Response of Barley Aleurone Layers to Abscisic Acid

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ABSTRACT

Cordycepin, an inhibitor of RNA synthesis in barley (Hordeum vulgare L.) aleurone cells, does not inhibit the gibberellic acid-enhanced α-amylase (EC 3.2.1.1.) synthesis in barley aleurone layers if it is added 12 hours or more after the addition of the hormone. However, the accumulation of α-amylase activity after 12 hours of gibberellic acid can be decreased by abscisic acid. The accumulation of α-amylase activity is sustained or quickly restored when cordycepin is added simultaneously or some time after abscisic acid, indicating that the response of aleurone layers to abscisic acid depends on the continuous synthesis of a short lived RNA.

By analysis of the newly synthesized proteins by gel electrophoresis with sodium dodecylsulfate, we observed that the synthesis of α-amylase is decreased in the presence of abscisic acid while the synthesis of most of the other proteins remains unchanged. From the rate of resumption of α-amylase production in the presence of cordycepin and abscisic acid, it appears that abscisic acid does not have a measurable effect on the stability of α-amylase mRNA.

Aleurone layers of barley seeds respond to GA₃ by synthesizing and secreting several hydrolases, beginning 8 to 10 hr after the addition of GA₃ (22). During this lag period GA₃ enhances membrane proliferation as shown by direct electron microscopic observation (15) and by biochemical determination of phospholipid synthesis in membrane fractions (5, 17). Two enzymes, phosphorylcholine-cytidyl (EC 2.7.7.15) and phosphorylcholine-glyceride (EC 2.7.8.2) transferases, in the phosphatidyl choline pathway appear to be activated within minutes after GA₃ administration (1, 14). Membrane bound polysome formation (6) and poly(A)-RNA² synthesis (10, 13) are promoted in the presence of GA₃. All these GA₃ effects are reversed or prevented by ABA. ABA does not have any effect on general cellular metabolism as measured by O₂ consumption (2).

Although ABA can prevent the response to GA₃, no direct effects of ABA in aleurone cells have been observed. The failure of the aleurone cells to respond to GA₃ in the presence of ABA does not result from simple competition between these two hormones because a high concentration of GA₃ cannot completely overcome the ABA effect (2, 12).

Because most of the effects of GA₃ in aleurone cells depend

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2 Abbreviation: poly(A)-RNA: RNA species containing polyadenyllic acid.

MATERIALS AND METHOD

Sources of Chemicals and Seed. Cordycepin, GA₃, and ABA (mixture of equal amounts of cis-trans and trans-trans isomers; all concentrations used in this study refer to that of cis-trans isomer only) were purchased from Sigma Chemical Co. Potato starch for the α-amylase assay was obtained from Nutritional Biochemical Co. 1-Leucine-4.5-³H (5 Ci/mmole) and uridine-³H (G) (5-15 Ci/mmole) were purchased from New England Nuclear. NCS tissue solubilizer was obtained from Amer sham/Searle Co. Barley seeds (Hordeum vulgare L. cv. Himalaya. 1969 crop) were supplied by Department of Agronomy, Washington State University, Pullman. in 1972 and stored in the cold room before use.

Preparation and Treatment of Aleurone Layer. Embryoless half-seeds were surface-sterilized by sodium hypochlorite (5-fold dilution of commercial bleach) for 20 min and rinsed several times with sterile deionized H₂O and further stirred in 0.01 M HCl for 10 min to destroy any remaining sodium hypochlorite. After thorough rinsing with H₂O and with 20 mM sodium succinate buffer, pH 5, containing 10 mM CaCl₂, the half-seeds were allowed to imbibe water under sterilized conditions on sand moistened with the same sodium succinate buffer. Aleurone layers were peeled from 3-day imbibed half-seeds and incubated in sodium succinate buffer containing different combinations of hormones and inhibitors, in a reciprocal metabolic shaker (120 oscillations/min) at 25 °C. The concentration of GA₃ used in this work was 2.5 μM.

Extraction and Assay of Enzyme. α-Amylase was extracted according to the methods of Crispeels and Varner (3), combined with enzyme in the medium, and assayed as described by Varner and Mense (21). The unit of α-amylase was defined as a change of 1 absorbance unit/min at 620 nm.

RNA Extraction and Fractionation. Total RNA from aleurone layers was extracted and poly(A)-RNA was separated from the other RNA species by oligo(dT) cellulose chromatography as described before (10).

