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Modes of Ethylene Action in the Release of Amylase from Barley Aleurone Layers[1]

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ABSTRACT

The development of xylanase activity by isolated barley (*Hordeum vulgare* cv. Himalaya) aleurone layers exposed to gibberellic acid was enhanced by ethylene, whereas the rate of glucanase synthesis was unaffected by ethylene. The elevated xylanase activity expressed in ethylene-treated tissue may be responsible for enhanced release of amylase in response to ethylene.

The redistribution of ionic species in response to gibberellic acid and ethylene was explored. The release of calcium was much more sensitive to ethylene than the release of any of the other ions investigated (potassium, magnesium, and phosphate). Ethylene induced a 101% increase in the release of calcium from gibberellic acid-treated aleurone layers. The possible relevance of this observation to the ethylene-enhanced release of amylase is discussed.

The aleurone cells of barley secrete substantial quantities of protein in response to GA_3 (12, 19). However, the massive cell walls of the aleurone layer pose a formidable barrier to the release of protein into the surrounding medium. It has been demonstrated that the passage of amylase through the cell wall material is diffusion-limited (27).

Extensive degradation of barley aleurone cell walls in response to GA_3 was detected by light microscopy (1) and scanning electron microscopy (20). Moreover, it has been shown that the diffusion of acid phosphatase activity from the aleurone cells, through cell walls, occurs in channels that have been depleted of carbohydrate (1). The carbohydrate composition of the barley aleurone cell wall material has since been established (18). The most prominent carbohydrates of the cell wall were arabinoxylans; β-1, 4-xylosyl linkages formed the basis of these polymers (18). Cellulose constitutes a much smaller fraction of the cell wall material. Xylanases, which hydrolyze the xylosyl linkages of arabinoxylans (5, 24), and endo-β-(1,3:1,4)-glucanase, which can hydrolyze the glucosyl linkages of cellulose (17) are found in germinating barley. The production and release of these enzymes, in response to GA_3, may fulfill an important role in the release of proteins from aleurone layers.

The availability of inorganic ions also may serve a regulatory function during the GA_3 induction of enzyme release from aleurone layers (4, 27). It was found that salts of Ca, Mg, and K were required for the release of amylase from isolated aleurone layers (27). The aleurone layer itself was the major source of inorganic ions in cereals (11, 16), and the release of large quantities of ions from isolated aleurone layers was dependent on the application of GA_3 (15).

We have studied the effect of exogenous ethylene on the cell wall-degrading enzyme systems of isolated barley aleurone layers and on the release of inorganic ions. These results are related to the stimulation, by ethylene, of the GA_3-enhanced release of amylase (8).

MATERIALS AND METHODS

Preparation of Samples. Seeds of barley (*Hordeum vulgare* cv. Himalaya, 1974 harvest) were used. The preparation of isolated aleurone layers and incubation conditions have been described in detail (8). Ten aleurone layers were incubated at 25°C in sterile culture tubes with 2 ml incubation medium (10 μM GA_3 [Grade III, Sigma Chemical Co.], 20 μM $CaCl_2$, 20 mM succinic acid, adjusted with NaOH to pH 5.5 at 25°C). The preparation of samples for the determination of xylanase and glucanase was performed at 0 to 4°C. The media and extracts from layers were first prepared in a final volume of 5 ml (8). Three volumes cold 3.9 M ammonium sulfate (special enzyme grade, Serva Feinbiochemica) were added and, after 30 min, the precipitate was collected by centrifugation at 15,300*g* for 30 min. The precipitates were each resuspended in 1 ml potassium acetate buffer (20 mM K-acetate, 20 mM $CaCl_2$, adjusted to pH 5.5 at 25°C with HCl), and dialyzed overnight against the same buffer. The samples were further diluted with the same buffer for the individual enzyme assays.

