Ethylene-forming Systems in Etiolated Pea Seedling and Apple Tissue

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ABSTRACT

Auxin-induced ethylene formation in etiolated pea (Pisum sativum L. var. Alaska) stem segments was inhibited by inhibitors of RNA and protein synthesis. Kinetics of the inhibition is described for actinomycin D, cordycepin, α-amanitin, and cycloheximide. α-Amanitin was the most potent and fast-acting inhibitor, when added before induction or 6 hours after induction of the ethylene-forming system. The ethylene-forming system of postelmacleristic apple (Malus sylvestris L.) tissue, which is already massively induced, was not further stimulated by auxin. Ethylene production in apples was inhibited least by α-amanitin and most by actinomycin D. The relative responses of the ethylene system in apples to RNA inhibitors were different from the ethylene system of pea stems. However, the protein synthesis inhibitor, cycloheximide, appeared to act equally in both tissue systems. The effect of cycloheximide on ethylene production in postelmacleristic apple tissue, already producing large quantities of ethylene, suggests a dynamic regulating system for the synthesis and degradation of the ethylene-forming system.

Two major systems for ethylene production have been studied in higher plants. One is the subhook stem section of the etiolated pea seedling (Pisum sativum L. var. Alaska), which is stimulated by auxin (1, 2, 9, 12), and the other is the mature and aging apple tissue, which is little influenced by auxins (vide infra). Both systems produce ethylene from methionine (4, 10) and are specifically inhibited by rhizobitoxine (13), an analogue of methionine. Therefore, they may have similar enzymes and substrates for ethylene production. The difference between these tissues may be their control or regulation, which depends on the age of the tissue.

This report describes and compares the effects of inhibitors of nucleic acid and protein synthesis on ethylene production in the two tissues. The results provide insight into the control and regulation of ethylene production in young, rapidly developing cells and in senescent cells.

MATERIALS AND METHODS

Plant Materials. Seeds of Pisum sativum L. var. Alaska were treated for 15 min with 1% sodium hypochlorite and soaked overnight in running, aerated tap water. The seeds were then grown in the dark for 6 to 8 days on wet “kimpack.” Stem segments (0.5 cm) were cut, in dim green light, from the region below the apical hook or the subhook region (15). Five subhook segments were placed in a 5-mL shell vial with 1 mL of H2O or 2% sucrose, capped with serum vaccine caps, and incubated at 20°C in the dark. Various additions were made to the incubating medium as shown in the table and figures.

Plugs of Golden Delicious apples (Malus sylvestris L.) were prepared as previously reported (10). Four apple plugs weighing about 1 g were incubated in a 25-mL Erlenmeyer flask containing 5 mL of 0.4 M sucrose and a small vial of 20% KOH (CO2 trap). The flasks, capped with serum caps, were shaken gently in a water bath at 30°C. Various additions were made to the incubation solution as indicated in the table and graphs.

Ethylene in the gas space above the incubating tissue was determined as previously reported (10). All experiments were repeated at least three times, and treatments within experiments were in triplicate or more. The results presented are averages of at least three experiments in which variations between experiments were not more than 15 to 20%. Additional experimental details are described in the figures and table where appropriate.

RESULTS

Stimulation of Ethylene Production in Pea Subhook Segments. Pea seedlings produce very little while subhook segments of etiolated peas produce very little no ethylene. As first noted by Zimmerman and Wilcoxon (17), applied auxin stimulates ethylene production considerably. This phenomenon was observed most vividly in experiments with subhook segments of etiolated peas (3) (Fig. 1). IAA in concentrations up to 0.1 mM stimulated ethylene production by more than 50-fold. At 1 mM the stimulation decreased markedly. In some IAA-induced systems ethylene production decreases after 6 or 8 hr due to conjugation or destruction of IAA (9). Relative suppression of ethylene production by 1 mM IAA may have been due to a toxicity effect at this high concentration. Similar results were observed with 2, 4-D in the same type of experiment.

