Differential Effects of Actinomycin D and Cordycepin in Lettuce Seed Germination and RNA Synthesis

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ABSTRACT

Intact lettuce seed germination was inhibited by cordycepin but not by actinomycin D; however, when seeds were clipped at the cotyledonal end, actinomycin D partially inhibited germination. Uptake studies with intact seeds using ³H-actinomycin D showed that it was unable to reach the embryo prior to radical protrusion. ³H-Cordycepin uptake studies using intact seeds showed that cordycepin was able to reach the embryo during the first 3 hours of incubation and at subsequent times. The pericarp and endosperm offered resistance to penetration of cordycepin into the embryo. In contrast to actinomycin D, cordycepin markedly inhibited ³H-uridine incorporation into RNA of intact seeds during the first 10 and 12 hours of incubation. About 60% of ³H-adenosine incorporation into poly A-RNA was inhibited by cordycepin during 12 hours of incubation, whereas actinomycin D had little effect. RNA synthesis appears to be essential for seed germination.

A number of studies have indicated that dry seeds contain preformed mRNA (2, 5, 23, 24). Since polyribosomes and polypeptide synthesis occur during imbibition (10, 12, 13), it has been suggested that germination proceeds while the genome is still inactive (2). It has recently been reported that synthesis of RNA, including mRNA, occurs in the first few hours of germination of wheat (15, 20), barley (22), and rye (16) grains. Thus, it is uncertain whether or not RNA synthesis is required for seed germination.

In the case of lettuce seed germination, the evidence for the involvement of RNA synthesis is particularly confusing because there are no conclusive data to show whether Act D is taken up by lettuce seeds and reaches the site of action prior to radical protrusion, although uptake of Act D into lettuce seedlings was reported (14). We have examined the penetration of Act D and CP into different seed parts and the effects of these inhibitors on RNA synthesis and lettuce seed germination.

MATERIALS AND METHODS

Seed Germination. Lettuce seeds (Lactuca sativa L. cv. Grand Rapids, 1974 harvest) were obtained from Ferry-Morse Seed Co. Fifty seeds were germinated in white light at 25 C in 2 ml of 20 mM HEPES (pH 7.6), or desired solutions (used 20 mM HEPES [pH 7.6] as a solvent) on a filter paper in a 5-cm Petri plate for various lengths of time. There is an empty space between cotyledons and the endosperm in the lettuce seed at the cotyledonal end (18). In some experiments, seeds were clipped at the cotyledonal end without causing injury to the embryos prior to germination. In other experiments, seeds were clipped after the removal of pericarp. All results reported are the mean of two or three replicates.

Uptake of ³H-Act D and ³H-CP. One hundred seeds were incubated in 2 ml of ³H-CP (1 mM, 0.1 μCi/μg from New England Nuclear), or ³H-Act D (80 μM, 0.1 μCi/μg, from Schwarz/Mann) solution on a filter paper in a 5-cm Petri plate at 25 C in white light. After various times, 10 seeds (two replicates) were removed and rinsed with distilled H2O. The pericarp, endosperm layer, and embryo were separated from each other on a paper towel. The different seed parts or intact seeds were homogenized with 4 ml of dioxane scintillator solution in a mortar, and the mortar was rinsed twice with 3 ml of scintillator solution. Radioactivity in the pooled 10-ml solution was determined with a Unilux II liquid scintillation system. The amount of CP uptake was calculated after sample quenching was corrected.

Isolation of DNA-³H-Act D Complex. DNA was isolated as described by Jaworski and Key (6) with some modifications. Intact seed or clipped seeds were germinated in ³H-Act D solution (0.2 mM, 0.2 μCi/μg) for 13 hr. One hundred endosperms or embryos were isolated and were ground in a mortar with 5 ml buffer containing 1% SDS, 0.1 mM Na2 EDTA, 0.45 mM NaCl, and 20 mM HEPES (pH 7.6). The homogenate was washed once with 5 ml of chloroform-isoamyl alcohol (24:1) mixture and twice with 5 ml of H2O-saturated phenol. The aqueous phase was then incubated with 500 μg RNase A at 25 C for 30 min. Under these conditions, RNase was able to hydrolyze RNA (250 μg/ml) at a rate of 2.2 μg/min. After an addition of 100 μg calf thymus DNA (from Sigma), DNA was precipitated with ethanol for 30 min at −10 C. The precipitate was pelleted by centrifugation at 5,000g for 10 min and washed once with 5 ml cold ethanol. The pellet was dissolved in 0.5 ml distilled H2O and its radioactivity was detected by a liquid scintillation system.

