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Abscisic Acid Is an Endogenous Inhibitor in the Regulation of Mannanase Production by Isolated Lettuce (*Lactuca sativa* cv Grand Rapids) Endosperms[1]

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ABSTRACT

The production of mannanase, a cell-wall-degrading carbohydrase, can be manipulated in isolated lettuce (*Lactuca sativa* cv Grand Rapids) endosperms by changes in the volume of buffer in which they are incubated. The enzyme is produced when endosperms are incubated in a large volume, but not when incubated in a small volume, which is suggestive that an endogenous, diffusible inhibitor of mannanase production is being lost from the endosperms in a large volume (JD Bewley, P Halmer 1980/1981 Israel J Bot 29: 118–132). We have investigated the possibility that the phytohormone abscisic acid (ABA) is involved in this regulation of mannanase production in isolated lettuce endosperms. We find several correlations between the presence of the endogenous inhibitor and of ABA, *i.e.* (a) a 'leachate' prepared from isolated lettuce endosperms induces synthesis of ABA-specific proteins in barley aleurone layers, indicating that incubation of endosperms in a large volume results in the diffusion of ABA therefrom into the surrounding medium; (b) fractionation of the components of a leachate by either polyvinylpyrrolidone-chromatography of C_{18} reversed-phase high performance liquid chromatography fails to separate the endogenous inhibitor from authentic ABA; and (c) changes in the incubation volume of endosperms result in changes in the amount of extractable ABA in the endosperms, as detected by ELISA. These results are consistent with a role for endogenous ABA in the regulation of mannanase production in isolated lettuce endosperms.

Endo-(1,4)-β-mannanase (EC 3.2.2.1) is produced in endosperms of lettuce achenes, wherein it acts to break down the mannose-based carbohydrates of the cell walls early after germination of the intact seed. The production of mannanase is regulated by a complex series of embryo-directed interactions, in which the red-light stimulus that breaks dormancy is transduced by the lettuce embryo into at least two promotive compounds, one of which acts on the endosperm to overcome an endogenous, diffusible inhibitor (5). Although the nature of neither the promotive compounds nor the endogenous inhibitor has been determined, a number of observations are suggestive that the endogenous inhibitor is ABA or ABA-like. Endosperms isolated from intact seeds will produce quantities of mannanase similar to those found in intact, germinated seeds, but only under appropriate incubation conditions. Specifically, mannanase levels increase when endosperms are incubated in a relatively LV[4] of buffer; enzyme production does not occur if endosperms are incubated in a volume of buffer that is just sufficient to keep the endosperms moist throughout incubation (*i.e.* SV). Mannanase can be produced in endosperms incubated in a SV, however, if the endosperms are first rinsed for 2 h in a LV prior to their transfer into the SV, thus indicating that incubation in a LV results in the loss of an inhibitor to enzyme production. Inclusion of ABA inhibits the production of mannanase by endosperms incubated in a LV of buffer (LV + ABA), and this inhibition similarly can be removed by rinsing. Phytohormones that may act as agonists of ABA action, *viz.* GA and cytokinin (specifically benzyl-amino-purine or BA), do not induce mannanase activity in isolated endosperms in a SV or in a LV + ABA when they are applied directly (1).

Based on these observations, it seems reasonable to expect that at least a component of the endogenous inhibitor that is leached from isolated endosperms in a LV is ABA. We have addressed this possibility by testing three requirements necessary to support such a conclusion: (a) that appreciable quantities of ABA be leached from endosperms incubated in a LV, (b) that ABA and the effective endogenous inhibitor co-elute when an endosperm leachate is subjected to chromatographic separation, and (c) that the changes in incubation volume that mediate enzyme production also cause changes in the content of endogenous ABA in isolated endosperms.

MATERIALS AND METHODS

Plant Material. Lettuce achenes (*Lactuca sativa* cv Grand Rapids) were obtained from the Ferry Morse Seed Co. (Mountain View, CA, 1982 harvest) and stored at 4°C until use. Endosperms were isolated under dim green light as previously described (5). Conditions of incubation typically were: (a) LV, 1 ml 10 mM Mes (pH 5.0) (Sigma, St. Louis, Mo.) or sterile water per 20 endosperms or 3 ml per 50; (b) SV, 20 μl buffer or water per 20 endosperms or 50 μl per 50; (c) LV + ABA, as described for LV but with addition of 10^{-6} M ± *cis,trans* abscisic acid (Sigma). All buffers were filter-sterilized prior to use. LV and LV + ABA samples were incubated in sterile 35-mm plastic Petri dishes (Fisher Scientific, Edmonton, Alta); SV samples were incubated in glass spot-plates that were surface-sterilized with ethanol just prior to use. Incubation was in darkness in a sealable plastic container lined with moistened paper toweling at 25°C.

