubated in air for 3 hours. B. Incubated in ethylene for 3 hours.

TABLE 3. EFFECT OF ETHYLENE AND INHIBITORS ON P^{32} INCORPORATED INTO NUCLEIC ACID FRACTIONATED ON MAK COLUMNS[a]/

Peak on MAK Column	% C_2H_4 Increased or Decreased over Control		% Inhibition of C_2H_4 Stimulation	
	3 hr	6 hr	6 hr C_2H_4+ 10^{-2} M 5-FU	6 hr C_2H_4+ 1 μg act. D
s-RNA	-2	58	49	87
DNA	62	62	46	95
r-RNA	72	84	53	94
m-RNA	45	75	35	83
Total Nucleic acids	42	77	51	94

a. Explants aged 18 hours at 400 ft-c and 25 C. Top 2 mm of pulvinus were cut off and the inhibitors were added as described in text. At 22 hours after excision (i.e., 4 hours after inhibitor treatment), P^{32} agar blocks (8 μc per agar block) were placed on the pulvinal surface and incubated with or without 2 ppm ethylene for 3 and 6 hours. The nucleic acids were extracted by the phenol method and separated on MAK columns with a linear gradient. Per cent abscission: 3-hour control, 0; 3-hour ethylene, 17; 6-hour control, 17; 6-hour ethylene, 80; 6-hour ethylene + 5-FU, 80; and 6-hour ethylene + actinomycin D, 10.

C. INHIBITOR TREATMENT

Actinomycin D (1 μg) injected into explants 4 hours before ethylene treatment blocks ethylene-stimulated abscission[3] and causes a 94% inhibition of the ethylene-enhanced P^{32} incorporation (Fig. 3b and Table 3). The inhibition is similar in all fractions and supports the concept that actinomycin D inhibits all DNA-dependent RNA synthesis.

The pyrimidine analogue 5-FU (added by agar droplet) showed different effects on abscission rates, depending on the time of application (Fig. 4). 5-FU (10^{-2} M) did not inhibit control or ethylene-stimulated abscission in senescent explants (those excised 18 to 20 hours). This seems to occur because 5-FU interferes with the aging process.* The greater inhibition with the pulvinal application of 5-FU appears to come from the shorter diffusion path because the explants are inserted pulvinal end down instead of petiole end down.

* Unpublished results.

The results for 5-FU shown in Figure 3a and Table 3 were obtained from explants treated 18 hours after excision and 4 hours before ethylene treatment. This treatment did not alter the subsequent ethylene-stimulated abscission, but it did inhibit the P^{32} nucleic acid stimulation of ethylene by 51% (Table 3). The greatest inhibition occurred in the s-RNA and r-RNA fractions, indicating that not all of the nucleic acid enhanced by ethylene was needed for abscission to occur.

D. BASE RATIO ANALYSIS OF EXPLANT RNA-P^{32}

Because ethylene caused a large stimulation of P^{32} nucleic acid synthesis (70 to 80%) by 6 hours, a preliminary study was undertaken to determine whether ethylene changed the base composition of the RNA synthesized. Experiments extracting total RNA-P^{32} from control and ethylene-treated explants showed slight or no differences (Table 4). Although there was slight increase in the s-RNA by ethylene at 3 hours and 5-FU inhibited the s-RNA and r-RNA to a greater extent than m-RNA (Table 3), the m-RNA fraction was examined for base composition differences at 6 hours. Table 4 shows some differences in the base ratios of the control and ethylene m-RNA fractions. The ratio of GMP to AMP in this fraction indicates contamination by r-RNA if one assumes that plant messengers have a high AMP content.[7] 5-FU, which inhibited r-RNA synthesis by 53% (Table 3), lowered the ratio of GMP to AMP of the ethylene-treated explants although the total counts were still above those for the controls.

E. DIFFERENTIAL EXTRACTION OF NUCLEIC ACIDS

An attempt was made to further characterize the nucleic acids produced under ethylene stimulation by differential extraction techniques that have been shown in other plant tissues to separate the m-RNA, DNA, and DNA-associated RNA from the bulk of the tissue RNA.[7,12] This technique involves homogenizing the tissue with buffered phenol and bentonite, centrifuging, and drawing off the aqueous phase (tris-extracted nucleic acid). The interphase and phenol residue are then re-extracted with buffer and SLS (designated SLS-extracted nucleic acid).