Isolation of RNA Including Poly A-RNA. For RNA studies, 120 seeds were incubated with ³H-uridine (20 μCi/ml, 26 Ci/ mol) or ³H-adenosine (20 μCi/ml, 8.9 Ci/mole) for various lengths of time at 25 C in light in the presence or absence of the RNA synthesis inhibitor. After the removal of pericarp, RNA was extracted from 100 seeds with 10 ml of phenol-buffer mixture (21) containing 10 mM HEPES buffer (pH 7.6), 60 mM KCl, 10 mM MgCl2, 1% SDS, 0.1% hydroxyquinoline, EDTA-washed bentonite (0.5 mg/ml), and 5 mM of H2O-saturated phenol. After washing twice with phenol, followed by ethanol precipitation, RNA was dissolved in 0.5 ml of 10 mM HEPES buffer containing 60 mM KCl and 10 mM MgCl2 and dialyzed overnight. RNA was then fractionated by 5 to 20% sucrose gradient centrifugation (11), and radioactivity in each fraction (26 drops) was measured by using dioxane liquid scintillator. For the poly A-RNA study, the dialyzed RNA solution was incubated with DNase (100 μg/ml from Sigma) at 37 C for 1 hr. Equal volume of 1 mM KCl in HEPES (pH 7.6, 10 mM) was added to each sample. Poly A-RNA was isolated on an oligo(dt)-cellulose column (0.5 × 2.5 cm) essentially as described by Ho.

1 Approved by the Director of the New York State Agricultural Experiment Station as Journal Series Paper No. 2283.
2 Abbreviations: Act D, actinomycin D; CP, cordycepin.
and Varner (4). The cellulose column was washed with distilled H₂O and then equilibrated with 0.5 M KCl-HEPES buffer. Following the application of RNA, the column was washed with 0.5 M KCl-HEPES and 0.1 M KCl-HEPES. Finally, the bound RNA was eluted with 1 ml of HEPES buffer.

RESULTS

Act D failed to inhibit lettuce seed germination in light at 25 C. However, when the seeds were clipped at the cotyledonary end, Act D partially inhibited germination (Fig. 1). A faster germination was observed when the seeds were clipped. A time course of ³H-Act D uptake into different seed parts and intact seeds is shown in Figure 2. Pericarp contained the largest amount of ³H-Act D among the seed parts. Almost no radioactivity was found in the embryo during the first 13 hr of germination. Since only the ungerminated seeds were used at 13 hr, the seed coats (endosperm plus pericarp) completely restricted the uptake of ³H-Act D by the embryo during this time. Following the rupture of the seed coats by radical protrusion, some ³H-Act D was found in the embryo at 19 hr.

In order to determine whether or not the Act D reached the site of action, DNA was isolated from the intact and clipped seeds after 13 hr of germination in ³H-Act D solution. Compared to no radioactivity (1 cpm) in the embryo DNA of intact seeds, a significantly higher level (286 cpm) was found in the embryo DNA of clipped seeds. The amounts of ³H-Act D associated with endosperm DNA from both seeds were low (31 cpm for intact and 64 cpm for clipped seeds). Similar distribution of radioactivity was observed in two separate experiments.

The effect of CP on the lettuce seed germination is shown in Table I. Germination was inhibited completely by 1 mM and partially by 0.5 mM. Concentration of 0.1 mM or lower had no effect. In contrast to the uptake of ³H-Act D into the embryo, ³H-CP was found to reach the embryo in the first 3 hr of soaking, and the radioactivity increased with an increase of soaking time (Fig. 3). There was no marked change between 13 and 19 hr since radical protrusion was inhibited at the concentration of CP used (1 mM).

The data in Table II show the amount of CP associated with different seed parts during 13 hr of soaking in ³H-CP at 25 C in light. At 1 mM concentration, there were only 14 ng of CP found in the embryo plus endosperm layer. At 0.5 mM CP, the amounts reaching the embryo and endosperm layer were about 50% of that in presence of 1 mM CP.

The barrier effect of pericarp and endosperm layer on CP
inhibition of lettuce seed germination is shown in Table III. At the inhibitory concentrations, such as 0.25 mM and 0.5 mM, the removal of pericarp caused decrease in germination percentage. When the endosperm was clamped, an additional decrease in germination was observed and at 0.5 mM CP germination was completely inhibited.

\[ ^3\text{H}-\text{Uridine incorporation into RNA during the first 10 and 12 hr of soaking, prior to radical protrusion, was markedly inhibited by CP but not by Act D (Table IV). The incorporation at 12 hr in the presence of Act D was slightly lower than the HEPES control, but it could be due to the experimental deviation. When RNA was fractionated by sucrose gradient centrifugation, the inhibitory effect of CN was largely in the region of sRNA and no significant difference was found between HEPES and Act D treatments (data not shown).] \]

The synthesis of poly A-RNA during the first 12 hr of germination was studied by using \(^{3}\text{H}\)-adenosine. Similar radioactivities were found in the poly A-RNA fractions from Act D-treated seeds (259 cpm) and from the control (288 cpm), but a lower level was found in the fraction from CP-treated seeds (108 cpm). It can be seen that the poly A-RNA synthesis is inhibited about 60% by CP but not by Act D.

**DISCUSSION**

It has been reported that germination of lettuce seeds is inhibited by nucleotide base analogs (3, 8, 17) but not by Act D (1, 9, 14). Our data show that Act D fails to reach the embryo of the intact seeds. The different responses of lettuce seed to Act D and CP could be explained by the failure of the seed to take up Act D into the embryo.