For the determination of the release of inorganic ions from aleurone layers, the $CaCl_2$ normally present in the incubation medium was omitted. After incubation for 24 h at 25°C in either hydrocarbon-free air or 12.4 nl/ml ethylene in air, the layers were rinsed twice with deionized glass-distilled water. The combined rinse and incubation solutions were cleared by centrifugation at 980*g* for 20 min. The supernatant layer was reserved and the pellet was resuspended in water and centrifuged. The supernatant layer from the rinse was combined with the first supernatant layer, and the solution was brought to 50 ml. The samples were further diluted for the analysis of each ion, as required. Control samples were prepared by incubating isolated aleurone layers in the absence of GA_3, and the background was determined by preparing samples without aleurone layers. All glassware was rinsed with HCl or HNO_3 to reduce contamination by inorganic ions. The culture tubes were further rinsed thoroughly with deionized, glass-distilled water.

Determination of Ion Release. Calcium, magnesium, potassium, and sodium were quantified by a Varian AA-475 atomic absorption spectrophotometer. Inorganic phosphate was determined spectrophotometrically at 660 nm by the method of Bartlett (2).

Enzyme Assays. The assay for glucanase activity was performed essentially as described by Taiz and Jones (25). The 0.1% (w/v)

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laminarin (purum grade, Fluka AG) solution was prepared in the K-acetate buffer. The assay was performed at 30°C and the incubation time with enzyme was extended to 60 min. After the reaction with the arsenomolybdate reagent was completed, samples were diluted with 5 ml water and the optical density of the sample was measured at 520 nm (Cary 219 spectrophotometer, Varian) against a zero time reference.

Xylanase activity was determined by the procedure described by Taiz and Honigman (24). Larchwood xylan (Sigma Chemical Co.) was purified by the classical method of alkaline copper precipitation (13). This procedure removed contaminating hexoses (7). The commercial xylan was regarded as the equivalent of the crude xylan obtained from the first precipitation step. The remainder of the purification followed the published procedure. The purified xylan was dissolved in the K-acetate buffer. The assay was performed at 30°C for 60 min. The release of reducing terminals was determined as described above.

The amylase assay has been described (8).

One enzyme unit of glucanase, xylanase, or amylase activity was defined as the amount of enzyme required to expose 1 μeq reducing terminals per min at 30°C.

Partial Purification of Xylanase. Methods for the purification of xylanase from various sources have been summarized (6), and on the basis of these procedures, the following protocol was developed to obtain a xylanase preparation free from amylase activity. A crude preparation was obtained by incubating 10 aleurone layers in each of 10 small culture flasks containing 2 ml

Table I. *Partial Purification of Xylanase from Isolated Barley Aleurone Layers*

The details of the purification procedure are given in the text.

	Volume	Total Enzyme Activity			
		Amylase	Glucanase	Xylanase	
	ml		*units*		*units/mg protein*
Initial Solution	16	191	11.2	0.573	0.13
Ammonium Sulfate ppt	2	0		0.079	
Final Product	1	0	0.1	0.071	0.39

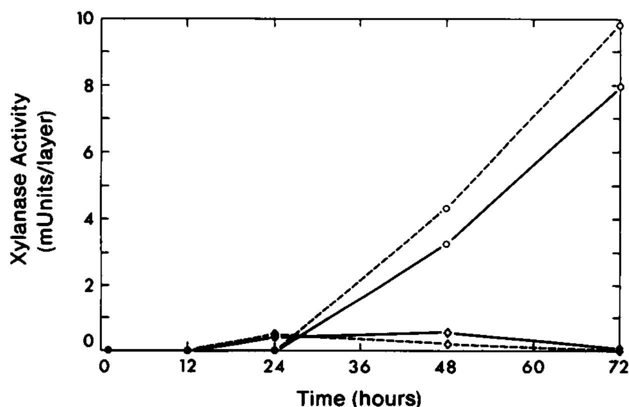

FIG. 1. Effect of ethylene on the time course of the GA_3-enhanced synthesis and release of xylanase by isolated barley aleurone layers. Enzyme activity was determined in the medium (●) and the extract (◆) after layers had incubated in a 10 μM GA_3 medium at 25°C for the indicated time. Each point represents the average of four samples that were either incubated in hydrocarbon-free air (——) or in air containing 30 nl/ml ethylene (- - -). Hollow symbols indicate points where significant differences (at 2.5% level) exist between data from control and ethylene-treated samples.

