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The Sensitivity of Barley Aleurone Tissue to Gibberellin Is Heterogeneous and May Be Spatially Determined[1]

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In cereals, gibberellin (GA) enhances the synthesis and secretion of hydrolytic enzymes from aleurone cells. These enzymes then mobilize the endosperm storage reserves that fuel germination. The dose-response curve of aleurone protoplasts to GA extends over a range of concentrations from 10^{-11} to more than 10^{-6} M. One hypothesis is that subpopulations of cells have different sensitivities to GA, with each cell having a threshold concentration of GA above which it is switched on. The dose-response curve therefore reflects a gradual recruitment of cells to the pool exhibiting a full GA response. Alternatively, all cells may gradually increase their responses as the GA level is increased. In the present study we found that at increasing GA concentrations, increasing numbers of barley (*Hordeum vulgare*) cells showed the enhanced amylase secretion and vacuolation characteristic of the GA response. We also observed that the region of aleurone tissue closest to the embryo contains the highest proportion of cells activated at the GA concentrations thought to occur naturally in germinating grain. These data indicate that an aleurone layer contains cells of varying sensitivities to GA and that recruitment of these differentially responding pools of cells may explain the broad dose response to GA.

The aleurone layer of the barley (*Hordeum vulgare*) grain secretes hydrolases that mobilize endosperm reserves during germination (for review, see Fincher, 1989; Jones and Jacobsen, 1991). The synthesis and secretion of these hydrolases (principally α-amylases) is under hormonal regulation. GA stimulates the synthesis and secretion of α-amylase and ABA reverses this effect (Fincher, 1989; Jones and Jacobsen, 1991). Therefore, barley aleurone has been used extensively as a model system for the study of signal transduction in response to GA and ABA. However, the molecular basis of GA and ABA signal transduction remains poorly understood (Bethke et al., 1997; Ritchie and Gilroy, 1998a).

The GA enhancement of α-amylase production increases with the time of exposure to the hormone. Thus, once exposed to GA, the cells of the aleurone layer show detectable stimulation of α-amylase secretion after a lag time of approximately 8 h (Varner and Chandra, 1964), and this increase continues for 2 to 3 d. This GA-induced enhancement of α-amylase secretion with time is thought to be due to the recruitment of more and more cells to a secreting population rather than to a gradual increase in the secretory activity of all of the cells (Hillmer et al., 1993).

In addition to a time-dependent increase in secretion, GA also elicits the stimulation of secretion over a broad range of concentrations. Increasing [GA] induces increasing α-amylase synthesis and secretion. Two possible models for the cellular basis of this dose-response curve can be proposed (Bradford and Trewavas, 1994). Increasing GA levels could recruit more and more cells to the secreting population, with each cell having an all-or-nothing secretory response. This would be analogous to the explanation of the time-related increase in secretion induced by GA proposed by Hillmer et al. (1993). An alternative explanation is that increasing levels of GA gradually increase the output of all of the secreting cells.

These two models suggest two very different modes of cellular regulation. The all-or-nothing recruitment model implies a hormonal response system in which GA throws a limited number of molecular "switches" that engage the cellular machinery, leading to activated hydrolase synthesis and secretion. Cells with different [GA] activation thresholds might have subtly different receptors with different GA affinities, different receptor numbers, or different requirements for receptor occupancy to elicit a response (Rodbard, 1973). However, once the receptor is triggered, the full activity of the GA signal transduction and response machinery would be elicited. In the second model, GA would continuously modulate the activity of each cell's secretory machinery over the entire dose-response range of the GA response. Thus, a single cell would have to possess a GA-receptor system capable of monitoring [GA] ranging from 10^{-11} to 10^{-8} M, as well as a signal transduction and response system capable of setting an appropriate intermediate secretory activity for each [GA].

The recruitment model of the GA-response time course proposed by Hillmer et al. (1993) suggested to us that a similar mechanism might underlie the dose-response curve of aleurone to GA. This idea implies heterogeneity in hormone sensitivity within cells of a single aleurone layer, with groups of cells exhibiting a different threshold concentration of GA above which they are activated (Bradford

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Abbreviation: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester.

and Trewavas, 1994). Thus, as GA levels are increased, more cells are activated. This population-based threshold model has been tested at the level of tomato seed germination. The addition of increasing concentrations of GA (10^{-7} to 10^{-4} M) to GA-deficient tomato seeds resulted in increasing numbers of germinated seeds, and hastened the rate at which germination proceeded (Ni and Bradford, 1993). These effects of GA on germination indicated that each seed had a threshold GA concentration below which germination failed to occur. Likewise, increasing the applied [GA] beyond this threshold resulted in a faster rate of germination. These observations have led to the concept of "GA time," in which a GA response is proposed to include components of the concentration of GA and the rate of the GA response. The rate of response depends on how much the [GA] is above the response threshold (Bradford and Trewavas, 1994).

The threshold/response model was originally proposed to explain the hormone and inhibitor responses in mammalian endocrine cells (Rodbard, 1973). This model of hormone responsiveness predicted that the behavior of a tissue reflects the average response of a heterogeneous population of cells. We report that individual aleurone cells and protoplasts respond to increasing GA concentrations in an all-or-nothing manner, which is consistent with such a threshold model of activation. As the level of GA increases, more cells exhibit the GA response, suggesting that this hormone acts to recruit cells with different thresholds for activation. In addition, the two ends of the aleurone layer are shown to have significantly different sensitivities to the endogenous levels of GA released from the embryo. These results suggest that position within the grain may be one factor that determines the GA sensitivity of an aleurone cell, and that developmental factors may play a role in determining this property.