It has been reported that Act D at low concentration (4 µg/ml) promoted dark germination of lettuce seeds, and evidence suggests that protein synthesis occurs most extensively in endosperms of dark germinating seed (1). \(^{3}\text{H}\)-Act D was detected in the endosperm extract (Fig. 2). It could be that the Act D effect on dark germination at low concentration is through its action on the endosperm.

The partial inhibitory effect of Act D on clamped seed suggests that pericarp and/or endosperm layers could work as barriers for the uptake of Act D. Lettuce endosperm cells are composed of thick cell walls and dense cytoplasm (7). Chemicals such as phosphate and arsenate are also unable to penetrate the intact seeds (3, 19). There was no \(^{3}\text{H}\)-Act D complexed with DNA in the embryo of intact seeds. Some labeled Act D was found in the embryo DNA of clamped seeds. This suggests that some Act D is able to reach the DNA of the embryo in these seeds. The possibility that formation of Act D-DNA complex occurred during isolation of DNA is not ruled out. Obviously, Act D could have little effect on \(^{3}\text{H}\)-uridine and \(^{3}\text{H}\)-adenosine incorporation into RNA of intact seeds prior to radical protrusion (Tables IV and text). In contrast to this, Act D is reported to have a marked inhibitory effect on RNA synthesis in lettuce seeds following radical protrusion (3, 11). Act D has been shown to be taken up by the lettuce seedling and to inhibit seedling growth (14).

In previous studies, Act D was used at 0.08 mM and little or no inhibition of radicle elongation was observed at 20 and 30 hr (9). In the present study using higher concentration of Act D (0.2 mM), the antibiotic did penetrate and partially inhibit germination. Thus, the previous conclusion (9) on Act D-resistant RNA synthesis controlling germination appears unjustified.

The germination of intact lettuce seeds was completely inhibited by 1 mM CP (Table I). Clipping, which presumably allows greater uptake of CP into the seed, caused inhibition at
lower concentration (Table III). Although CP acts at the transcriptional level, we cannot rule out the possibility that it might affect germination by other mechanisms.

Cordycepin had a marked inhibitory effect on 3H-uridine and 3H-adenosine incorporation into RNA in lettuce seeds including poly A-RNA (Tables IV and text). Poly A-RNA is regarded as a mRNA (4, 20). These data, together with the studies with base analogs (3, 8, 17), suggest that RNA synthesis is essential for lettuce seed germination. This is consistent with the recent evidence that RNA synthesis occurs during imbibition in other seeds (15, 16, 20, 22).

**Table III.** Barrier Effect of Pericarp and Endosperm on Cordycepin Inhibition of Lettuce Seed Germination

Intact seeds, seeds without pericarp and seeds without pericarp plus clipped endosperm layer were germinated in light at 25°C for 24 hr. Values in columns and rows followed by the same letter are not significantly different at 5% level.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact</th>
<th>Minus pericarp plus clipping</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPE</td>
<td>94.8</td>
<td>100.8</td>
</tr>
<tr>
<td>HEPE + 0.1 mM</td>
<td>95.6</td>
<td>95.6</td>
</tr>
<tr>
<td>HEPE + 0.25 mM</td>
<td>97.0</td>
<td>93.5</td>
</tr>
<tr>
<td>HEPE + 0.5 mM</td>
<td>104.0</td>
<td>96.6</td>
</tr>
<tr>
<td>HEPE + 1 mM</td>
<td>94.8</td>
<td>94.8</td>
</tr>
</tbody>
</table>

**Table IV.** Effects of Actinomycin D and Cordycepin on 3H-uridine Incorporation

Intact lettuce seeds were germinated in 3H-uridine solution in the Presence or Absence of the RNA Synthesis Inhibitor. Radioactivity in the RNA fraction was measured. RNA content was determined by U.V. absorption, assuming A260 = 25 for a solution containing 1 mg of RNA/ml.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H-uridine incorporation</th>
<th>Specific radioactivity</th>
<th>HEPE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation time (hr)</td>
<td>cpm x 10⁻³</td>
<td>cpm x 10⁻³/μgRNA</td>
<td>%</td>
</tr>
<tr>
<td>HEPE</td>
<td>10</td>
<td>3.6</td>
<td>0.7 ± 0.07</td>
</tr>
<tr>
<td>Act D 100 μg/ml</td>
<td>10</td>
<td>4.1</td>
<td>0.8 ± 0.08</td>
</tr>
<tr>
<td>CP 1 mM</td>
<td>10</td>
<td>2.6</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>HEPE</td>
<td>12</td>
<td>10.5</td>
<td>1.5 ± 0.12</td>
</tr>
<tr>
<td>Act D 100 μg/ml</td>
<td>12</td>
<td>8.8</td>
<td>1.1 ± 0.10</td>
</tr>
<tr>
<td>CP 1 mM</td>
<td>12</td>
<td>3.7</td>
<td>0.6 ± 0.06</td>
</tr>
</tbody>
</table>

1 Standard error

**LITERATURE CITED**