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EFFECT OF ETHYLENE AND METABOLIC INHIBITORS ON ANTHOCYANIN BIOSYNTHESIS

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Abstract—The use of metabolic inhibitors indicated that ethylene-enhancement of light-induced anthocyanin biosynthesis in *Sorghum vulgare* is through promotion of enzyme synthesis. Ethylene treatment had no effect on the amount of cyanidin synthesized in sorghum tissue infiltrated with actinomycin D to inhibit RNA synthesis. Treatment of sorghum tissue with ethylene in the dark for 24 hr prior to light-induction of anthocyanin biosynthesis reduced the ability of cycloheximide to inhibit anthocyanin formation in the tissue. Ethylene treatment promoted the biosynthesis of two 3-deoxyanthocyanidins in sorghum for which light-induced RNA synthesis is not necessary.

INTRODUCTION

Developmental studies have demonstrated that light-induced anthocyanin biosynthesis in plants is through gene activation of specific RNA and enzyme systems [1–4]. Although the metabolic processes which control the formation of these systems are unknown, they may be regulated by plant hormones [5]. Ethylene has been shown to have both promotive and inhibitive effects on anthocyanin biosynthesis in plants [6, 7], and to influence RNA and enzyme systems in plant tissues [8]. The purpose of these experiments was to determine whether ethylene control of anthocyanin biosynthesis is through control of RNA and enzyme systems.

RESULTS AND DISCUSSION

Ethylene has previously been shown to enhance light-induced anthocyanin biosynthesis in many plant tissues [7]. A similar promotion of anthocyanin formation was observed in these studies, using the tissue sections cut from ethylene-treated sorghum seedlings (Table 1). Data in Table 2 agrees with the work of Stafford [9], indicating that infiltration of sorghum sections with metabolic inhibitors as the tissue is placed in the light can effectively inhibit biosynthesis of anthocyanin in the tissue. Ethylene treatment of sorghum tissue for 24 hr prior to infiltration with the inhibitors did not appear to prevent a decrease in anthocyanin biosynthesis. These results are consistent with the idea that tissue treated with ethylene in the dark does not produce all the systems necessary for light-induced anthocyanin biosynthesis. The failure of chloramphenicol to inhibit anthocyanin biosynthesis in these studies cannot be explained at this time, but may be due to differences in infiltration or timing as compared with other work using chloramphenicol as an inhibitor of anthocyanin biosynthesis [9].

Table 1. The effect of ethylene and water infiltration on anthocyanin accumulation in sorghum tissue sections

	Anthocyanin content (A at 525 nm)	
	Control	Ethylene-treated
Control	0·537 ± 0·034	0·938 ± 0·105
Water-infiltrated	0·325 ± 0·018	0·403 ± 0·048

Sections were exposed to light for 24 hr. Means ± s.e.

In addition to cyanidin, sorghum seedlings produce two 3-deoxyanthocyanidins for which neither light nor light-induced RNA synthesis is necessary [9, 10]. Ethylene treatment in itself did not initiate the biosynthesis of these two pigments in either dark- or light-treated tissue (Tables 3 and

Table 2. Metabolic inhibition of anthocyanin synthesis in sorghum tissue sections

Inhibitor	Anthocyanin content (A at 525 nm) Control	Ethylene-treated
Chloramphenicol	0·418 ± 0·037	0·685 ± 0·114
Ethionine	0·262 ± 0·042	0·538 ± 0·097
Puromycin	0·219 ± 0·029	0·412 ± 0·055
Actinomycin D	0·078 ± 0·029	0·146 ± 0·014
Cycloheximide	0·024 ± 0·019	0·041 ± 0·034
H_2O control	0·443 ± 0·099	0·650 ± 0·104

Metabolic inhibitors were vacuum infiltrated into sections from ethylene-treated or control plants and the sections then exposed to light for 24 hr. Means ± s.e.

4). However, there were indications that once biosynthesis was initiated, ethylene had an effect on 3-deoxyanthocyanidin biosynthesis. In cycloheximide and actinomycin D-treated tissue (Tables 3 and 4) the biosynthesis of luteolinidin and apigeninidin was increased in ethylene-treated tissue. Their biosynthesis in tissue treated with actinomycin D is probably due to an inhibition of RNA synthesis for other plant processes, thus causing a build-up of their substrates. Ethylene appears to enhance their biosynthesis in tissue treated with actinomycin D in the dark (Table 4). The ethylene-enhancement of biosynthesis is slight for both 3-deoxyanthocyanidins in the dark-incubated tissue and for apigeninidin in light-treated tissue, but has a large effect on the biosynthesis of luteolinidin in light-treated tissue. The notable ethylene enhancement of luteolinidin biosynthesis in light-treated tissue agrees with previous work indicating that ethylene promotes the light-reactions of normal anthocyanin biosynthesis [11, 12]. Since RNA synthesis is not necessary for the production of luteolinidin and apigeninidin [9], the ethylene promotion of these two anthocyanins is obviously due to its effect on some other metabolic process.

Table 3. Anthocyanins in sorghum tissue sections treated with metabolic inhibitors

Inhibitor	Anthocyanin Cyanidin	Luteolinidin	Apigeninidin
Chloramphenicol	+	0	0
$+C_2H_4$	+	0	0
Ethionine	+	0	0
$+C_2H_4$	+	0	0
Puromycin	+	+	0
$+C_2H_4$	+	+	0
Actinomycin D	+	+	0
$+C_2H_4$	+	+	+
Cycloheximide	0	0	0
$+C_2H_4$	tr	tr	tr
H_2O Control	+	0	0
$+C_2H_4$	+	0	0

Metabolic inhibitors were vacuum infiltrated into sections cut from the control or ethylene-treated plants and placed in light for 24 hr. Extracts were chromatographed for detection of different anthocyanins. Scale: +, positive; tr, trace; 0, no indication. Data from 3 chromatographs.