MATERIALS AND METHODS

Plant Material and Aleurone Cell Preparation

Barley (*Hordeum vulgare* cv Himalaya, provided by the Department of Agronomy, Washington State University, Pullman) grains were de-embryonated and prepared for aleurone layer or protoplast isolation as described by Deikman and Jones (1985) and Hillmer et al. (1993). Aleurone layers or protoplasts were incubated for 16 h with 10 mM $CaCl_2$ and GA_3, ABA, or GA_3 plus ABA at various concentrations. α-Amylase secretion was assayed as described by Bush and Jones (1988). For preparation of the different sections, de-embryonated seed halves were cut into either proximal and distal regions (relative to the embryo), or left and right sides of the grain. To control for variations in the size of the pieces used, the layers for each sample were weighed after removal of the starch and also at the end of each treatment. Proximal and distal tissues were routinely produced with similar weights (±3%) from an individual seed. Although dorsal/ventral sections of aleurone layer were also taken, these proved difficult to prepare reproducibly. In addition, the ventral section contained a large region of suture tissue that is very different from the rest of the aleurone layer (Cochrane and Duffus, 1980; Olsen et al., 1992). Because of these difficulties, we restricted our analysis to the proximal/distal and left/right regions.

Embedding Protoplasts for Monitoring α-Amylase Secretion from Individual Protoplasts

Single aleurone protoplasts were embedded in a gel matrix according to the method of Gilroy and Jones (1994). The gel matrix contained 3% (w/v) ultra-low-melting-point agarose (Sigma) and 3% (w/v) soluble potato starch (Baker Chemical, Philadelphia, PA) in Gamborg's B5 medium supplemented with 0.5 M mannitol. Single-cell secretion assays were carried out as described previously (Hillmer et al., 1993).

Zymograms and IEF Immunoblotting

Glycerol was added to a final concentration of 10% (v/v) to samples of incubation medium from layers treated for 16 h with various concentrations of GA_3 (as described in the figure legends). Polyacrylamide IEF gels (1 mm) were cast onto the hydrophobic side of film (Gelbond PAG, FMC Bioproducts, Rockland, ME). The gels consisted of 5% (w/v) acrylamide/bis (37.5:1), 10% (v/v) glycerol, 0.93% (v/v) Ampholine (Amersham Pharmacia Biotech, Piscataway, NJ), pH 3.5 to 10.0, 0.067% (v/v) Ampholine, pH 4.0 to 6.0, 0.067% (v/v) Ampholine, pH 5.0 to 7.0, and 0.044% (w/v) Glu, giving an overall pH range of 3.5 to 10.0. Polymerization was initiated by the addition of 600 μL of 1% (w/v) ammonium persulfate, 20 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and 20 μL of 1% (w/v) riboflavin in a final volume of 40 mL. Five microliters of each sample was loaded onto application wicks at positions equivalent to a final pH of 6.0. The gels were run at a constant 1 W/cm gel for 30 min, after which time the wicks were removed and the gel was run for a further 45 min using an IEF apparatus (LKB Multiphor, Amersham-Pharmacia Biotech).

After focusing, a piece of chromatography paper was pressed evenly onto the gel surface, and the paper was peeled back with the gel attached and removed from the backing sheet. The gel was equilibrated for 10 min in 0.3% (v/v) acetic acid and then blotted onto PVDF membranes (Immobilon-P, Millipore) in the same buffer for 1 h at 100 V using a TransBlot Cell (Bio-Rad). Immunodetection of α-amylase was carried out using an α-amylase antibody, as described previously (Ritchie and Gilroy, 1998b). For detection of amylase activity after focusing, the gels were incubated in 50 mM KH_2PO_4, pH 5.7, 5% (w/v) starch, and 20 mM $CaCl_2$ for 20 min at room temperature with gentle shaking. The starch solution was poured off and the gels were washed with water. The remaining starch was stained for 5 min using 0.03% (w/v) I_2, 0.3% (w/v) IKI, and 0.05 N HCl. The stain was washed off with water and regions of amylase activity were revealed as cleared areas of the gel devoid of stained starch. Gels and immunoblots were imaged using a scanner (ColorOne, Apple Computer, Cupertino, CA) and densitometry was performed using image-analysis software (Spectrum, IPLabs Signal Analytics, Vienna, VA).

(1→3,1→4)-β-Glucanase Assays

Samples of medium from layers treated for 16 h with various GA_3 concentrations were centrifuged for 5 min at 16,000*g* (model 415C Eppendorf centrifuge, Brinkmann) to remove particulate matter. A 50-μL sample was then added to 500 μL of 0.1 M succinate and 1 mM EGTA, pH 5.8, containing 250 μg of β-glucan from barley (Sigma), vortexed, and left at room temperature for 1 h. Then, 250 μL of 1 mg/mL Congo red dye (Sigma) was added, the mixture was vortexed and centrifuged for 5 min at 16,000*g*, and the supernatant was removed. The Congo red precipitated the β-glucan and a pellet was formed after centrifugation (Wood, 1982). Because the amount of Congo red remaining in the supernatant is inversely proportional to the amount of β-glucan in the sample, the more (1→3,1→4)-β-glucanase activity in the sample, the more Congo red remaining in the supernatant. The amount of dye in the supernatant was assayed spectrophotometrically (model Du7500, Beckman) at A_{550}.

A calibration curve was constructed using a range of β-glucan concentrations from 100 to 300 μg per assay (see Fig. 4A), and the activity of (1→3,1→4)-β-glucanase from *Bacillus subtilis* (Fluka) was used as the standard enzyme activity. To determine whether the α-amylases produced by aleurone would interfere with this assay as a result of contamination of the β-glucan with polymer containing α-bonds, up to 10 μg of purified *Bacillus licheniformis* α-amylase (Sigma) was added to the assay, but did not yield detectable glucan hydrolysis. Similarly, when 100 μg of the amylase/subtilisin inhibitor (Sigma) was added to 1 mL of medium from 25 aleurone layers that had been treated for 16 h with 5 μM GA_3, it reduced the activity of amylase in the sample by 60%. This amylase/subtilisin-inhibitor treatment had no detectable effect on the glucanase activity measured in the same sample using this Congo red assay, suggesting that the α-amylase present in the samples from aleurone would not contribute significantly to the (1→3,1→4)-β-glucanase activity monitored. Thus, the glucanase assay appears selective for monitoring glucanase activity in the background of aleurone α-amylase.