TABLE 4. EFFECT OF ETHYLENE ON RNA-P^{32} BASE COMPOSITION OF MESSENGER-RNA AND TOTAL RNA

Treatment	P^{32} Mole Fraction				GMP/AMP	Total Counts
	CMP	AMP	GMP	UMP		
Total RNA[a]						
3-hr control	21.9	25.7	30.7	21.7	1.19	96,545
3-hr C_2H_4	22.8	25.2	30.1	21.9	1.19	136,749
m-RNA[b]						
3-hr control	18.8	28.4	31.8	21.0	1.12	25,232
3-hr C_2H_4	20.7	27.1	32.2	20.0	1.19	36,582
6-hr control	20.7	27.6	32.9	18.8	1.19	148,236
6-hr C_2H_4	22.1	26.1	32.0	19.8	1.23	251,467
6-hr C_2H_4 + 5-FU	20.9	29.1	31.8	18.2	1.09	194,131
Total explant RNA	23.6	23.8	31.5	21.1	1.32	

a. Prepared by the perchloric acid method.
b. Precipitated from the MAK column fraction as described in text.

Table 5 shows that the tris buffer extracted 76% of the explant nucleic acids, although it did not show an ethylene enhancement. The SLS-extracted nucleic acids, however, contained ethylene-stimulated fraction and contained the highest specific activity RNA-P^{32}. Separation of the nucleic acids on MAK columns (Fig. 5 and 6) shows that there is little difference in the control and ethylene treatments with tris extraction (Fig. 5), but that ethylene enhancement is observed in the SLS-extracted fractions (Fig. 6). Table 6 indicates that the major ethylene enhancement occurs in the messenger and ribosomal regions.

TABLE 5. DIFFERENTIAL EXTRACTION OF NUCLEIC ACID WITH TRIS AND SODIUM LAURYL SULFATE[a]

Treatment	Extraction Medium	Total Counts from Column Peaks[b]	mg NA[c] Extracted[d]	cpm[e] per mg NA	% Increase
3-hr control	SLS	117,456	0.122	962,758	
3-hr C_2H_4	SLS	253,950	0.116	1,497,429	+56
3-hr control	Tris	320,136	0.488	656,016	
3-hr C_2H_4	Tris	306,648	0.459	668,078	+2

a. Conditions for the experiment were the same as those described in Table 6.
b. Determined from Figures 5 and 6.
c. Nucleic acid.
d. From the amount extracted by each technique and before 1 mg of carrier nucleic acid was added for column separation.
e. Counts per minute.

TABLE 6. EFFECT OF DIFFERENTIAL EXTRACTION ON ETHYLENE-STIMULATED P^{32} NUCLEIC ACID[a]/

Peak on MAK Column	% C_2H_4 Increase or Decrease over Control	
	SLS-Extracted NA	Tris-Extracted NA
s-RNA	20	4
DNA	24	12
r-RNA	55	-12
m-RNA	62	-6
Total NA	48	-4

a. Explants were aged for 22 hours before the top 2 mm of pulvinal tissue were removed and an agar block (10 μc per agar block) was placed on top. The explants were treated, with or without 2 ppm ethylene, for 3 hours and the nucleic acids were differentially extracted as outlined in text. The nucleic acids were separated on MAK columns using linear NaCl gradients. The percentage increase or decrease by ethylene was calculated from the total counts in each of the peaks described.

F. EXAMINATION OF DNA SYNTHESIS DURING ABSCISSION

Data in Tables 3 and 6 show that ethylene caused enhancement of P^{32} incorporation into the DNA peak although this peak is known to contain RNA also, probably as a natural hybrid.[13] The use of P^{32} did not rule out the possibility of ethylene-enhanced DNA synthesis. Experiments were first performed to see if the inhibitors of DNA synthesis (mitomycin C, PEA, and methotrexate) would inhibit abscission. Nitsan and Lang[14] have shown that these compounds inhibit DNA synthesis and cell elongation in peas and lentils. The results shown in Table 7 indicate that only PEA at the highest concentration used inhibited ethylene-stimulated abscission. It is known that PEA also inhibits RNA synthesis to some extent,[15] so this result can not be taken as completely valid because we have shown that some inhibitors of RNA synthesis also inhibit abscission.[1] 5-FDU also tried at various times after excision (either by agar droplet or by injection), had no effect on abscission.