Sodium Dodecyl Sulfate Gel Electrophoresis. The procedures
used were modification of those of Laemmli (18). Gels composed of 2.5 cm of stacking gel (4%), and 10.5 cm of separation gel (12%) were used. Samples for gel electrophoresis were prepared by grinding 30 to 40 aleurone layers with 0.5 ml 0.2 M NaCl, containing 10 mM KBrO$_3$, with a mortar and pestle. The mortar was rinsed with another 1 ml of NaCl-KBrO$_3$ solution and the solutions were combined. After centrifuging for 30 min at 12,000g, the supernatant was decanted and 1 ml of SDS reagent containing 0.125 M tris-HCl, pH 6.8 4% SDS. 10% mercaptoethanol was added. This supernatant is referred to as salt-soluble supernatant. To the washed pellet 1 ml of SDS reagent was added. After storage at room temperature for several days, the pellet with SDS reagent was centrifuged. The resulting supernatant was diluted with equal volume of distilled H$_2$O and referred to as salt-insoluble protein preparation. A 100-μl sample was applied to each gel and electrophoresis was carried out at 80 v (constant) and an initial current of 40 mamp/12 gels.

RESULTS

Effect of Cordycepin on GA$_3$-enhanced α-Amylase Formation. Cordycepin is believed to act as a chain terminator during RNA synthesis and inhibits the formation of both poly(A)-RNA and RNA sequences not containing a poly(A) segment in barley aleurone layers (Fig. 1). Cordycepin is still effective as a transcription inhibitor when it is added 12 hr after GA$_3$ (Fig. 1). The inhibitory effect of cordycepin on GA$_3$-enhanced α-amylase synthesis is less and less as cordycepin is added later and later after the hormone. and no inhibitory effect is observed if cordycepin is added 10 hr or later after the addition of GA$_3$ (Table I). In fact, an enhancement of α-amylase formation by cordycepin added 12 hr after GA$_3$ was occasionally observed (Table I). Because α-amylase is synthesized at the time the increasing activity of this enzyme is observed. and cordycepin has no effect on the degradation of the enzyme (Ho and Varner, manuscript in preparation), it is concluded that α-amylase is translated from stable mRNA (10).

Effect of ABA on α-Amylase Formation. Abscisic acid (5 mM), inhibits α-amylase formation if it is added at the same time as GA$_3$. Higher concentrations of ABA (10–25 μM) are needed to reduce α-amylase synthesis when ABA is added 12 hr after GA$_3$. It has been reported that ABA, at a concentration of 38 μM forms a complex with fungal α-amylase, resulting in inhibition of the enzyme activity (19, 20). ABA at concentrations up to 50 μM has no significant effect on barley aleurone α-amylase activity in a cell-free enzyme preparation after 23 hr of incubation at 25 C (Table II). Since the concentration of ABA we used here was 25 μM (Figs. 2 and 3) and the time span of the experiment was no longer than 12 hr (from 12–24 hr after addition of GA$_3$), we conclude that the ABA effect on α-amylase production must be via a physiological process.

Effect of Cordycepin on Action of ABA. ABA added 12 hr after GA$_3$ gradually inhibits further accumulation of α-amylase (Fig. 2). Because the accumulation of α-amylase activity in response to GA$_3$, both before and after 12 hr, is due to de novo synthesis (i.e., there is no evidence for accumulation of an inactive precursor of α-amylase. Ho and Varner, manuscript in preparation) and because α-amylase accumulation after 12 hr of GA$_3$ is not inhibited by cordycepin, it is clear that ABA inhibits the synthesis of α-amylase posttranslationally. When ABA is added 12 hr after GA$_3$ and cordycepin is added at the same time or later, the accumulation of α-amylase either does not stop, or is quickly resumed (Figs. 2 and 3). The effect of cordycepin cannot be due to a generally toxic effect because α-amylase production remains normal in the presence of cordycepin without ABA. The effect of ABA seems to depend on the continuous synthesis of a short lived RNA which is inhibited by cordycepin. Because the rate of α-amylase accumulation after cordycepin addition is close to that of tissue treated with GA$_3$ alone (Figs. 2 and 3), it appears that the amount of α-amylase mRNA is not limiting in the presence of ABA and cordycepin. i.e. ABA does not reduce the stability of α-amylase mRNA.

In order to determine whether ABA specifically prevents the

![Figure 1](https://www.plantphysiol.org/figure/1)