Visualizing Vacuolation

Aleurone layers were incubated in 10 μM BCECF-AM (Molecular Probes, Eugene, OR) prepared from a 1 mM stock in DMSO (final DMSO concentration, 0.2%, v/v) for 1 to 2 h, after which time the BCECF was loaded into the vacuoles (Swanson and Jones, 1996). BCECF fluorescence was visualized using an inverted epifluorescent microscope (Diaphot 300, Nikon) using a ×40, dry, 0.7 numerical aperture fluor objective, with 480 nm excitation, a 500-nm dichroic mirror, and >530 nm emission. Images were captured using a cooled CCD (charge-coupled device) camera (CH250A, Photometrics, Tucson, AZ), and the size of vacuoles was measured using image-analysis software. For each vacuole the microscope was focused to the plane of the largest cross-sectional area before the image was digitized and the vacuole size was measured. The degree of aleurone cell or protoplast vacuolation was assigned to stages I to IV as defined by Bush et al. (1986). Aleurone cells show a progressive vacuolation in response to GA from many small vacuoles (stage I) to extensive vacuolation (stage IV). Vacuolation to stage IV is thus indicative of a maximal GA response. Individual aleurone cells are between 25 and 30 μm in diameter. Cells with a large vacuole (>20 μm in diameter) were scored as having reached stage IV. Cells were scored as stage III if one or more vacuoles were 15 to 20 μm, and as stage II if one or more vacuoles were 7 to 15 μm. Cells with small vacuoles only (<7 μm) were scored as stage I.

Vacuolation of protoplasts was also scored based on the stages described by Bush et al. (1986); vacuolar development is easily visible using light microscopy. At stage I the cytoplasm is very dense and vacuoles are barely visible. Once the GA response is initiated, the vacuoles enlarge and become much more visible. Protoplasts of stage III or IV (as defined by Bush et al., 1986) were scored as vacuolated, clearly exhibiting the GA response.

RESULTS

Individual Aleurone Protoplasts Do Not Respond to GA as a Homogeneous Population

We first asked whether all or only some aleurone cells respond to increasing [GA] by increasing their levels of secretion. Therefore, we monitored the GA response in isolated aleurone cell protoplasts. Aleurone protoplasts respond similarly to GA, as do cells of the aleurone layer (Hillmer et al., 1993). However, the use of protoplasts allowed us to analyze the GA response of individual cells using a series of single-cell response assays developed for use with protoplasts. GA-induced secretion can be assayed from single aleurone protoplasts using thin starch films in which a secreting protoplast digests the starch in its immediate vicinity. This region of starch digestion is then visualized as a cleared "halo" after the starch in the gel is stained blue with iodine reagent (Hillmer et al., 1993). Another single-cell measure of the GA response is the development of prominent vacuolation in protoplasts (Bush et al., 1986; Gilroy, 1996).

Figure 1 shows that at low concentrations of GA only a small percentage of cells exhibited secretory activity or vacuolation by 48 h of GA treatment, whereas at increasing GA concentrations more cells exhibited a detectable GA response. These observations are consistent with increasing GA levels recruiting more cells to a full GA response. However, this result could also arise from protoplasts being differentially damaged or desensitized during the process of cell wall removal. Therefore, we repeated these assays in intact aleurone cells, in which there would be no potential artifacts due to protoplast formation.

Vacuole Development in Aleurone Tissue in Response to GA

The cells in an intact aleurone layer cannot be assayed by the single-cell secretion assay described above for protoplasts. However, as with protoplasts, development of vac-

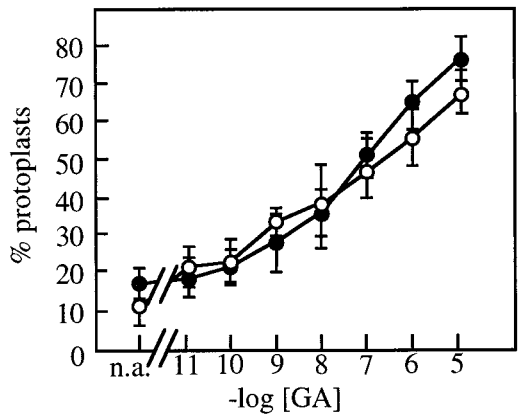

Figure 1. Dose-response curve of aleurone protoplasts to GA assayed by single-cell secretion (●) and vacuolation (○). Freshly released protoplasts were treated for 48 h with various [GA], after which single-cell secretion assays were carried out and vacuolation was assessed. The data show mean percentages ± SE from three separate experiments. For single-cell secretion, at least 60 protoplasts were examined per treatment per experiment. For vacuolation, at least 300 protoplasts were examined. Protoplasts were classified as exhibiting GA-induced vacuolation if they were at stage III or IV (as defined by Bush et al., 1986) and as secreting if they had digested a clear zone of at least 100 μm in diameter in the single-cell secretion assay. n.a., No GA added.

uolation can be used to monitor the GA response in intact aleurone layers. Vacuole size was determined using the fluorescent dye BCECF-AM, which is readily taken up into the vacuole of aleurone cells (Swanson and Jones, 1996). We recorded fluorescence images of layers incubated in the BCECF-AM and visualized vacuole size in the cells of intact aleurone layers after treatment with various concentrations of GA. Cells were assigned to one of four categories as described by Bush et al. (1986). As shown in Figure 2A, at stage I the cells contained small vacuoles characteristic of untreated aleurone cells, at stages II and III the cells had increasingly larger vacuoles, and at stage IV the volume of the cell was almost entirely occupied by one or two large vacuoles. This increasing vacuolation is characteristic of GA-responding cells. Figure 2, B to E, shows that, similar to protoplasts, as the [GA] was increased the proportion of vacuolated cells in the aleurone layer also increased.