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[4] Abbreviations: LV, large volume; SV, small volume; ELISA, enzyme-linked immunosorbent assay.

Mannanase Activity Determinations. Endosperms were extracted in 1 ml of ice-cold 0.1 M Tris-glycine (pH 7.8) in a Kontes-Duall (Vineland, NJ) ground-glass homogenizer (size 20) on ice, and the extracts were clarified by low-speed centrifugation. Each extract was combined with the medium in which the endosperms had been incubated. Mannanase activity was determined by one of two methods. (a) When accurate determination of enzyme activity was required, activity was measured as the rate of decrease in viscosity of a solution of galactomannan. Galactomannan was purified from commercially available locust bean flour (Sigma) essentially as described by McCleary (11). Mannanase activity was measured as previously described (5). Samples were assayed at least in duplicate. A unit of enzyme activity was defined as a decrease in initial flow-time of 1% h^{-1}. Initial flow times were calculated by extrapolation of the mean change in flow time measured at each 6 min interval and were usually 300 to 360 s. (b) Where a quick determination of the presence or absence of mannanase was desired, enzyme activity was detected by a 'microassay' measurement of viscosity changes devised by P. Halmer (personal communication). The assay buffer was 0.4% galactomannan in one-fifth strength McIlvaine's buffer (pH 5.0) to which was added 0.1 ml of sample. The reaction was allowed to proceed for 15 min at room temperature, then was stopped by addition of 0.4 ml of 75 mM Na_2CO_3. A 'microviscometer' was assembled by inserting a 5 μl capillary pipette into the end of a 1.0 ml tip for an Eppendorf pipettor (Fisher Scientific). This tip acted as a funnel in which was placed 200 μl of the assay solution. Changes in viscosity were determined by timing the descent of this solution through markings of 1 μl on the capillary pipette and comparing this time with that for a solution in which the endosperm extract was boiled prior to addition to the assay buffer.

Barley Aleurone Layer Bioassay. Barley grains (*Hordeum vulgare* cv Betze's) were deembryonated and incubated as described by Chrispeels and Varner (2). Ten aleurone layers were incubated in 2 ml of appropriately supplemented buffer for 16 h at 30°C with constant stirring in 35-mm sterile plastic Petri dishes. The buffer was 20 mM sodium succinate (pH 5.0) with 10 mM $CaCl_2$. When appropriate, the buffer was made to either 10^{-7} M, 10^{-6} M, or 10^{-5} M ± *cis,trans*-ABA (Sigma). Alternatively, 2 ml of buffer were used to redissolve the residue from a lyophilized sample of medium in which 100 or 200 isolated lettuce endosperms had been incubated. The endosperms were isolated as described above, then incubated in 3.5 ml sterile water for 12 h. The incubation medium was collected, passed through a 5 μm syringe filter (Schleicher and Schull, Keene, NH), frozen, and lyophilized to dryness. This residue was taken up in a minimum volume of 1 M NaOH, then made up to 2 ml with the barley aleurone incubation buffer. All buffer preparations were filter-sterilized immediately prior to use.

After incubation, aleurone layers were quickly rinsed three times with 1 ml sterile water, then transferred for 1 h to fresh buffer containing 15 μCi [^{35}S]methionine (>800 Ci/mmol, NEN, Boston, MA). ABA or endosperm diffusate was included in the labeling buffer as well. Layers were then rinsed with sterile water and frozen in liquid N_2, and constituent proteins were extracted and prepared for SDS-PAGE (13). Separation was on a 17% polyacrylamide gel, which was then impregnated with fluor (EN^{3}Hance, NEN), dried, and exposed to preflashed x-ray film (X-Omat, Kodak).