**Fig. 1.** Effect of cordycepin on RNA synthesis. Two samples, 80 aleurone layers each, were pretreated with GA$_3$ for 12 hr and cordycepin was then added to one of the samples. Label (3H-uridine, 5 μCi/ml) was introduced between 13.5 and 18 hr after GA$_3$. RNA was extracted and fractionated by oligo(dT) cellulose chromatography as described previously (8). The first peak (fractions 1–4) eluted by 0.5 M KCI consists of RNA species containing poly(A)-RNA. The peak (fractions 13–15) eluted in the absence of KCl contains poly(A)-RNA. Sometimes a tiny peak was eluted by 0.1 M KCl. Control (+ GA$_3$) only (C--O); cordycepin (0.1 mM) treated (X---X).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α-Amylase Activity</th>
<th>Increase over control</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA$_3$ only</td>
<td>27.7</td>
<td>26.6</td>
</tr>
<tr>
<td>Control (No GA$_3$)</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>GA$_3$ + cordycepin</td>
<td>6.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Added 4 hr after GA$_3$</td>
<td>10.9</td>
<td>9.8</td>
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<tr>
<td>Added 8 hr after GA$_3$</td>
<td>25.0</td>
<td>23.9</td>
</tr>
<tr>
<td>Added 10 hr after GA$_3$</td>
<td>27.6</td>
<td>26.5</td>
</tr>
<tr>
<td>Added 12 hr after GA$_3$</td>
<td>31.2</td>
<td>30.1</td>
</tr>
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</table>
synthesis of α-amylase (and perhaps other GA_3-enhanced hydro-
lases as well) or whether ABA slows down protein synthesis in
general, the profile of newly synthesized proteins was examined
by SDS gel electrophoresis. α-Amylase is detected as the pre-
dominant radioactive band on SDS gel with a mol wt of about
50,000 daltons. As shown in Figure 4 for salt-soluble proteins,
the synthesis of α-amylase is substantially decreased in the
presence of ABA, while the amount of radioactivity in most of

Table II. Lack of Direct Effect of Abscisic Acid on α-Amylase Activity

<table>
<thead>
<tr>
<th>Incubation Time (at 25 °C)</th>
<th>α-Amylase</th>
<th>No ABA</th>
<th>10 μM ABA</th>
<th>50 μM ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td>unit %</td>
<td></td>
<td>unit %</td>
<td>unit %</td>
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<tr>
<td>0</td>
<td>41.0 100</td>
<td>41.0 100</td>
<td>41.0 100</td>
<td>41.0 100</td>
</tr>
<tr>
<td>3</td>
<td>41.6 101</td>
<td>40.9 99.8</td>
<td>40.8 99.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>40.2 98</td>
<td>39.5 96</td>
<td>37.2 91</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>38.6 94</td>
<td>37.2 91</td>
<td>37.5 91</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Effect of midcourse addition of (25 mM) ABA and 0.1 mM
cordycepin on the synthesis of α-amylase. GA_3 only (O—O); GA_3
and ABA (●—●); GA_3, ABA, and cordycepin (3-dA) (X—X—X).

Fig. 3. Effect of cordycepin added at different times on the synthe-
sis of α-amylase in the presence of both GA_3 and ABA. ABA was added
12 hr after GA_3. Arrows indicate the time of cordycepin addition. GA_3
only (O—O); GA_3 and ABA (●—●); GA_3, ABA, and cordycepin
(X—X—X).

Fig. 4. Profile of newly synthesized salt-soluble proteins on SDS
gel. Aleurone layers (30-40) were labeled with [3H]-leucine (15 μCi/ml)
for 2 hr (18-20 hr after GA_3). Salt-soluble proteins were extracted as
described under "Materials and Methods." One mm thick gel pieces
were sliced and digested in 0.5 ml of NCS solubilizer (9 parts of full
strength NCS solubilizer and 1 part of distilled H_2O) at 50°C for 2 hr.
Ten ml of toluene-based scintillation fluid (6 g of PPO and 75 mg of
POPOP) were used in each sample.

the minor bands remains essentially the same as in control
tissue (+GA_3 only).

DISCUSSION

Although the synthesis of α-amylase after 12 hr of GA_3 treat-
ment is no longer subject to transcriptional control, the inhibi-
tory effect of ABA on α-amylase production at this same stage
apparently depends on the continuous synthesis of a short-lived
RNA (regulator RNA in Fig. 5). Apparently, this regulator
RNA, or its translation product, can decrease the rate of synthe-
sis of α-amylase without influencing protein synthesis in gen-
eral. The mRNA of α-amylase is stable, at least after 12 hr of
exposure of the tissue to GA_3, and its stability is maintained in
the presence of ABA.

Ihle and Dure (11), working with precociously germinating
cotton embryos, obtained evidence that the translation of car-boxyypeptidase mRNA was inhibited by ABA. Because they
found that actinomycin D prevented the ABA inhibition, they
proposed that a suppressor molecule had to be formed to bring
about the ABA inhibition (11).

The action of ABA thus appears to be similar in the two
systems, the precociously germinating cotton embryos where
gibberellins probably have no regulatory role, and the mobiliza-
tion of reserve nutrient in the germinating barley seed where
ABA prevents the GA_3-enhanced α-amylase production in the
aleurone cells. It seems reasonable to suggest that many ABA
effects depend on transcription, although there is no evidence