In conducting these assays we noted a spatial component to the GA-regulated vacuolation patterns in the aleurone layer. As shown in Figure 2, B to E, in the absence of added GA the proportions of vacuolated cells were the same at both ends of the layer. Similarly, at saturating GA concentrations ($>10^{-6}$ M) all regions of the layers appeared similar. However, between 10^{-9} and 10^{-6} M GA, although the proportions of cells showing vacuolation to stages II, III, and IV increased with increasing [GA], this increase was greater in the proximal region of the layer (nearest the embryo in the intact grain) than in the distal region (farthest from the embryo in the intact grain). To ensure that the difference in the responses of the two ends of the layer was not due to differential damage during the preparation of the layer, we used the vital dye fluorescein diacetate (Huang et al., 1986) to determine the number of dead cells in freshly stripped layers, and found no difference in the proportion (95%) of living cells between the two ends of the layer.

We also determined the number of vacuolated cells after 5 d of treatment with GA. Under these conditions all viable, responsive cells should have completed their response to GA. At this time there was no difference in the proportion of stage IV cells (92% ± 5%) at either end of the layers. Finally, we examined the vacuolation of aleurone cells directly beneath the testa pericarp. After imbibition the testa pericarp can be removed with forceps from an intact half seed, revealing the aleurone cells beneath. These cells were not subjected to the starch-removal protocol of a standard aleurone tissue preparation, which would affect cells on the other side of the three-cell-thick aleurone layer. The range and degree of vacuolation and GA dose response of these aleurone cells just below the seed coat were identical to those of the innermost layer of cells examined (Fig. 2), the cells on the other face of the aleurone layer. The proximal/distal difference in vacuolation was also evident in aleurone cells just below the testa pericarp (data not shown). These results suggested that the difference in the response to GA of the proximal and distal regions of the aleurone layer was not an artifact of tissue preparation.

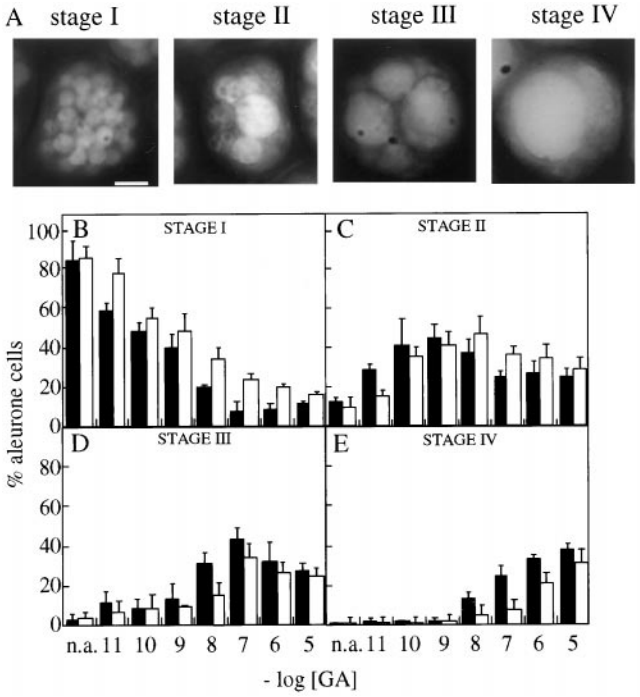

Figure 2. The effect of GA on the vacuolation of aleurone cells. A, Fluorescence micrographs of aleurone cells loaded with BCECF-AM showing vacuolation from stages I to IV. Bar = 10 μm. B to E, Comparison of stages of vacuolation of cells proximal (black bars) and distal (white bars) to the embryo treated with a range of [GA] for 16 h. Layers were loaded with BCECF-AM as described in "Materials and Methods," and vacuole size was monitored by fluorescence microscopy. The data represent means ± SE for three experiments ($n \geq 3$ aleurone layers and 150 cells per experiment). n.a., No GA added.

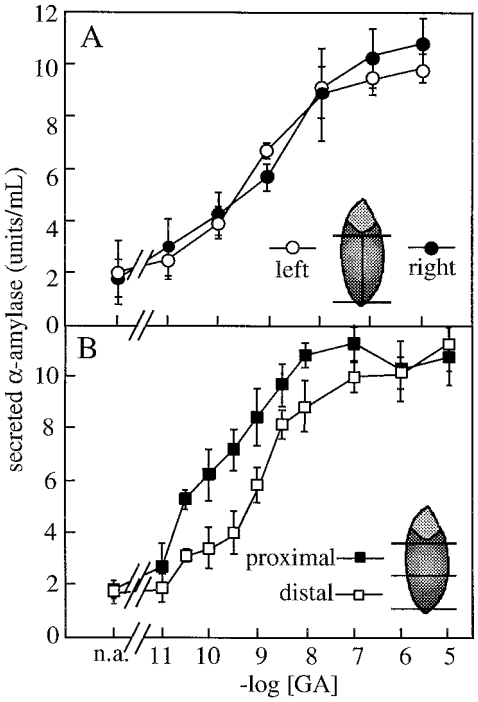

Figure 3. Dose-response curve of aleurone layers to GA assayed by secreted α-amylase activity. A, Comparison of the α-amylase secreted in response to different [GA] by aleurone tissue from the left (○) and right (●) sides of the grain relative to the embryo. B, Comparison of the α-amylase secreted in response to different [GA] by aleurone tissue proximal (■) and distal (□) to the embryo. Layers were prepared and cut as depicted in the diagrams included with the graphs. After treatment with various concentrations of GA for 16 h, secreted α-amylase was assayed. Data are means ± SE from at least three separate experiments. n.a., No GA added.