HPLC of Endosperm Diffusate. Diffusate was prepared by incubating 300 isolated endosperms in 3.5 ml distilled, deionized H_2O for 12 h. The endosperms then were washed five times with 1 ml dH_2O, and the incubation medium and rinse water were combined, passed through a 5 μm syringe filter (Schleicher and Schull), frozen, and lyophilized to dryness. The residue from lyophilization was taken up in 300 μl 10% methanol (in 1% aqueous acetic acid) and approximately 40,000 dpm of ^{14}C-ABA (15 Ci/mmol, a gift from R. P. Pharis, Calgary) was added as an internal standard. This solution then was passed through a 0.5 μm filter (FHUP, Millipore). All manipulations with diffusate were carried out under reduced light.

HPLC was performed on a Waters Assoc. (Milford, MA) ALC/GPC R-401 liquid chromatograph with two model 6000 pumps, a model 660 solvent flow programmer, and a model U6K universal injector, Schoeffel models GM 770 and SF 770 UV monitors, and a Berthold HPLC radioactivity monitor (LB 503). A Waters Assoc. μBondapak C_{18} column of dimensions 300 × 7.9 × 3.0 mm i.d. was employed. Methanol was commercially available HPLC grade (Fisher). Both solvent (*i.e.* methanol and 1% aqueous acetic acid) were filtered through 0.5 μm FHUP Millipore filters and degassed prior to use. Chromatography was performed as described by Koshioka *et al.* (8). Two min fractions were collected. Each fraction was tested for radioactivity by scintillation counting a 100 μl aliquot in dioxane-based scintillation cocktail (Omnifluor, NEN) to confirm results from the on-line radioactivity detector. UV and/or radioactive peaks were combined, and these samples then were taken to dryness on a centrifuge-lyophilizer. Samples were stored in darkness at −70°C in the lyophilized state until required.

'Bioassay' Procedure for the Presence of the Endogenous Inhibitor. The residues from the lyophilized HPLC fractions were resuspended in 1 ml of dH_2O when required, and twofold series of dilutions of these solutions was prepared. Samples of 10 isolated endosperms were incubated in 200 μl of each fraction (or dilution thereof) for 30 h, at which time copious amounts of mannanase were produced in control samples (*i.e.* samples incubated in a LV of water). Endosperms were then extracted as previously noted, and the presence or absence of mannanase was determined in each sample by the microassay. For those fractions in which inhibition of mannanase production was retained throughout dilution, mannanase levels were quantitated by the viscometric assay of Halmer and Bewley (5).

ABA Quantitation. For quantitation of ABA in isolated endosperms, 200 endosperms were incubated in either 200 μl (a SV) or 10 ml (a LV) of sterile water for 12 h. The endosperms were then washed three times with 1 ml of sterile water and frozen in liquid N_2. The incubation medium and rinse water were combined, passed through a 5 μm syringe filter (Schleicher and Schull), and frozen. Both the medium and the endosperms were then lyophilized to dryness. When ABA levels in endosperms from intact seeds were to be measured, the seeds were imbibed in water for 2 h and then were either exposed to white light for 5 min prior to incubation at 25°C in darkness or incubated immediately. Endosperms were dissected from the seeds 28 h later and frozen and lyophilized as above.

Extraction of ABA was based on the procedure of Weiler (15). Lyophilized endosperms were ground twice in 2 ml 80% acetone containing 20 mg/L butylated hydroxy-toluene as an antioxidant. Then 10^5 cpm of ^{3}H-ABA (Amersham, Dorval, P.Q.) was added as an internal standard. The solvent was made to 10 ml, and the ground endosperms were extracted for 16 h at 4°C. The sample was then briefly centrifuged, and the pellet was reextracted with a further 10 ml of acetone solution. Samples of the incubation medium were similarly extracted but without grinding. The combined extracts from each sample (total volume approximately 20 ml) were then evaporated under air to dryness. Immediately prior to assay, samples were taken up in 600 μl of 100% methanol and made to 2 ml with dH_2O, thus giving a final concentration of 30% methanol. They were loaded onto a pre-packed C_{18} column (PrepSep, Fisher) which subsequently was washed twice with 2 ml of 30% methanol. The ABA was eluted from the column by 2 ml of 70% methanol. All manipulations were carried out under reduced light.

ABA in each sample was quantified by the ELISA kit marketed by Idetek (San Bruno, CA), as recommended by the manufacturer. This kit employs the monoclonal antibodies against cis-ABA described by Mertens *et al.* (12). Standards were included with each experiment.