Table II. *Effect of Ethylene on Amylase Synthesis and Release in Presence and Absence of Exogenously Applied Xylanase*

Ten aleurone layers were incubated for 24 h as described under "Materials and Methods". Partially purified xylanase was added where indicated. Samples were equilibrated with either hydrocarbon-free air or 12.4 nl/ml ethylene. Values are the mean of four replicates.

Treatment	Xylanase Added	Amylase Produced	
		Extract	Medium
	milliunits/layer	*units/layer*	
Control	0	0.48	1.43[a]
Ethylene	0	0.43	1.69[a]
Control	1.1	0.51	1.03
Ethylene	1.1	0.46	0.91

[a] Difference between control and ethylene treatment is significant at the 5% level.

incubation medium. Ten layers were incubated for 96 h at 25°C on a gyratory shaker. The medium was collected and centrifuged at 980*g* for 20 min. The supernatant layers were combined, and 0.1 M HCl was added dropwise to the solution, with constant stirring, to reduce the pH to 3.0. Stirring was continued for 20 min at room temperature. The pH was returned to 5.0 by the addition of 0.1 M sodium hydroxide. Although the low pH treatment reduced the over-all yield of xylanase from 90% to 12%, it effectively eliminated amylase activity in the sample.

All subsequent steps were performed at 0 to 4°C. One-quarter volume cold 3.9 M ammonium sulfate was added to the sample and, after 30 min, the precipitate was removed by centrifugation for 30 min at 15,300*g*. Another 3.75 volumes 3.9 M ammonium sulfate were added to the supernatant and the suspension was centrifuged as above. The pellet thus obtained was resuspended in 2 ml 1 mM sodium phosphate buffer (pH 6.2) and dialyzed overnight against the same buffer. The sample was applied to a column (15 × 0.9 cm i.d.) packed with hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) that had been equilibrated with 1 mM sodium phosphate buffer (pH 6.2). The sample was washed into the column with 2 bed volumes 1 mM sodium phosphate buffer (pH 6.2) followed by 2 bed volumes 40 mM sodium phosphate buffer (pH 6.2). The xylanase was eluted from the column with 160 mM sodium phosphate buffer (pH 6.2). After the void volume of elution buffer had passed through the column, the next 2 bed volumes were collected. Xylanase was precipitated from the column effluent by the addition of 4 volumes cold 3.9 M ammonium sulfate and the precipitate was collected as previously described, and then resuspended in 20 mM succinic acid, 20 mM $CaCl_2$, adjusted to pH 5.5 at 25°C with NaOH. The suspension was dialyzed overnight against the same buffer. The results of the purification procedure are given in Table I.

To differentiate between endo- and exo-xylanases, the products of xylan hydrolysis by the enzyme preparation were evaluated by paper chromatography. Results of this evaluation (7) indicated that endoxylanase was the prevalent enzyme in this preparation although free xylose did accumulate after prolonged incubation periods. These results are similar to the observations of Dashek and Chrispeels (5).

Scanning Electron Microscopy. Isolated barley aleurone layers were dehydrated, at 0°C, through a series of ethanol solutions (6.7%, 13.6%, and 20.0%, v/v). After 2 h in 20.0% ethanol, the aleurone layers were removed and frozen in liquid freon, fractured, then freeze-dried. The specimens, mounted on aluminum studs, were coated with a gold layer (150–200 Å), and examined with a Cambridge Stereoscan 150 scanning electron microscope operated at 20 kv. At least four specimens from each set of treatments were examined.