Table 4. Production of anthocyanins as influenced by actinomycin D and ethylene

Treatment	Anthocyanin Cyanidin (A at 525)	Luteolinidin (A at 495)	Apigeninidin (A at 480)
Control, dark	0	0	0
$+C_2H_4$	0	0	0
Actinomycin D, dark	0	0·060	0·130
$+C_2H_4$	0	0·082	0·167
Actinomycin D, dark, light	0	0·310	0·760
$+C_2H_4$	0	0·720	0·800
Actinomycin D, light	0·085	0·075	0
$+C_2H_4$	0·083	0·165	0
Control, light	0·100	0	0
$+C_2H_4$	0·395	0	0

Sections of tissue were treated with ethylene, actinomycin D and light as indicated. Treatments labeled actinomycin D and dark were treated and left in the dark for 24 hr before harvest or light-treatment. The treatment labeled actinomycin D and light was treated as the tissue was placed in the light. Extracts of the tissue were chromatographed; the anthocyanins were identified, removed and their absorbance measured. Means of three samples from three different trials.

Delaying treatment with actinomycin D until the tissue is placed in the light allowed the biosynthesis of cyanidin, probably due to incomplete inhibition of light-induced RNA formation. Ethylene treatment did not increase the amount of cyanidin synthesized under these conditions. These results indicate that ethylene neither increased the rate of RNA synthesis before actinomycin D-treatment nor enabled the plant tissue to utilize the synthesized RNA more efficiently for cyanidin production.

Inhibition of *de novo* light-induced enzyme synthesis with cycloheximide at different times after the tissue is placed in the light was accompanied by changes in the amount of anthocyanin accumulated (Table 5).

Table 5. Inhibition of anthocyanin synthesis with cycloheximide

Time of treatment (hr)	Anthocyanin content ($A_{525} \times 10^{-3}$) Control	C_2H_4 treated
0	55	75
2	80	105
4	80	155
6	115	145
8	130	240
24	325	405

Sections of control or ethylene-treated tissue were infiltrated with cycloheximide at times indicated and the anthocyanin content of tissue measured 24 hr after they were placed in the light. Tissue placed in light at 0 hr.

Delaying infiltration of the cycloheximide apparently allowed formation of the enzymes necessary for biosynthesis of light-induced anthocyanin, resulting in anthocyanin being synthesized during the 24 hr light treatment. Treatment of the tissue with ethylene in the dark for 24 hr prior to the tissue being placed in the light reduced the ability of cycloheximide to inhibit anthocyanin biosynthesis. These results indicate that ethylene treatment increased the rate of light-induced enzyme synthesis as compared with controls and that the increased enzyme synthesis increased the rate of light-induced anthocyanin formation within the tissue.

EXPERIMENTAL

Plant material. Dark grown *Sorghum vulgare*, c.v. DeKalb E57, seedlings were used in these studies. Seeds were surface sterilized with 0·5% sodium hypochlorite, rinsed with distilled water, and planted on 10 ml of sterile agar in 125 ml flasks as previously described [6, 12]. The flasks were wrapped with aluminum foil to exclude light, stoppered with cotton plugs to prevent airborne contamination, and placed in a dark incubator at 28 ± 1° for germination and growth in the dark for 4 days.

Experimental procedure. Those flasks containing seedlings for treatment with ethylene were sealed with rubber vaccine caps 4 days after planting. Ethylene was injected into the gaseous atmosphere around the seedling tissue with a hypodermic needle and syringe to a concentration of 10 μl/l and the flasks containing the seedlings were then returned to the dark incubator for 24 hr. Cotton plugs were left in the control flasks to provide for air exchange. In all cases, ethylene treatment was for 24 hr in the dark with the ethylene removed from the flasks by flushing with compressed air for 1 min as they were placed in the light. All light treatments were by cool white fluorescent tubes at 10^4 ergs cm^{-2} sec^{-1}. Temperature was maintained at 28 ± 2 during the light treatment. For treatment with metabolic inhibitors, each sample consisted of 10 mesocotyl tissue sections (10 mm in length) cut under a green safe-light immediately proximal to the first node of the seedlings. The sections of tissue were placed in a small rubber-stoppered vial containing the inhibitor and a vacuum pulled with a syringe or water aspirator [9]. Molar concentrations of inhibitors were: chloramphenicol, 5×10^{-3}; ethionine, 5×10^{-3}; puromycin, 5×10^{-4}, cycloheximide, 1×10^{-4}, actinomycin D, 5×10^{-5}. Water was vacuum infiltrated into control plant tissue. Infiltrated sections of tissue were placed on agar (1·5%) in covered Petri plates and incubated in light or dark. Anthocyanin content was measured in a 4-ml extract (MeOH–HCl, 99:1) of 10 sections. For chromatography, the extract was evaporated at room temp to *ca* 0·2 ml and spotted on a 19·5 × 19·5 cm sheet of Whatman No. 1 chromatograph paper, and run by ascent first in HOAc–H_2O–HCl (10:87:3) and then in *n*-BuOH–H_2O–HOAc–HCl (60:20:10:1) [9]. The three anthocyanins were identified by R_f and color comparison with previously reported data [9, 11]. Luteolinidin and apigeninidin were present in the free state; the acylated and deacylated cyanidin glycosides which separated chromatographically were combined for all measurements.

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