RESULTS

ABA is Leached from Lettuce Endosperms Incubated in LV. The presence of endogenous ABA in the incubation medium (leachate) from isolated endosperms was demonstrated by application of a bioassay specific to ABA. Barley aleurone layers produce a group of specific proteins in response to ABA (7). Accordingly, leachates from 100 and 200 lettuce endosperms were filtered to remove particulates, lyophilized, and resuspended in buffer suitable for the incubation of barley aleurone layers (see "Materials and Methods"). Aleurone layers, isolated aseptically from ungerminated barley grains (2), were incubated in buffer only, in buffer to which known quantities of authentic ABA (Sigma) were added, or in buffer in which the lyophilized leachate from lettuce endosperm was resuspended.

Fluorographs of the aleurone layer proteins separated by SDS-PAGE reveal at least two proteins which are induced and/or enhanced by authentic ABA; they are marked by dashed lines in Figure 1 (lanes 1–4). Both of these proteins are also induced by endosperm diffusate (lanes 5 and 6), indicating that quantities of ABA sufficient to stimulate the barley aleurone layers are leached from lettuce endosperms incubated in a LV.

ABA and the Endogenous Inhibitor Coelute in Endosperm Diffusate Fractionated by Either PVP-Column Chromatography or by HPLC. Leachate from isolated endosperms was fractionated by PVP adsorption and by reversed-phase HPLC to test whether the inhibitor(s) of mannanase production could be resolved.

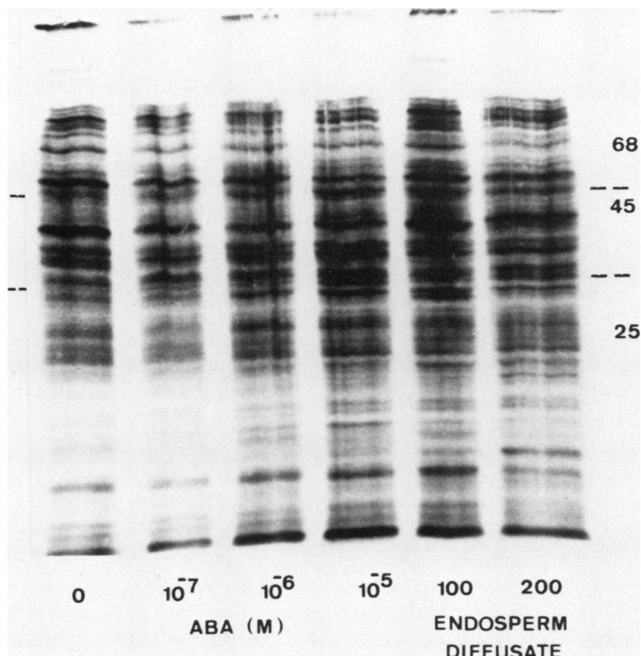

FIG. 1. Barley-aleurone-layer bioassay for the presence of ABA. Aseptically isolated aleurone layers were incubated in buffer only (lane 1), increasing concentrations of authentic ABA (lanes 2–4), or in buffer in which the residue from lyophilization of the incubation medium from 100 (lane 5) or 200 (lane 6) isolated lettuce endosperms had been resuspended. The dotted lines mark the positions of two ABA-specific proteins produced by the aleurone layers. The numbers at the right side refer to the mol wt in kD of marker proteins included on the gel.

A major component of the leachate of isolated endosperms in any volume is phenolics, evidenced by their characteristic brown coloration. Since these compounds can be inhibitory to plant cell metabolism at high concentrations (9), their contribution to inhibition of mannanase production was evaluated. Preliminary experiments in which a leachate was adsorbed with bulk PVP revealed that only approximately 20% of inhibitor activity was removed from the leachate, an indication that phenolics play only a minor role in mannanase regulation.

Further analysis of the endogenous inhibitor was obtained by dialysis, followed by chromatography of a leachate on a column of PVP. ^{14}C-±ABA (40,000 dpm of a 15 Ci/mmol sample) was included in the leachate upon its collection to estimate any loss of this compound. This was insufficient ABA to cause any detectable inhibition of mannanase production. Dialysis overnight in acidified water removed any larger components of the incubation medium (such as proteins) from the initial leachate. Greater than 99% of the ABA standard was recovered outside of the dialysis bag. The leachate components that were retained within the dialysis membrane made only a minor contribution to inhibition of mannanase production, since serial dilution of this fraction quickly overcame the observed inhibition (Table I, part A).