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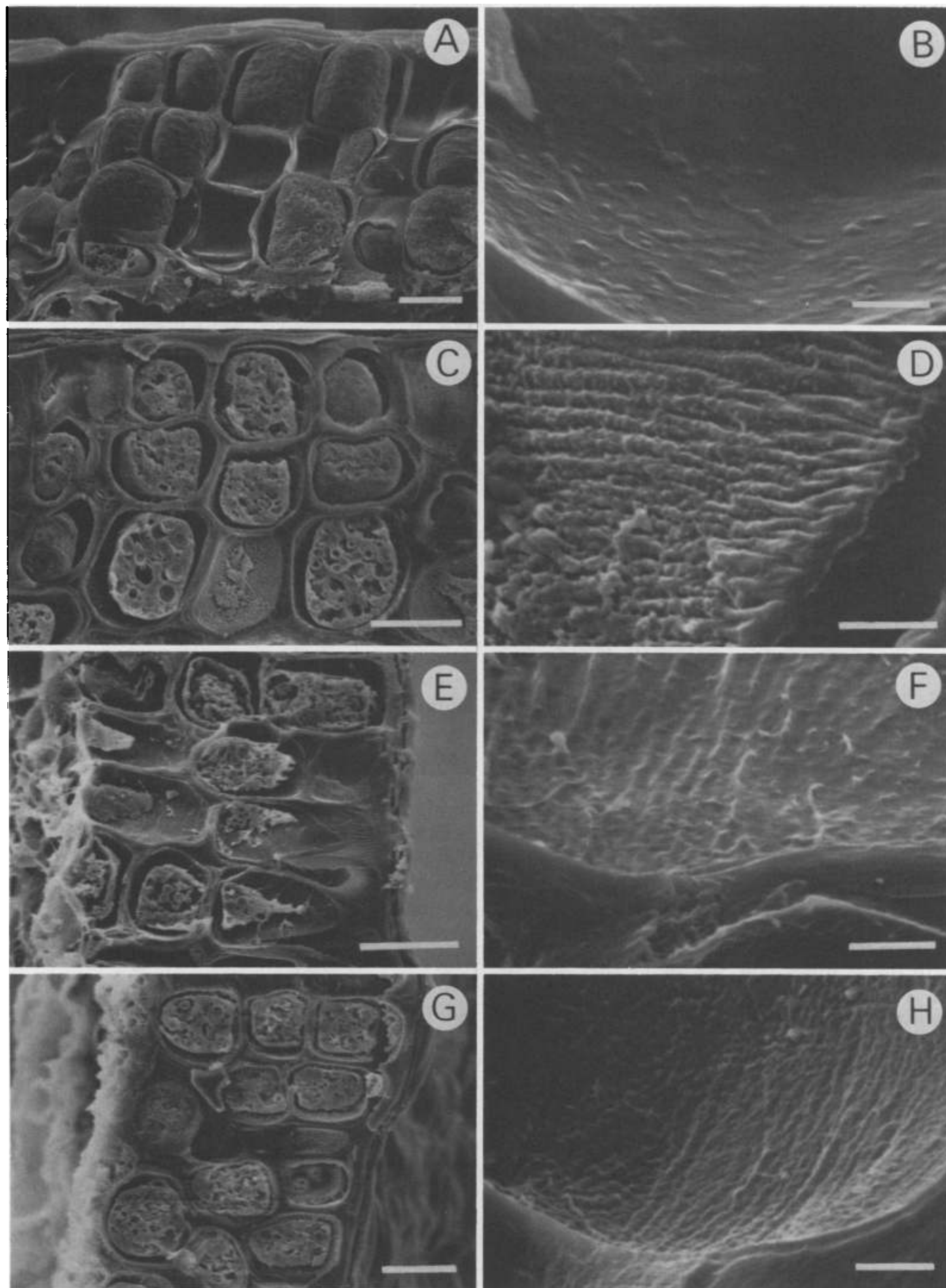

FIG. 2. Scanning electron micrographs of isolated barley aleurone layers. Layers were incubated in incubation buffer for 24 h at 25°C, and the medium was equilibrated with hydrocarbon-free air except as noted. A, B, no GA_3; C, D, 10 μM GA_3; E, F, 10 μM GA_3 plus 1.1 milliunits/layer xylanase; G, H, 10 M GA_3 plus 12.4 nl/ml ethylene. The scale bars represent 20 μm in micrographs A, C, E, G, and 2 μm in micrographs B, D, F, H.