TABLE 7. EFFECT OF DNA SYNTHESIS INHIBITORS ON ETHYLENE-STIMULATED ABSCISSION[a]

Treatment	Per Cent Abscission at Given Molar Concentration of Inhibitor					
	5×10^{-3}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}
Control			97			
PEA	57	80	97	100		
Mitomycin C		100	100	100		
Methotrexate			100	100	100	100

a. Explants were cut and aged for 20 hours at 25 C. At that time 1 µl of the inhibitor was injected through the petiole. At 22 hours the explants were given 2 ppm ethylene. Abscission was measured at 28 hours.

Results shown in Table 8 indicate that although there is a considerable amount of thymidine-C^{14} incorporation into the DNA, ethylene does not enhance the amount incorporated. Similar results were obtained with perchloric acid and phenol extraction methods.

TABLE 8. EFFECT OF ETHYLENE ON THYMIDINE-C^{14} INCORPORATION INTO BEAN EXPLANT DNA[a]

Treatment	Per Cent Abscission at 29 hr	µg DNA per 20 Explants	cpm[b] per mg DNA
Control	27	36.3±1.0	22,000±200
2 ppm C_2H_4	87	37.2±0.8	20,900±300

a. Senescent explants had the top 2 mm of pulvinal tissue removed and agar blocks containing 0.78 µc thymidine-C^{14} (2×10^{-4} M) were placed on the pulvinal stumps. Explants were treated for 7 hours.
b. Counts per minute.

G. ENHANCEMENT OF RNA SYNTHESIS IN BANANA PEEL

Because ethylene is thought to be responsible for the respiratory climacteric in banana fruit,[16] it could cause an induction of RNA synthesis in this system.

Green bananas were divided into two groups, one of which was given a 2-day ethylene pretreatment. The peels were then removed and incubated in a buffered medium containing P^{32} in the presence or absence of 30 ppm ethylene. The green peels that had not been previously treated with ethylene showed an enhancement of RNA-P^{32} synthesis. The previously induced peels that were yellowing showed a smaller enhancement (Table 9).

TABLE 9. EFFECT OF ETHYLENE ON P^{32} INCORPORATION INTO RNA OF YELLOW OR GREEN BANANA PEELS

Peel	Atmosphere	µg RNA	cpm[a] per mg RNA	Per Cent Increase
Green	control	602±6	72,475±1,111	
Green	C_2H_4	576±5	95,676±1,151	+32
Yellow	control	232±3	68,346±3,688	
Yellow	C_2H_4	249±4	76,658±5,978	+12

a. Counts per minute.

IV. DISCUSSION

In earlier papers we presented evidence that ethylene stimulated RNA-P^{32} synthesis in explant tissue[1] and that ethylene enhanced L-leucine-C^{14} protein formation in the separation layer and not in the surrounding tissue.[3] As reported here, the ethylene enhancement of RNA-P^{32} was confined to the 2-mm area on each side of the abscission zone (Table 1). The ethylene enhancement is not caused by increased isotope uptake into the tissue or by decreased inorganic phosphate pool size. However, the gross measurement of pool sizes may be misleading because of unknown effects of cell compartmentalization and the possibility of more than one phosphate pool.

The 3- and 6-hour labeling periods were chosen for studying two different time intervals during ethylene-stimulated abscission. At 3 hours, the enhancement of P^{32} was 30 to 40% greater than that of the controls, but abscission had not started. At 6 hours, the enhancement reached 70 to 80%, and the ethylene-treated explants had abscised 70 to 90%, the controls 5 to 10%.

The differential effect of ethylene after 3 hours' treatment on the stimulation of P^{32} into the nucleic acid fractions indicates that ethylene may be rather specific in regulating the production of certain types of RNA that in turn produce the protein (enzymes) necessary for abscission. Kinetin caused more labeled orotate in the m-RNA fraction than it did in the r-RNA or s-RNA fractions in radish leaf discs.[17] In peanut cotyledons Carpenter and Cherry,[18] however, found general stimulation of P^{32} into all MAK fractions with low concentrations of benzyladenine. Ingle and Key[19] have shown that 2,4-D preferentially stimulated r-RNA during growth of excised soybean hypocotyl tissue. The problem of comparing ethylene with other plant growth regulators that appear to act through control of nucleic acid metabolism is difficult. Other plant growth regulators increase total RNA levels in growing tissue or delay the decrease of nucleic acids in senescent tissue. Ethylene, however, stimulated P^{32} incorporation into the nucleic acids of explants while the total levels of RNA and DNA were decreasing.* These results further indicate that abscission is an active process requiring energy and the synthesis of RNA and protein[1,3] rather than a passive process that releases preformed degradative enzymes.