The kinetic curve for IAA-induced ethylene production showed a lag period of about 2 hr, followed by a linear production of ethylene. Preincubation in actinomycin D followed by auxin treatment prevented the induction of ethylene formation in the pea sections (data not shown). The initial lag period, therefore, was attributed to the time required to activate RNA and protein synthesis mechanisms necessary for biosynthesis of the ethylene-forming enzymes.

Inhibition of IAA-induced Ethylene Formation in Pea Subhooks during Induction. Pea segments were incubated in solutions of IAA with different inhibitors, and the course of ethylene production was followed with time. These studies provided information about the nature and mechanisms of IAA-induced ethylene production. Actinomycin D, which inhibits DNA-directed RNA synthesis, inhibited ethylene formation 33% during the first 4 hr (Fig. 2). The inhibition increased...
with time and reached 77% in 24 hr. Cordycepin (3′-deoxyadenosine), an inhibitor of post-transcriptional processing of RNA in the nucleus (5), inhibited 44% in the first 4 hr and 59 to 72% in 6 to 8.5 hr. Inhibition of ethylene formation increased to 74% after 24 hr. Cordycepin and actinomycin D inhibited about equally in the long term, but cordycepin was a more effective inhibitor initially. The RNA synthesis inhibitor which was the most potent inhibitor of ethylene formation was α-amanitin, which inhibits nuclear RNA polymerase II in mammalian (8) and plant cells (16). α-Amanitin inhibits 83% of ethylene production in 4 hr, 88% in 6 hr and 78% in 24 hr. Decreased effectiveness of α-amanitin with time has also been noted in other experiments (11).

Cycloheximide, an inhibitor of RNA-directed protein synthesis (14), was extremely potent in inhibiting IAA-induced ethylene production in pea segments. Inhibition was 85% in 4 hr and increased to 97% in 6 to 8 hr (Fig. 2). After 24 hr ethylene formation was essentially completely inhibited by cycloheximide.

**Inhibition of IAA-induced Ethylene Formation in Pea Subhooks after Induction.** The same ranking of inhibitor potencies was observed with RNA synthesis inhibitors, when IAA induction of ethylene was allowed to proceed for 6 hr, before addition of the inhibitors. α-Amanitin was the most effective and actinomycin D the least effective inhibitor, during the first 7 hr of incubation (Fig. 2).

Cycloheximide inhibited only 5% in the first 2.5-hr period of incubation after the 6-hr induction period. However, in the next 5 to 15 hr it became the most potent inhibitor (Fig. 2).

The kinetic curves for these data (Fig. 2) show that once the ethylene-forming system was induced by IAA, actinomycin D and cycloheximide did not inhibit ethylene production significantly in the first 2.5 hr. Cordycepin and α-amanitin were more effective during this time period. The most effective inhibitor during the first 7 hr after induction of the ethylene-forming system was α-amanitin.

**Inhibitors of DNA Synthesis in Pea Subhooks.** 5-Fluoro-

deoxyuridine, an inhibitor of nuclear DNA synthesis, did not inhibit auxin-induced ethylene synthesis. This indicated that DNA synthesis is not directly involved in auxin-induced ethylene production in the pea subhooks (7). In contrast, ethidium bromide, an inhibitor of mitochondrial DNA (18) inhibited auxin-induced ethylene biosynthesis about 40%. The significance of this finding is not yet known.

**Ethylene Production in Apple Tissue Slices: Effect of Auxin.** Auxin did not stimulate ethylene production in tissue from fully grown preclimacteric apples which were not producing ethylene (Fig. 3) and slightly inhibited ethylene production in tissue from postclimacteric apples stored at 0 C for 27 weeks (Fig. 4). However, very young intact apples, whose cells were still enlarging, were stimulated to produce ethylene when vacuum-infiltrated with 2,4-D.