Leachate components that were dialyzable were collected, lyophilized, and resuspended in the original volume of the leachate from which they were obtained. This suspension was loaded onto a PVP column that was developed as described by Glenn *et al.* (4). The eluate contained approximately 80% of the ^{14}C-ABA initially added. The highest dilution of the eluate tested still inhibited enzyme activity by greater than 75% (Table I, part B). Hence, the inhibitor was inseparable from ABA by either bulk adsorption with PVP or by PVP column-chromatography.

Further resolution of the components of the incubation medium was achieved by reversed-phase HPLC of the medium by the methods described by Koshioka *et al.* (8). Initial testing of the resultant fractions for inhibition of mannanase production in isolated endosperms revealed some inhibitory activity in all fractions prior to any dilution, but was strongest about the region in which authentic ABA eluted from the column (30–32 min, Table II). When diffusate fractions were diluted in series, inhibition of mannanase activity was retained most strongly by that fraction which coeluted with [^{14}C]ABA standard (Table III). The fraction in which most of the authentic [^{14}C]ABA eluted from the column is marked by an asterisk, although much lower levels of radioactivity eluted at 26 to 30 min and at 40 min. The 'microassay,' which is only sufficiently accurate to allow a plus/minus estimate of mannanase activity, was used for initial survey of all the fractions from the HPLC column. Mannanase activity was then quantified by the standard viscometric assay in fractions of interest.

Some inhibition of mannanase activity was also retained with dilution in fractions that eluted earlier (20 min) or later (36 min) than the ABA standard. This may be related, in part, to the difficulty we had in obtaining a sharp elution of the ABA standard. The goal of this experiment was to identify components of a diffusate that specifically inhibit mannanase activity and then to assess their chromatographic similarities to ABA. Since any 'clean-up' procedure for the sample would have selected for particular classes of molecules, and hence could have caused unwitting removal of the endogenous inhibitor (or a part thereof), this was kept to a minimum. The necessary compromise between chromatographic resolution and the experimental goals resulted in a spreading of ABA about the central peak which may have accounted for the inhibitory activity of other fractions. Alternatively, ABA may not be the only compound in the diffusate that can cause mannanase inhibition. Indeed, previous workers

Table I. *PVP-Column Separation of Components of the Incubation Medium from 300 Isolated Endosperms Incubated in a LV of Sterile Water for 12 h*

The incubation medium was first dialysed overnight against acidified water. The material that passed through the dialysis membrane (approximately 25 ml) was then concentrated and applied to a column (1.0 × 3.0 cm) of PVP. The column effluent was collected, frozen, and lyophilized, then resuspended in a final volume of 6.0 ml, which was the starting volume of the incubation medium. The inhibitor activity of this fraction is compared with that which remained in the dialysis bag (1.05 ml). Inhibitor activity was tested by incubating 10 isolated endosperms in 200 μl of either fraction (or a dilution thereof) for 28 h, then extracting the endosperms and quantifying mannanase activity. Control values are for mannanase activity produced by endosperms incubated in a LV of sterile water for up to 28 h (those used for A were incubated 2 to 3 h less than those used for B). [^{14}C]ABA was included as a tracer, and was found in the PVP-column effluent. A unit of mannanase activity is defined as a decrease in initial flow time of a 0.1% galactomannan solution of 1% h^{-1}.

A. Fraction retained within the dialysis membrane

Sample Dilution	Mannanase Activity	Control Activity
	units × 10^{-3}/10 endosperms	*%*
No dilution	0.5	32
2 times	0.7	48
4 times	0.8	59
8 times	2.0	100
16 times	1.4	100
Control	1.4	100

B. Fraction eluted from PVP column

Sample Dilution	Dilution Relative to Solution Internal to Dialysis Membrane	Mannanase Activity	Control Activity
		units × 10^{-3}/10 endosperms	*%*
No dilution	6 ×	0	0
2 times	12 ×	0	0
4 times	24 ×	0	0
8 times	48 ×	0	0
16 times	96 ×	0.8	14
32 times	192 ×	1.3	23
Control		5.6	100

have described a number of compounds that inhibit mannanase production when added to the incubation medium (6). Despite these limitations, however, this experiment shows a strong correlation between the chromatographic behavior of at least a major part of the endogenous inhibitor and ABA.