After 3 or 6 hours of ethylene treatment, the enhancement of P^{32} incorporation was greatest in the r-RNA fraction. At 3 hours there was no effect on s-RNA synthesis but at 6 hours there was an increase. Because the ethylene-treated explants have almost completely abscised at 6 hours, the increase in s-RNA could result from the degradation of other classes of RNA, or it could be a net synthesis. A chase experiment with P^{31} after 3 hours' treatment with P^{32} would be needed to test the latter possibility. This is not easily done because of the time involved in manipulating the agar blocks and the disruption of the ethylene atmosphere around the explants. Actinomycin D inhibited 94% of the nucleic acid synthesis (Table 3) and abscission[1] stimulation by ethylene at 6 hours, and the inhibition was similar in all nucleic acid fractions. 5-FU added at 18 hours after excision inhibited the ethylene enhancement of nucleic acid synthesis 50%, yet it did not alter the abscission rate. The greater inhibition of s-RNA and r-RNA with 5-FU compared with that of m-RNA is analogous to other plant systems where 5-FU has been tested.[20-22] Key and Ingle[22] found that 5-FU could inhibit more than 50% of the total nucleic acid synthesis in excised soybean hypocotyl without growth inhibition. The greatest inhibition was in the ribosomal and soluble fractions of RNA. Cherry and van Huystee[20] noted that 5-FU inhibited r-RNA and DNA to a greater extent than m-RNA in Xanthium buds. They concluded that 5-FU did not influence cell activity by the production of nonfunctional m-RNA.

* Unpublished results.

In an attempt to determine whether ethylene was altering the nucleotide composition of the RNA that was synthesized, base ratio analyses were performed on the newly synthesized RNA-P^{32}. The total RNA-P^{32} showed no difference between control and ethylene-treated explants. Because 5-FU showed greater inhibition of s-RNA and r-RNA syntheses without affecting abscission, the m-RNA fraction was examined. Although slight differences were noted (Table 4) at 3 and 6 hours, the effect was slight. The higher ratios of GMP to AMP for the ethylene treatments probably resulted from r-RNA contamination of the m-RNA fraction, because the greatest ethylene stimulation is in the ribosomal fraction. The ethylene and 5-FU combination showed a decreased ratio of GMP to AMP, which also indicates ribosomal contamination. The total counts for the m-RNA for ethylene and 5-FU were still higher than those of the controls.

Assuming that explant m-RNA had a ratio of GMP to AMP of 0.60 as the DNA-like RNA in soybean hypocotyl tissue,[7,19] then the m-RNA fraction in our separation contains only 18 to 20% m-RNA after 3 hours of ethylene, comprising only 4 to 5% of the total RNA-P^{32} synthesized. This is apparently different from other plant tissues,[19,20] where excision results in greatly reduced r-RNA synthesis, thereby magnifying the m-RNA production. Cherry and van Huystee[20] found that excising Xanthium buds caused 50% more m-RNA-P^{32} to be formed than was present in intact buds. They likened excision of plant parts to bacterial step-down cultures[23] that preferentially synthesize informational RNA. Examination of RNA metabolism in abscission zone explants immediately after excision instead of at 25 hours could alter these conclusions. However, our work is limited to the period in which explants respond to ethylene in terms of accelerated nucleic acid metabolism and protein synthesis, and this does not occur until the explants have aged for about 24 hours.

Differential extraction of the nucleic acids from explants at 3 hours showed the enhancement by ethylene in the SLS fraction (Table 5). Other investigators[7,12] have shown that tris extraction with phenol removes the bulk of the s-RNA and r-RNA. Re-extraction of the interphase and phenol residue with SLS and tris removes the DNA and rapidly labeled RNA (i.e., m-RNA). The separation of explant nucleic acids by MAK chromatography, however, showed that the tris extraction yielded some DNA as well as r-RNA and s-RNA (Fig. 5). The specific activities of the individual fractions could not be determined accurately because the amounts extracted (0.1 to 0.5 mg) could not be run on the column without added carrier nucleic acid. The specific activities (Table 5) were determined from the total counts from the MAK column peaks and the total amount of nucleic acid extracted before addition of the carrier. The ethylene enhancement in the SLS fraction was again a differential effect (Table 6) on the fractions, as it had been in the total extraction (Table 3), although the greatest stimulation in the SLS extraction was in the m-RNA.