α-Amylase and (1→3,1→4)-β-Glucanase Secretion Differs between Proximal and Distal Aleurone Tissue

Vacuolation is only one measure of the GA response of the aleurone layer. Therefore, we asked whether the spatial difference in responsiveness was seen in other aspects of the GA response. We compared the GA dose-response curves of the left versus the right side of the aleurone layer, and proximally versus distally (depicted schematically in Fig. 3) with respect to GA-induced α-amylase production. Figure 3A shows that the left and right sides of the aleurone layer did not differ in their GA dose-response curves. However, as shown in Figure 3B, the proximal part of the aleurone showed a statistically significantly higher level of secreted amylase activity than the distal part (in the range of 3×10^{-11} to 1×10^{-8} M GA; $P < 0.05$ by t test). Outside of this range the responses were identical.

The α-amylase synthesized and secreted by aleurone cells of cereals is made up of many different isoforms that are enhanced to different degrees by GA (Jacobsen et al., 1970; Jacobsen and Higgins, 1982; Lazarus et al., 1985; Rogers, 1985; Huttly et al., 1988; Karrer et al., 1991). Therefore, we separated the isoforms produced at each end of the layer at particular GA concentrations by IEF to determine whether different α-amylases might be secreted by the proximal and distal tissues. Figure 4 shows that when visualized either by zymogram (Fig. 4A) or immunoblot (Fig. 4B), the low-pI α-amylase isoforms did not appear to vary significantly with GA level. However, in the range of 10^{-11} to 10^{-8} M GA, the increase in production of high-pI α-amylase isoforms was more prominent at lower GA levels in the proximal tissue. This is shown quantitatively below Figure 4B as densitometry of representative high- and low-pI bands scanned from the immunoblot.

We next assayed another enzyme activity up-regulated by GA, (1→3,1→4)-β-glucanase II (Mundy and Fincher, 1986; Stuart et al., 1986), to determine if the difference in GA response between the proximal and distal regions of the layer was specific to α-amylase. An assay was developed based on the property of β-glucan binding to, and precipitating, the dye Congo red (Wood, 1982). As shown in Figure 5A, there was a nearly linear relationship between precipitation of Congo red and the amount of β-glucan, in the range of 150 to 250 μg/assay. Therefore, we used this relationship to assay for (1→3,1→4)-β-glucanase activity in the aleurone samples. Figure 5B shows the GA dose-response curve of proximal and distal tissue assayed for (1→3,1→4)-β-glucanase secretion. This

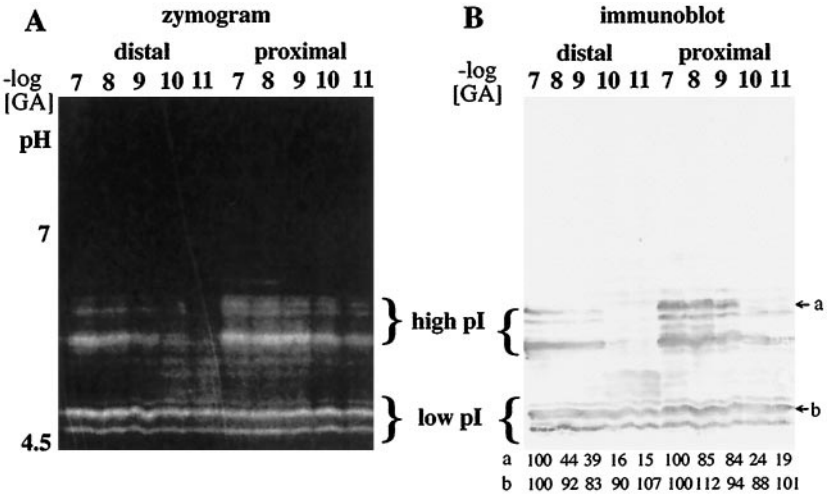

Figure 4. Analysis of secreted α-amylase isoforms in response to different [GA] from aleurone layers isolated from distal and proximal portions of the grain relative to the embryo. Samples are from one representative experiment of four carried out as described for Figure 3B, in which aleurone layers were treated with different [GA] for 16 h. A, IEF zymogram showing negatively staining bands of α-amylase activity. B, Immunoblot detecting isoforms of α-amylase. The figures beneath B show the densitometry of representative high-pI (a) or low-pI (b) bands normalized to 100% for 10^{-7} M GA treatment.

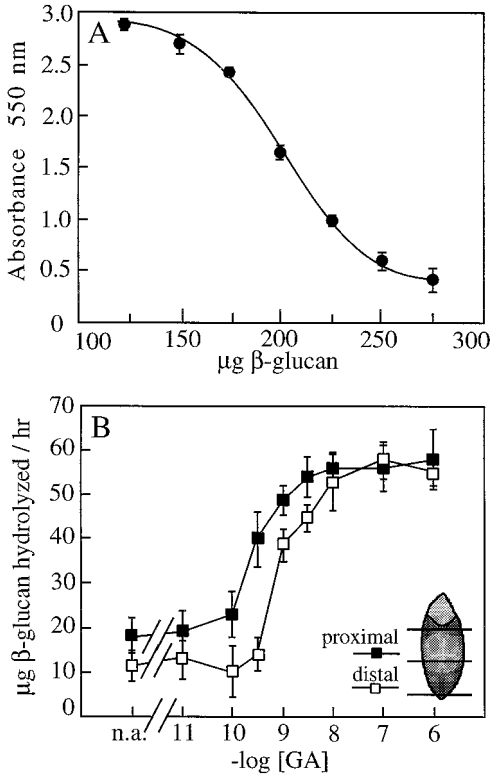

Figure 5. (1→3,1→4)-β-Glucanase assay and secreted (1→3,1→4)-β-glucanase from aleurone layers proximal and distal to the embryo in response to different [GA]. A, Standard curve of the relationship between the amount of β-glucan added per assay and unprecipitated Congo red as expressed as A_{550} of the assay supernatant. Data are means ± SE from one representative experiment of three. Note that as the glucan level increased more Congo red was precipitated. B, Assay of (1→3,1→4)-β-glucanase activity secreted from aleurone layers proximal (■) and distal (□) to the embryo and treated with different [GA] for 16 h using the Congo red precipitation assay. Data are means ± SE from three separate experiments. n.a., No GA added.

proximal/distal dose-response curve is similar to that of the α-amylase graph (Fig. 3B) in that the proximal layers showed a statistically significantly ($P < 0.05$ by t test) greater response than the distal cells at 10^{-10} to 10^{-8} M GA, whereas outside of this range the levels of secreted (1→3,1→4)-β-glucanase were similar.