RESULTS AND DISCUSSION

Inasmuch as cell wall-degrading enzymes have been implicated in the promotion of protein release from barley aleurone layers, the effect of ethylene on the synthesis and release of these enzymes was examined. Progressive increases in the activity and release of xylanase by aleurone layers in response to GA_3 were observed (Fig. 1). Only trace quantities of xylanase were released into the medium during the first 24 h, although activity had accumulated within the layers. After the same time interval, 49% of the ultimate amylase activity had been produced (8). This contradicts previous reports that xylanase release closely parallels the release of amylase (24). It was also reported (5, 24) that the accumulation of xylanase activity ceased after 36 h. In the present study, xylanase activity continued to increase throughout the duration of the experiment, up to 72 h. This disparity may be a consequence of the improvements in the incubation procedure used in the current investigation. The flowthrough air supply would prevent the accumulation of CO_2 and other volatiles and the concurrent depletion of oxygen during the incubation period.

Ethylene caused a significant increase in the accumulation of xylanase within the tissue during the first 24 h of the response to GA_3 (2.5% level of significance, t test). These small differences in xylanase activity elicited by ethylene at 24 h may be critical as,

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Table III. *Effects of GA_3 and Ethylene on Release and Adsorption of Inorganic Ions by Isolated Barley Aleurone Layers*

Ten aleurone layers were incubated in 2 ml 20 mM sodium succinate adjusted to pH 5.5 at 25°C with HCl. The layers were exposed to 13.1 nl/ml ethylene and/or 10 μM GA_3 for 24 h at 25°C. The amounts of the individual ions released were determined by atomic absorption analysis with the exception of phosphate, which was determined spectrophotometrically. Each value is the mean of four samples.

Treatment	Change in Ion Concn. of Medium				
	Na^+	K^+	Ca^{2+}	Mg^{2+}	PO_4^{3-}
	μmol/layer				
Control	−0.26[a]	0.53	0.020	0.028	0.11
+ GA_3	−1.94[a]	1.16	0.081[b]	0.624	1.14[b]
+ GA_3 + C_2H_4	−2.10[a]	1.26	0.163[b]	0.632	0.98[b]

[a] A negative value indicates adsorption from the medium. The incubation medium initially contained 40 mM sodium or 8.00 μmol/layer.

[b] The difference between +GA_3 and + GA_3 + C_2H_4 is significant at the 2.5% level.

during these initial hours, the enzyme is confined within the small volume of the aleurone layers (9 μl/layer), in close proximity to the substrate. These data do not demonstrate, however, whether the arabinoxylans of the cell wall were accessible to xylanase retained by the aleurone layers at this time. Samples of media assayed for xylanase 48 h and 72 h after exposure to GA_3 showed that ethylene promoted 32% and 23% increases in activity, respectively. This was concomitant with only a small reduction of xylanase activity in the extract. Thus, a net increase in total xylanase activity was evident in response to 30 nl/ml ethylene, even after prolonged incubation. In contrast, the stimulatory effect of ethylene on amylase synthesis was maximal at 24 h, and ethylene treatment led to reduced amylase activity at 48 h and 72 h (8). This reduction in amylase activity after longer incubation periods may reflect the release of inhibitory or inactivating substances (manuscript in preparation).

Possible changes in the synthesis and release of glucanase in response to ethylene were also examined. Thirty nl/ml ethylene had no significant effect on the synthesis or release of glucanase by isolated aleurone layers. These results indicate that the increased production of enzyme activity induced by ethylene was a selective event, as the syntheses of all enzymes were not escalated by the ethylene treatment.

Since the production of xylanase activity has been implicated in the response of aleurone layers to ethylene, its role was examined further. The exogenous application of xylanase to the aleurone layers should reduce the restriction of amylase release imposed by the cell walls and, hence, mimic the action of ethylene. Table II shows that, when GA_3 and exogenous xylanase were applied simultaneously, ethylene had no significant effect on amylase synthesis and release. However, the addition of the partially purified xylanase to the medium inhibited the amount of amylase synthesized in 24 h. In an attempt to reduce this inhibition of amylase synthesis, a parallel series of experiments was conducted in which the isolated aleurone layers were first incubated with xylanase before exposure to GA_3. After 36 h, the layers were washed thoroughly with buffer for 2 h, then transferred to incubation medium containing GA_3 for an additional 24 h. This treatment resulted in a reduction in total amylase activity recovered from treated samples to 0.42 unit/layer, compared to 1.43 units/layer from samples that were not treated with exogenous xylanase. If a similar preincubation was performed in buffer alone, however, the effect on amylase synthesis was minimal, and 1.16 units/layer were recovered. The latter observation was anticipated as it has been found that the length of the incubation period for half-seeds had no effect on subsequent amylase synthesis or release (26). The addition of an osmoticant (0.2 M sorbitol) could not reduce the inhibition of amylase synthesis after incubation with xylanase. Prolonging the dialysis of the xylanase preparation and introducing several buffer changes during dialysis to reduce low molecular contaminants also failed to prevent the inhibition of amylase synthesis by incubation with the xylanase preparation.