**Inhibitors of DNA Synthesis on Ethylene Production of Apple Tissue.** FUDR1 at 0.1 mM inhibited ethylene production by about 20% in postclimacteric apple tissue in 6 hr of incubation and by 30% in 24 hr (Table I). Ethidium bromide inhibited 26% in 6 hr and 69% in 24 hr. The strong inhibition of ethylene production by ethidium bromide in apples was greater than that observed in pea segments. FUDR also caused fairly significant inhibition of ethylene production in apple slices in contrast to its total ineffectiveness in the pea segments.

**Inhibitors of RNA Synthesis in Apple Tissue.** The production of large amounts of ethylene by postclimacteric Golden Delicious apples was significantly inhibited by inhibitors of RNA synthesis only after 24 hr of incubation (Table I). Actinomycin D inhibited ethylene production 0 to 6% in the

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1 Abbreviation: FUDR; 5-fluorodeoxyuridine.
first 3.5 hr of incubation, 25% in 6 hr, and 53% in 24 hr. Cordycepin and α-amanitin were less inhibitory than actinomycin D, causing virtually no inhibition in 6 hr of incubation and 43 and 42% inhibition, respectively, in 24 hr. The relative potencies of these inhibitors on ethylene production in apples differed from that in the pea system, where α-amanitin and cordycepin were far more effective than actinomycin D.

Inhibitors of Protein Synthesis in Apple Tissue. Inhibitors of protein synthesis, cycloheximide, pactamycin, and sparsomycin (14) all effectively inhibited ethylene production by apple tissue in 6 hr (Table 1). These inhibitors were much more effective than inhibitors of RNA synthesis or of DNA template activity in suppressing ethylene production (Table 1). Generally, protein synthesis inhibitors acted similarly in the apple ethylene-forming system and in the auxin-induced pea subshub ethylene-forming system.

Decay of Ethylene-forming System. Ethylene production in plant tissues is governed by the rate of biosynthesis of the ethylene-forming enzyme system, the rate of degradation of this system, and the availability of substrates, cofactors, and effectors. Ethylene production in the pea sections was not limited by methionine, its substrate, addition of which did not influence the rate of ethylene production (data not shown). The limiting factor appeared to be the rate of formation of the ethylene enzyme system which seemed wholly dependent on the presence of high levels of auxin. The decay of this enzyme system, as measured by changes in ethylene production, was determined by treating pea stem tissue with cycloheximide to prevent renewal of the enzyme-forming system. Ethylene production rapidly declined in the presence of cycloheximide, reaching half its maximum rate in approximately 1 hr and 40 min (Fig. 5). The control tissue, without cycloheximide, produced ethylene at an undiminished rate. The decay curve suggests that the pea stem ethylene-forming enzyme system has a short half-life, and the enzymes of this system must be continually renewed to keep pace with its rapid degradation.

The decay of the apple ethylene-forming enzyme system was determined similarly (Fig. 5). Ethylene-forming system of summer Rambo, harvested and stored 2 days at 5 C before use, reached half its initial rate of production in approximately 3 hr and 26 min. This apple variety senesces rapidly and does not store well. The time to reach half maximal rate of ethylene production in the control system (without cycloheximide) was 7 hr and 28 min. This suggested that as the tissue aged during incubation, for time periods exceeding 4 hr, the rate of degradation of the ethylene-forming system accelerated and exceeded the rate of biosynthesis. Addition of methionine to these aged systems did not stimulate ethylene production (data not shown).