Proximal Aleurone Protoplasts Show Enhanced Sensitivity to GA

As noted previously, aleurone protoplasts responded to GA in the same manner as aleurone layers. Therefore, we looked at protoplasts isolated from proximal and distal regions of the aleurone layer at 48 h after treatment with various GA concentrations to determine if the difference in GA responsiveness was also evident in protoplasts. As shown in Figure 6, protoplasts isolated from proximal regions showed a statistically significant increase in response to GA relative to those isolated from distal regions ($P < 0.05$ by t test), confirming the results from the aleurone layers. It is important to note, however, that protoplasts require higher GA levels to yield the same response as intact layers. This is in keeping with previous reports indicating that protoplasts respond qualitatively identically to intact cells but that the speed of their response is slower (Jacobsen and Beach, 1985; Hillmer et al., 1993; Bethke et al., 1997). In addition, the dose-response curve of protoplasts to GA is broader than that of layers (Fig. 3). This altered dose response may reflect a subtle change in the GA-response system upon protoplast formation. Alternatively, the more rapidly responding cells of the intact layer may have more fully completed their GA response at the time of the assay than the slower protoplasts. Assaying the protoplasts while they were still progressing toward a maximal GA response could have led to the broader apparent dose-response curve, because even the less-sensitive cells of the layer would have completed an obvious GA response at the time of assay. In contrast, the less-sensitive pool of protoplasts (i.e. the slower-responding group) would still be developing the full response and so would appear as a broadening in the dose response of the protoplast population.

The ABA Response of Proximal and Distal Layers

During seed development the levels of ABA increase dramatically and then later decline as the seed reaches maturity (King, 1976). ABA also inhibits the GA response

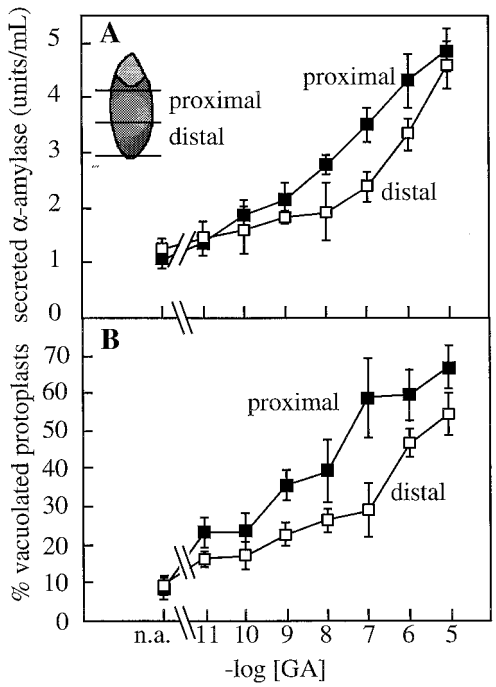

Figure 6. Dose-response curves to GA of protoplasts prepared from aleurone layers proximal (■) and distal (□) to the embryo. A, Secreted α-amylase activity. Data are means ± SE from three separate experiments. B, Vacuolation of protoplasts. Freshly isolated aleurone protoplasts were treated with various [GA] and after 48 h secreted α-amylase activity and the degree of vacuolation were assessed. Percentages are shown from three replicates of at least 200 protoplasts per treatment per experiment. n.a., No GA added.

of aleurone cells. Therefore, we wanted to determine if higher residual levels of ABA could account for the decreased sensitivity to GA in the distal versus the proximal tissue. We first determined whether proximal and distal tissue showed marked differences in ABA sensitivity. Figure 7 shows that at 1×10^{-9} M ABA there was no inhibition of α-amylase secretion from the proximal or distal layers elicited by 1×10^{-6} M GA. Increasing the ABA concentration from 3.3×10^{-9} to 1×10^{-7} M resulted in an increasing inhibition of α-amylase production, but this inhibition was not different between the proximal and distal tissues. Thus, the proximal and distal tissues did not exhibit a pronounced difference in ABA sensitivity at saturating (1×10^{-6} M) GA levels.

Figure 3 shows that at much lower GA levels (1×10^{-9} M), proximal tissue secreted more α-amylase than distal tissue. Therefore, we exposed proximal tissue treated with 1×10^{-9} M GA to a range of ABA concentrations to determine if we could reduce the level of α-amylase produced to that of the distal tissue. Figure 8 shows that at between 1×10^{-11} and 1×10^{-8} M GA, 3.3×10^{-9} M ABA inhibited the α-amylase produced by the proximal tissue to a level resembling that produced by the distal tissue. This suggests that if the lower response to GA of the distal tissue is due to endogenous ABA levels, these would likely be approximately 3.3×10^{-9} M. We reasoned that if this was true, incubation of the distal tissue in a medium containing 3.3×10^{-9} M ABA should have no effect on the GA response, because the tissue must already contain this concentration of ABA. Mixing two solutions of equal [ABA] will not alter the final [ABA] of either, and thus incubation of aleurone tissue already containing 3.3×10^{-9} M ABA in a solution of 3.3×10^{-9} M will not alter the [ABA] of the tissue. Figure 8 shows that there was a statistically significant decrease ($P < 0.05$ by t test) in α-amylase activity secreted by the distal tissue treated with 1×10^{-9} M GA and 3.3×10^{-9} M ABA, which is inconsistent with the idea that distal tissue already has an internal concentration of 3.3×10^{-9} M ABA.