The extent of cell wall modification that occurred in aleurone layers exposed to GA_3 alone, or in combination with exogenous xylanase or ethylene, was examined directly by scanning electron microscopy. In the absence of any of these additions, the cell wall appeared very smooth (Fig. 2 A and B). This was consistent with the observations made in a previous study (21). After exposure to GA_3, however, the cell wall appeared deeply furrowed (Fig. 2 C and D), suggesting dissolution of the cell wall material. The exposure of the tissue to either ethylene or exogenous xylanase in combination with GA_3 had no visible effect on apparent cell wall structure beyond the changes induced by GA_3 alone (Fig. 2, E, F, G, H).

The effect of GA_3 and ethylene on ion movement was also studied. The flux of all five ionic species was dependent on the presence of GA_3 in the incubation medium (Table III). However, the change in the distribution of calcium induced by ethylene was much more pronounced than the minor changes in the flux of the other ions. In the presence of GA_3, 13.1 nl/ml ethylene resulted in a 101% increase in the amount of calcium released into the medium compared to layers incubated in hydrocarbon-free air.

The influence of plant growth regulators on calcium, magnesium, and potassium is of particular importance. Calcium has been assigned a central role in the accumulation of amylase in barley malt. It has been recognized that in addition to conferring stability to barley α-amylase (10), calcium also enables the synthesis and release of hydrolytic enzymes (4, 27). Magnesium and potassium are also active in the release of amylase (27). Therefore, the ability of ethylene to promote the redistribution of any of the cations, as it markedly does for calcium, may be important in regulating the processes of enzyme synthesis and release.

Although the consequence of the release of calcium induced by ethylene may be prominent during the development of amylase activity within intact seedlings, it may be of less significance in the current study. In the presence of GA_3 and ethylene, 0.163 μmol calcium/layer was released, whereas, under routine experimental conditions, 4.0 μmol calcium/layer was present in the incubation buffer. This relatively high concentration may overshadow any effect that the release of calcium may have on the synthesis and release of amylase. However, if the enhanced release of calcium reflects an increased availability of soluble calcium within the cells, other biochemical or physiological conditions may be altered. These include membrane structure and integrity, flux of other ionic species, and the activity of enzymes (for a summary, see 22).

The primary source of calcium is from the hydrolysis of phytin by phytase (23). It is conceivable that ethylene promotes the release of soluble calcium by stimulating the development of phytase activity in the aleurone layers.

In summary, the data accumulated from enzyme analyses support the hypothesis that ethylene promotes the release of amylase from isolated aleurone layers by enhancing the production of the cell wall-degrading enzyme, xylanase. Although ethylene caused an increase in amylase and xylanase production, a general increase in the synthetic capacity of aleurone layers, with respect to the production of hydrolases, was not indicated. Glucanase activity was unaltered by exposing tissue to ethylene, and other workers have found that acid phosphatase also was not affected by ethylene (Ho, Abroms, Varner, personal communication). It should be noted that the synthesis of xylanase and amylase is much more dependent on GA_3 than the synthesis of acid phosphate or glucanase. The constitutive levels of glucanase (3, 14, 25) and acid

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phosphatase (Ho, Abroms, Varner, personal communication) are high, whereas very little amylase or xylanase are synthesized in the absence of GA_3. Of these four enzyme systems, ethylene affects the production of only those enzymes that require GA_3 for their synthesis. This implies that ethylene may interact directly at the primary site of GA_3 action.

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