Table 1. Inhibition of Ethylene Production in Postclimacteric Golden Delicious Apple Tissue Slices by Inhibitors of Replication

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>3.5 Hr</th>
<th>6 Hr</th>
<th>24 Hr</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-template inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide (0.1 mM)</td>
<td>3</td>
<td>26</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>FUDR (0.1 mM)</td>
<td>19</td>
<td>19</td>
<td>30</td>
<td></td>
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<tr>
<td>Transcription inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actinomycin D (50 µg)</td>
<td>6</td>
<td>25</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Cordycepin (50 µg)</td>
<td>0</td>
<td>3</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>α-Amanitin (10 µg)</td>
<td>5</td>
<td>2</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Translation inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheximide (0.1 mM)</td>
<td>10</td>
<td>44</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Sarcosyn (0.1 mM)</td>
<td>10</td>
<td>44</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Pactamycin (0.1 mM)</td>
<td>8</td>
<td>40</td>
<td>79</td>
<td></td>
</tr>
</tbody>
</table>

Golden Delicious apples removed from storage after 4.5 months at 0 C were used for these experiments. Tissue slices were prepared as described in “Materials and Methods.” Data were calculated from Figures 6 and 7.
**DISCUSSION**

Auxin-induced ethylene production in subhook sections of etiolated peas appeared to be brought about by the synthesis of an ethylene-forming enzyme system which required for its maintenance the continued presence of auxin (9). The RNA synthesis inhibitors α-amanitin, cordycepin, and actinomycin D were effective in inhibiting ethylene formation in the IAA-induced ethylene-forming pea system. Cycloheximide and other inhibitors of cytoplasmic polypeptide synthesis were also very effective in inhibiting and preventing auxin-induced ethylene production in the pea subhook system. In contrast, FUDR, an inhibitor of nuclear DNA synthesis (7), did not prevent auxin-induced ethylene production in pea subhooks. The inhibition of ethylene production by inhibitors of RNA and protein synthesis suggested that the induction process was initiated at the level of RNA synthesis. On the other hand, ethidium bromide, which inhibits mitochondrial DNA-dependent processes (17), inhibited the auxin-induced ethylene-forming system about 40%. This suggested a possible involvement of mitochondrial DNA at some stage in auxin-induction of the ethylene-forming system. However, more direct evidence, other than inhibitor effects, is necessary to establish that RNA and mitochondrial DNA are involved in auxin-induced ethylene production in pea segments.

The rapidity and extent of α-amanitin inhibition of the auxin-induced ethylene-forming pea stem system, relative to those of the other RNA inhibitors used, suggested that auxin-induced ethylene formation may involve an indirect interaction with the α-amanitin-sensitive site. This would place this manifestation of auxin action in the pea seedling stem at the level of nucleoplasmic RNA polymerase II. Auxin treatment of seedlings has been shown to increase total RNA polymerase activity and rates of RNA synthesis (6).

The apple ethylene-forming system differed from the auxin-induced pea system in three respects. The apple system was (a) not stimulated by auxin, (b) inhibited less by inhibitors of RNA synthesis than the pea system, and least of all by α-amanitin, and (c) moderately inhibited by FUDR, which had no effect on the pea system. These differences between the two systems may have their origins in the ages of the tissues. This suggests that the systems differ in their regulatory mechanisms of ethylene production.

Analyses of ethylene production and the influences of inhibitors in the pea and apple tissues lead to the speculation that these ethylene-forming enzyme systems exist in a dynamic state in which they are produced and degraded simultaneously. When cycloheximide was added to tissues evolving ethylene, the ethylene-forming system was blocked and the curve for ethylene production decayed, presumably because of the reactions which degraded the ethylene-forming system.

Ethylene production appears to be controlled by the rates of formation and degradation of the ethylene-forming enzyme systems. Auxin induced the ethylene-forming system in pea seedlings. Possibly other plant hormones are indirectly involved in ethylene production, by stimulating either biosynthesis or degradation of the ethylene-forming enzyme system.

Interpretation of these experiments depends on the assumption that the known effects of the RNA and protein inhibitors used also apply in systems for ethylene production, and there were no side effects of these chemicals which influenced ethylene production. We appreciate the necessity for more direct evidence linking IAA induction of the ethylene-forming system to RNA synthesis.

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**LITERATURE CITED**

6. _Guthrie_ and J. B. Hanson. 1974. Greater length of RNA synthesized by...