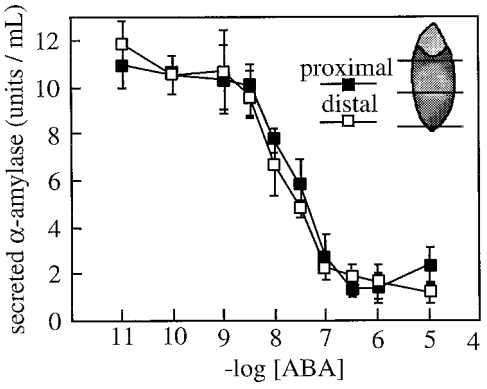

Figure 7. Dose-response curve of aleurone layers to ABA as assessed by ABA suppression of GA-stimulated α-amylase secretion. Aleurone layers isolated from regions proximal (■) and distal (□) to the embryo were treated with 1×10^{-6} M GA and different [ABA] for 16 h, after which time secreted α-amylase was assayed. Data are means ± SE from three separate experiments.

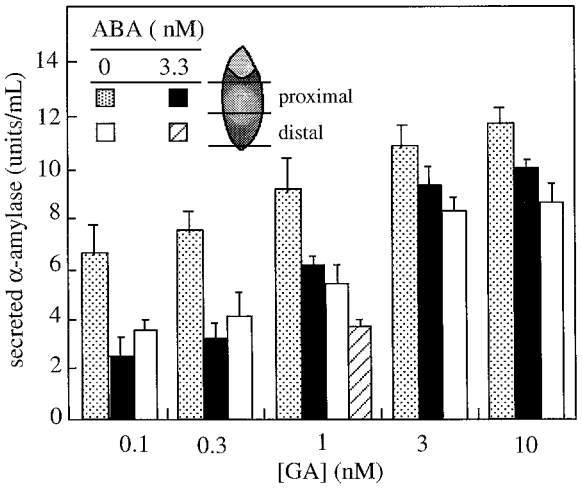

Figure 8. The effect of ABA on the inhibition of the GA response of proximal and distal aleurone tissues. Proximal and distal aleurone layers were prepared and treated with various [GA] with or without 3.3×10^{-9} M ABA (as indicated) for 16 h, after which time secreted α-amylase activity was assayed. Data are means ± SE from duplicate samples from three separate experiments.

DISCUSSION

Our results support the model for the GA response of the barley aleurone layer whereby different populations of aleurone cells have different thresholds for the GA response (Bradford and Trewavas, 1994). This suggests that GA does not exert a continuous, graduated control of cellular functions but, rather, that above a critical threshold its signal is transduced via a molecular switch(es) that triggers subsequent GA responses. Such a switch presumably lies close to the initial GA-perception event. Our observations concur with the threshold model originally developed by Rodbard (1973) for mammalian cells, which has also been suggested to be applicable to plant cells (Bradford and Trewavas, 1994). Underlying these models for a threshold basis of dose-response curves is a requirement for the cell-to-cell variation in hormone sensitivity that we observed in both aleurone protoplasts and the intact aleurone layer. Therefore, the aleurone layer contains cells with thresholds for GA activation that span the entire dose-response range of the tissue, and the effect of increasing hormone concentration is to recruit cells with progressively lower sensitivities to GA to the secreting population. Responsiveness to a broad range of hormone concentrations is not unique to aleurone tissue; for example, inhibition of root elongation is sensitive to auxin through approximately 3 orders of magnitude in concentration (Evans et al., 1994). Therefore, a threshold-based recruitment phenomenon could explain many plant hormone-response systems. Testing the generality of this model in plants must await collection of data on hormone sensitivity at the single-cell level for these other hormone responses.

The differential sensitivity to GA is reflected spatially in the aleurone layer. The proximal end of the aleurone layer (that nearest the embryo in the intact seed) shows a higher sensitivity to GA than the distal region. We assayed for GA

responsiveness in three ways: secreted α-amylase activity, (1→3,1→4)-β-glucanase activity, and vacuolation. In all three assays the proximal cells showed a greater response to GA concentrations in the range of 10^{-10} to 10^{-8} M than cells from the distal region of the aleurone layer. The proximal and distal tissues had the same non-GA-treated α-amylase activity and the same maximum capacity for α-amylase secretion at saturating levels of GA ($\geq 1 \times 10^{-6}$ M). This means that there are probably no inherent differences in the metabolic or secretory capacity of these regions, but more likely a genuine difference in the proportions of GA-sensitive cells.

From Figure 4 it is clear that the high-pI α-amylase isoforms are the cause of the differences in total secreted α-amylase activity. This group of α-amylase isoforms is thought to be much more highly induced by GA than the low-pI isoforms (Fig. 4; Jacobsen and Higgins, 1982; Rogers, 1985), so the difference in isoforms produced provides further evidence that the distal cells do not have a lower capacity for secretion but, rather, that regulation of specific GA-sensitive genes is responsible for the difference. Studies of the production and secretion of (1→3,1→4)-β-glucanases in barley seed tissue have demonstrated that the aleurone layer produces only isoform II (Stuart et al., 1986), and that secreted activity is enhanced significantly by the application of GA. Therefore, the differential levels of secreted (1→3,1→4)-β-glucanase activity seen in Figure 5 suggest a differential GA response, with the proximal region responding at lower GA levels with enhanced (1→3,1→4)-β-glucanase secretion.