Changes in Incubation Volume of Lettuce Endosperms Modulate *in Vivo* ABA Levels. If endogenous ABA regulates mannanase synthesis in isolated endosperms, then the changes in incubation conditions that modulate enzyme synthesis should also cause changes in the amounts of ABA in the endosperms. To test this directly, acetone extracts of isolated endosperms and of the media in which the endosperms had been incubated were assayed for *cis*-ABA levels by ELISA, which incorporates monoclonal antibodies against *cis*-ABA. ^{3}H-(±; *cis*/*trans*) ABA (Amersham) was added as an internal control for ABA recovery at the start of extraction: recovery was generally 75 to 95%. Although the cross-reactivity of these antibodies to ABA derivatives is reported to be at most <0.10%, nonetheless, a sample of extracted endosperms was tested for the presence of cross-

Table II. *Inhibitor Activity of HPLC Fractions of a Diffusate from 300 Endosperms Incubated in a LV for 12 h*

UV-absorbing peaks were bulked, lyophilized, and resuspended in 0.5 ml water, then 200 μl aliquots of each fraction were used to incubate 10 isolated endosperms for 28 h. The endosperms were then extracted and the mannanase activity of the extracts determined by the viscometric assay of Halmer *et al.* (6). A unit of enzyme activity is defined as a decrease in the initial flow time of the assay solution of 1% h^{-1}. Control values are for mannanase activity produced by endosperms incubated in a LV of sterile water for 28 to 30 h.

Retention Time	Activity
min	*units/10 endosperms*
2	186
4	966
6	385
8	242
10	430
12	186
14	110
16	230
18	134
20	102
22	0
24–28	90
30–32	0
34	339
36–38	220
40	117
42–44	5070
46	4299
50	7202
Control	9540

reacting components since significant quantities of these contaminants may interfere with the assay of a particular sample type. A constant amount of endosperm extract was included in wells containing varying known amounts of authentic (mixed isomer) ABA. As seen in Figure 2, addition of the endosperm extracts always increased the assayed quantity of ABA by exactly the same amount above the known quantity added. Any component of the endosperm extract reacting with the antibody, therefore, is either authentic *cis*-ABA or, less likely, is a cross-reacting compound that responds to the antibody with precisely the same kinetics as authentic ABA (16).

Samples from isolated endosperms were diluted to be within the bounds of the standard curve and were assayed for ABA at least in duplicate. For each experiment, three samples of each treatment of endosperms were prepared, and two separate experiments were performed. The results of these experiments are presented in Figure 3. Absolute values for quantitation of ABA varied from plate to plate in the commercial kits but were repeatable within plates. Accordingly, any samples that were to be directly compared were assayed all on the same plate. In both experiments, there was more endogenous ABA (approximately 2 times more) in extracts of endosperms incubated in a SV than in endosperms incubated in a LV. This difference is significant in both experiments at $P < 0.05$ by the Mann-Whitney test for nonparametric data (17). The absolute amount of ABA in the incubation medium was too variable to detect any trend (not shown).

ABA Levels in Endosperms in Intact Seeds. It was of interest to determine if ABA levels changed in endosperms in intact seeds upon germination. Seeds were imbibed for 2 h, then either incubated in darkness at 25°C immediately or exposed to white light for 5 min to induce germination prior to incubation. Endosperms were isolated from the seeds 28 h later, since germi-

Table III. *Retention of Inhibitor Activity through Dilution in Fractions from HPLC of the Incubation Medium from 300 Endosperms Incubated in a LV for 12 h*

Fractions were obtained and resuspended as previously described, then diluted as noted (relative to initial resuspension volume). Inhibitory activity of each fraction was assessed as the ability to repress mannanase activity in isolated endosperms incubated in a LV. Mannanase activity was tested first by a microassay procedure that allowed an approximate determination of enzyme levels. Fractions that retained significant inhibitor activity with dilution were then further tested by the viscometric assay to accurately quantify mannanase activity. The microassay is fully described in "Materials and Methods."