DNA synthesis does not appear necessary for abscission. The inhibitors of DNA synthesis had no effect on ethylene-stimulated abscission (Table 7), and ethylene showed no stimulation of thymidine-C^{14} incorporation into DNA (Table 8). However, because there was incorporation of thymidine-C^{14}, there was some turnover or synthesis of DNA. Cherry[13] has shown that the DNA peak from peanut cotyledons on MAK columns contains 24% RNA. Ingle et al.,[7] however, reported that the DNA peak from soybean tissue contained only 5% RNA. In our experiments, the amount of RNA-P^{32} in the DNA peak ranged from 5% for controls to 20% for ethylene-treated. This was determined by KOH hydrolysis and subsequent perchloric acid hydrolysis of the DNA peak. Ethylene, however, always caused a slight increase over control DNA-P^{32} by this method. The explanation of differences in results with P^{32} and thymidine-C^{14} will have to await further knowledge of the nature of the DNA peak as separated on the MAK column.

That ethylene may act through RNA regulation in other systems where it serves as a hormone is shown in the experiment with green and yellow (ripening) banana peels (Table 9). The ethylene enhancement of RNA-P^{32} was greatest in the peels that had not been previously induced to ripen by ethylene, indicating possible nucleic acid control and limited additional response once the ripening process was initiated. Stahmann et al.[24] have shown that ethylene stimulated the production or activity of the enzymes peroxidase and polyphenoloxidase in connection with disease resistance of sweet potato [Ipomoea batatas (L) Lam.] to black rot. Presumably this induction may be similar to the stimulation of leucine-C^{14} into protein from abscission zones,[1,3] although a specific enzyme was not found in that investigation and the RNA levels were not examined in the sweet potatoes.

The addition of ethylene to explant tissue seems only to speed the natural abscission process that appears to occur with endogenous ethylene levels. Stimulation of explant abscission by ethylene appears analogous to banana ripening as reported by Burg and Burg,[18] in which added ethylene only shifted the normal respiratory climacteric, which is caused by endogenous ethylene production, to an earlier time. During our experiments, the gas-collection bottles were sealed the same way for both treatments except that 2 ppm of ethylene were injected into one set. The control explants produced enough ethylene to allow an accumulation of 0.05 to 0.10 ppm in the atmosphere, which is enough to cause abscission. There appears to be a 3- to 4-hour lag between the control and ethylene-treated explants. As shown in Figures 1 and 2, the distribution of P^{32} on the MAK columns for the 6-hour control and the 3-hour ethylene explants appears similar. The specific activities for the total nucleic acids are also similar. Table 4 also shows that the base composition of the m-RNA fraction for the 6-hour control and the 3-hour ethylene explants is similar, as are the abscission rates (Table 3).

It is impossible at this time to show an absolute requirement for ethylene in the abscission process because there is no way known to inhibit ethylene production. It appears from our work that ethylene, which controls abscission at the RNA level, causes the production of the enzymes needed for the final separation process.

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13. ABSTRACT

Ethylene stimulated the incorporation of P^{32} into ribonucleic acid (RNA) in the abscission zone of bean explants (Phaseolus vulgaris L. var. Red Kidney). The enhancement was observed in all four peaks (soluble RNA, deoxyribonucleic acid, ribosomal RNA, and messenger RNA) separated by methylated albumin kieselguhr column chromatography, although the increase was not the same for each fraction. Differential extraction of the nucleic acids indicated that the ethylene stimulation was confined to the fraction extracted with sodium lauryl sulfate, with the increase mainly in the ribosomal and messenger RNA. Actinomycin D, which blocks ethylene-stimulated abscission, inhibited P^{32} incorporation into all column fractions. 5-Fluorouracil, which blocked 50% of the ethylene-enhanced P^{32} incorporation, did not inhibit ethylene-enhanced abscission. The results indicate that ethylene may regulate abscission through control of specific RNA.

14. Key Words

Abscission	Beans
RNA	Radiological agents
Synthesis	Chromatography
Ethylene	Actinomycin D
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