There was no apparent difference in sensitivity to ABA between the ends of the aleurone layer (Fig. 7). The method we used to assess ABA sensitivity was inhibition of GA-stimulated α-amylase activity at saturating GA levels (1×10^{-6} M). We used this saturating [GA] to ensure that the levels of α-amylase production were the same throughout the layer before ABA treatment. Therefore, we cannot rule out the possibility that at lower [GA] there is a differential response to ABA between the proximal and distal regions of the layer. However, such analysis of the ABA response would be complicated by the differential GA response between the ends of the layer under these subsaturating [GA]. Another aspect to the ABA response of the aleurone layer is that a range of genes are up-regulated after ABA application (Bethke et al., 1997). It will be interesting to see if measurement of the transcription of these ABA-regulated genes in the proximal and distal tissue reveals spatial heterogeneity in ABA sensitivity in this alternative aspect of the ABA response of the aleurone layer.

It is possible that the spatial patterning of the GA responsiveness in the aleurone layer could reflect the patterns set up during aleurone development. During seed formation, the aleurone starts to form from the endosperm cells around the ventral groove, and then in other locations around the periphery of the endosperm. The left and right sides and the dorsal region of the aleurone layer originate separately (Kowles and Phillips, 1988; Bosnes et al., 1992). Therefore, the patterns of aleurone development might predict a dorsal/ventral or left/right difference in aleurone layer physiology, but not the proximal/distal differences seen in this study.

An alternative explanation for the differences in GA responsiveness between the different ends of the layer might be different residual levels of ABA between the ends. Levels of ABA increase during cereal seed development, and then decline as maturation occurs (King, 1976). ABA inhibits the GA response and so higher residual ABA levels in distal tissues could desensitize this region to GA. We think this hypothesis is unlikely because the distal region of the layer still responds to 3.3×10^{-9} M ABA. This is the concentration required to inhibit the proximal tissue to the level of the distal tissue and thus the level predicted to already be in the distal tissue (Fig. 8). This interpretation assumes that no active ABA uptake processes are accumulating ABA to levels above the 3.3×10^{-9} M in the medium. However, ABA levels have also been measured in aleurone tissue (Napier et al., 1989), and in fully mature seeds there was no detectable ABA in the aleurone despite the immunoassay used being sensitive to concentrations of 1×10^{-9} M. Taken together, these observations suggest that different levels of ABA are unlikely to account for the proximal-to-distal difference in GA sensitivity in the aleurone.

The heterogeneity of cereal aleurone cells in response to GA has already been suggested by several kinds of data. Immunolocalization of α-amylase in aleurone layers revealed that of the three layers of cells, the central layer gave the most intense signal after 16 h of treatment with 1×10^{-6} M GA (Jacobsen and Knox, 1973). In addition, both wheat aleurone cells and barley aleurone protoplasts showed several different patterns in the cytoplasmic calcium increase in response to 5×10^{-6} M GA (Bush, 1996; Gilroy, 1996). Hillmer et al. (1993) noted that after 48 h of treatment with 5×10^{-6} M GA, individual aleurone protoplasts were gradually recruited to secrete α-amylase, suggesting a heterogeneous population of cells. Schuurink et al. (1997) used a range of microscopic techniques to show patches of GA-responding cells in layers of a dormant barley variety. Finally, Hoecker et al. (1995) found spatial patterning in transcription factor activities in aleurone. In maize aleurone the transcription factor VP1 is involved in ABA-related repression of α-amylase production (Hoecker et al., 1995). In a line that contains a somatically unstable mutant allele of VP1, a mosaic pattern of precociously activated aleurone cells was found (Hoecker et al., 1995). The aleurone nearest the embryo contained more of these mutant sectors than areas away from the embryo, suggesting that these regions are somehow inherently different.

During germination of the cereal grain, GA is released from the embryo into the starchy endosperm. Thus, in a whole-seed scenario the aleurone cells nearer the embryo will be exposed to GA sooner than those farther away. It has been demonstrated using the accumulation of both α-amylase (Sugimoto et al., 1998) and (1→3,1→4)-β-glucanase (McFadden et al., 1988) mRNA as markers for the GA response that there is a wave of activation that travels through the seed after imbibition. Our results suggest that this wave of activation is imposed not only by the GA level but also by the proximal/distal gradient in aleurone sensitivity to GA. Endogenous GA levels in the ger-

minating seed (2–3 d after imbibition) have been estimated at 1 $\times 10^{-9}$ M to 20 $\times 10^{-9}$ M (Paleg et al., 1962; Cohen and Paleg, 1967; Radley, 1967; Murphy and Briggs, 1973; Gaskin et al., 1984), the range in which GA shows quantitative regulation of α-amylase production (Fig. 3) and the proximal/distal difference. The proximal/distal differential GA sensitivity is therefore likely to operate in the intact germinating grain, and most likely reflects a steady and continuous decline in GA sensitivity, from highly sensitive cells near the embryo to less-sensitive cells in the distal regions of the aleurone layer. Such a gradient may help to impose a steady and progressive mobilization of endosperm reserves starting near the embryo and progressing distally, and so ensure an extended supply of fuel for the processes of seed germination and early seedling growth.

The question remains regarding the cause of differential sensitivity between cells. At a molecular level, differential sensitivity could arise from different cells having different receptor types with varying affinities for GA, receptors with altered requirements for the time of receptor occupancy, or GA receptors that are posttranscriptionally or posttranslationally modified in some way (Rodbard, 1973; Hausdorff et al., 1990; Freedman and Lefkowitz, 1996). Alternatively, differential hormone sensitivities could be generated by cells containing different numbers of receptors (Rodbard, 1973). In the latter scenario, a GA receptor need not bind GA over the broad concentration range known to elicit tissue responses in plants, because it would be its abundance in the cell that determines each cell's threshold for response. Yet it may be a component downstream of the receptor that is variable and so leads to heterogeneity in the hormone response. There are some clues to the components of the signal transduction elements in the GA response of aleurone (Ritchie and Gilroy, 1998a), but we await identification of a GA receptor to probe the molecular basis of the sensitivity modulation in the aleurone cell.

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