Microassay: Time(s) for Assay Volume to Travel 1 cm down Capillary Tube		Retention Time	Macroassay: Units × 10^{-3} per 20 Endosperms
1:40 dilution	*1:80 dilution*	*min*	*1:80 dilution*
6.35	6.35	Control	18.5
6.26		2	
6.32		4	
6.53		6	
6.33		8	
6.34		10	
6.81		12	
6.41		14	
6.85		16	
7.01		18	
7.43	6.69	20	13.3
6.89		22	
7.59	6.03	24	20.2
6.09		26	
7.35	6.30	28	19.5
7.54	6.19	30	
8.81	6.93	32*	11.0
6.85		34	
7.44	6.33	36	14.4
7.63	6.30	38	15.9
7.56	6.77	40	19.3
7.10		42	
7.21		44	
7.33		46	

* Retention time of [^{14}C]ABA.

nated seeds produce copious quantities of mannanase at this time (6). The endosperms were then extracted, processed, and assayed for *cis*-ABA by ELISA as described above. The amounts of ABA detectable in endosperms from ungerminated and germinated seeds were not significantly different, the values being 53 ± 9 and 47 ± 8 nmol per 50 endosperms, respectively.

DISCUSSION

We have demonstrated an inverse correlation between endogenous ABA and mannanase production by isolated lettuce endosperms under three experimental conditions. First, significant quantities of ABA are leached from endosperms when they are incubated in a LV, since such a leachate induces the synthesis of ABA-specific proteins in barley aleurone cells (Fig. 1). Endosperm leachates similarly prevent mannanase production in other lettuce endosperms, in a similar manner to exogenously applied ABA (5). That ABA can be readily leached from plant tissues by incubation in liquid has also been observed in bean mesophyll tissue (10) and spinach leaf slices (3). ABA thus leached from the cells accumulates due to the lack of ABA-catabolizing enzymes in the liquid medium (ibid). Second, fractionation of endosperm leachates by either PVP-chromatography or HPLC

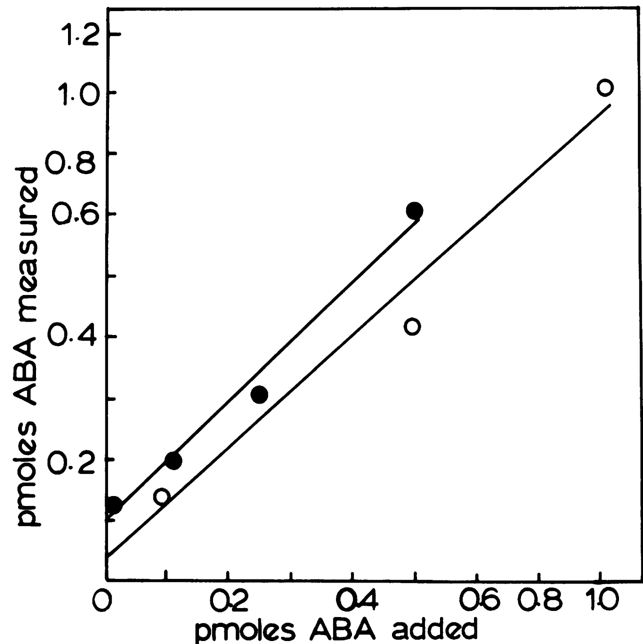

FIG. 2. Effect on ELISA-detectable ABA (○) of inclusion of 50 μl of an extract of endosperms incubated in a LV with 50 μl of samples of known concentrations of authentic ABA (●). The extract and standard samples were added together at the start of the assay.

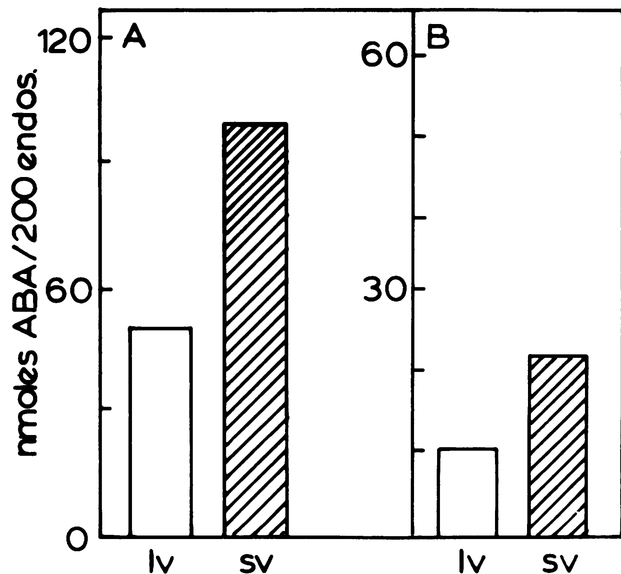

FIG. 3. Quantitation of endogenous *cis*-ABA in isolated endosperms incubated in either a SV or a LV for 12 h. Endosperms were washed, frozen, and lyophilized immediately after incubation. The lyophilized residues were extracted for ABA as described by Weiler (16). ^{3}H-ABA was added to each sample at the beginning of extraction for estimation of recovery. After passage of the extracts over a C_{18} column, they were assayed for *cis*-ABA by ELISA, using a commercial kit (Idetek, CA) based on the monoclonal antibodies to *cis*-ABA described by Mertens *et al.* (12). Results are the means of three separate samples, assayed at least in duplicate. The experiment was performed two times; since plate-to-plate variability did not allow comparison between experiments, the results of each are presented separately (A and B).

results in the coelution of ABA and that leachate component which gives the strongest inhibition of mannanase production (Tables I and II). PVP binds phenolic compounds present in plant extracts, yet gibberellins, ABA, and indole acetic acid (IAA) may be readily eluted from a PVP column. This method is commonly used as a preliminary cleanup of samples in which phytohormones are to be identified or quantified, or both. ABA recovery of approximately 99% can be achieved in some cases (4). Due to the high production during endosperm incubation of phenolics, which were visibly more concentrated in the SV than the LV, we were concerned that the observed inhibition of mannanase production was an artifact resulting from these compounds, rather than a molecule more likely to have a physiological role. Since high recovery of both ABA and the endogenous inhibitor from the PVP column was achieved, phenolic compounds are not a major contributor to inhibition of mannanase production in isolated endosperms. It should also be noted that phenolics do not cause inhibition of mannanase activity per se, since incubation of an enzyme extract with an endosperm leachate in a viscometric assay does not result in any loss of activity (not shown).

Resolution of leachate component by HPLC similarly resulted in coelution of inhibitor activity and ABA. This experiment, however, was not so clear-cut, since other fractions also inhibited mannanase activity to some degree (Tables II and III). This may be due either to the concentrating of otherwise noninhibitory sample components (nonspecific effects), or to the presence of more than one inhibitor in the lettuce endosperms. Specifically, Halmer and Bewley (5) report that Ca^{2+} can inhibit mannanase production. These ions are likely also to be leached from the endosperm cells and then concentrated in a particular fraction by HPLC, and therefore act as an inhibitor of enzyme production in the endosperm bioassay. No attempts to identify the elution patterns of ABA metabolites were made, since it appears that ABA metabolism is unique in lettuce seeds (14), and such a study was outside the scope of this work. Nevertheless, these considerations do not diminish the strength of the observation that the greatest degree of inhibition is found in that fraction that coelutes with authentic ABA (Table III). This supports our suggestion that the inhibition of mannanase production that occurs when incubation takes place in a reduced volume is primarily due to the presence of ABA.

This idea is further supported by the third correlation presented herein. Differential loss of ABA with incubation conditions is a necessary result if this inhibitor regulates mannanase production in isolated endosperms. Changing the volume of the incubation medium indeed results in changes in amounts of ABA extractable from the endosperms. There is twofold less ABA in endosperms that produce mannanase (*i.e.* those incubated in a LV) than in those in which mannanase production is inhibited (*i.e.* those in a SV). Therefore, ABA has a role in the regulation of mannanase production from isolated lettuce endosperms.

Control of mannanase production in intact seeds is more complex. There is no detectable loss of ABA from the endosperms in germinated seeds that are actively synthesizing mannanase. Since ABA represses mannanase production until the appropriate stage after germination of the intact seed, then its inhibitory effects must be overcome before enzyme synthesis can occur. Because ABA levels remain unchanged, the release of inhibition of enzyme synthesis cannot be due to its catabolism. Processes such as compartmentation of ABA, changes in ABA-sensitivity, or the action of some antagonist are more likely to be involved. Alternatively, it is possible that *in vivo* changes in ABA levels have been masked by the wounding that necessarily occurs during isolation of the endosperm, a process that may result in ABA production within the time required to complete sample collection. It may be inappropriate, therefore, to extrapolate the results from studies on isolated endosperms to explain the situation in intact seed endosperm.

Isolated lettuce endosperms offer an attractive experimental model in which the effects of ABA can be studied in that simple volume manipulations cause changes in endogenous ABA levels and in a readily measurable physiological response. We are using mannanase production in isolated lettuce endosperms to examine how ABA regulates this enzyme and to gain insight into the mechanism of ABA action.

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