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Visualizing Enzyme Secretion from Individual Barley (*Hordeum vulgare*) Aleurone Protoplasts[1]

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A method was developed to detect α-amylase gene expression and α-amylase secretion from individual barley (*Hordeum vulgare* L. cv Himalaya) aleurone protoplasts. Protoplasts are incubated in liquid media with or without hormones and embedded in a thin film of agarose and starch, where they remain viable for up to 24 h. α-Amylase secreted by individual protoplasts digests the starch, and starch hydrolysis is visualized after 45 min by staining the preparation with I_2KI. After I_2KI staining, secreting protoplasts are surrounded by a clear, starch-free halo visible by light microscopy. The formation of starch-free halos is dependent on the synthesis and secretion of α-amylase and is not caused by carry-over of preformed enzyme from incubation media. Treating protoplasts with inhibitors of protein synthesis or exposing them to anaerobic conditions for 2 h before embedding them in agarose prevents the formation of halos. When α-amylase secretion is observed by counting the percentage of secreting protoplasts, the data are comparable to that obtained by measuring α-amylase secretion from a population of cells. The response of individual protoplasts to gibberellic acid (GA_3) and abscisic acid measured by the thin-film method is almost identical to the response of populations of protoplasts to these hormones, validating the utility of this method. Although not generally practical for quantifying secretion, the thin-film method is uniquely useful in distinguishing secreting from nonsecreting protoplasts. In none of our experiments did more than 60% of the protoplasts secrete α-amylase when exposed to GA_3, even though more than 95% of the protoplasts in the preparations were viable. Similar results were obtained when the response to GA_3 was assayed at the level of gene transcription by visualizing the transient expression of a plasmid containing the promoter from α-amylase fused to the reporter gene glucuronidase in single protoplasts. The thin-film secretion assay also revealed that the response of a population of protoplasts to GA_3 was not uniform with time. The effect of GA_3 treatment was to gradually increase the percentage of responding protoplasts up to a maximum of 50 to 60%. Abscisic acid, which inhibits α-amylase secretion by GA_3-treated protoplasts, reduced the proportion of protoplasts that secrete the enzyme.

The aleurone layer of cereal grains is a digestive tissue that secretes a wide spectrum of acid hydrolases into the starchy endosperm (reviewed in Fincher, 1989). In the aleurone layer of barley (*Hordeum vulgare*), oat (*Avena sativa*), and wheat (*Triticum aestivum*), the synthesis and secretion of these hydrolytic enzymes is regulated by calcium and the plant hormones GA_3 and ABA. GA_3 stimulates the synthesis and secretion of hydrolases, including the α-amylases (EC 3.2.1.1), which make up as much as 70% of the newly synthesized proteins in the aleurone cell, and ABA inhibits these processes (Fincher, 1989; Jones and Jacobsen, 1991).

Protoplasts isolated from the aleurone layer of barley and oat are a useful experimental system for the study of hormone-regulated enzyme production because they retain their responsiveness to GA_3 and ABA (Hooley, 1982; Jacobsen and Beach, 1985) and are amenable to manipulations that are not possible with walled cells. These manipulations include the isolation of functional, intact nuclei (Jacobsen and Beach, 1985; Zwar and Hooley, 1986), the introduction of macromolecules such as DNA and RNA by permeabilization with PEG (Huttley and Baulcombe, 1989; Gopalakrishnan et al., 1991; Jacobsen and Close, 1991), the patch clamping of the plasma membrane (Bush et al., 1988b), and other experimental approaches that require direct access to the plasma membrane, such as probing for surface receptors (Hooley et al., 1991). The ability to transform aleurone protoplasts with reporter-gene constructs, for example, has led to the identification of both GA_3- and ABA-regulatory sequences in gene promoters (Huttley and Baulcombe, 1989; Gopalakrishnan et al., 1991; Jacobsen and Close, 1991; Lanahan et al., 1992). Protoplasts have also been used to show that the receptors for GA_3 are likely localized at the plasma membrane of oat aleurone cells (Hooley et al., 1991).

Although protoplasts offer several unique advantages, they suffer from certain limitations, most notably that, as with intact aleurone tissue, measurements of the synthesis and secretion of α-amylase must be made on populations of cells (Bush et al., 1988a). The secretory behavior of a single cell must, therefore, be extrapolated from the behavior of the population. This poses a serious limitation on experiments where parameters in single cells are being determined. For example, where changes in the concentrations of Ca^{2+} and H^+ in an individual protoplast following hormone treatment are measured (Bush and Jones, 1987, 1990; Bush et al., 1988a; Gilroy and Jones, 1992), the secretory status of the individual protoplast is not known. Although the viability of protoplasts

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Abbreviations: B5/mannitol, modified Gamborg's B5 medium containing 0.6 M mannitol; CH, cycloheximide; CHA, cycloheptaamylose; FDA, fluorescein diacetate; GM, gel matrix; GUS, glucuronidase; LUX, luciferase; RT, room temperature.

in a freshly isolated population is around 70% (Huang et al., 1986), it is not known whether all or a subpopulation of these protoplasts respond to GA_3 and ABA, or whether the time course of the response to these hormones is the same among individual protoplasts and the population. There are now many examples from both animals and plants where the response to hormones of apparently identical cells is asynchronous or heterogeneous when visualized at the single-cell level (Downton et al., 1988; Cheek et al., 1989; Terashami et al., 1989; Tsien and Tsien, 1990; Gilroy et al., 1991).

In this paper, we describe a method that permits the visualization of α-amylase secretion from individual barley aleurone protoplasts. Protoplasts are incubated in liquid media with or without hormones and then immobilized in an agarose gel that contains starch. The α-amylase secreted from individual protoplasts diffuses into the gel, digesting the starch. Starch hydrolysis is visualized after 45 min by staining the preparation with I_2KI, which stains undegraded starch. Protoplasts that secrete α-amylase are surrounded by a clear, starch-free halo. Because our method immobilizes aleurone protoplasts in an agarose matrix, it greatly facilitates other manipulations of single cells, such as microinjection. This has allowed us to transfect protoplasts by microinjecting them with a plasmid containing the α-amylase promoter fused to the reporter gene GUS. We have used this transient expression system to visualize the regulation of α-amylase gene transcription in response to GA_3 in single protoplasts. Thus, the thin-film method permits measurement of the hormonal response of individual barley aleurone protoplasts at the level of transcription and protein secretion.

MATERIALS AND METHODS

Protoplast Isolation

Barley grains (*Hordeum vulgare* L. cv Himalaya; Department of Agronomy, Washington State University, Pullman, WA) were deembryonated, cut into quarters, and prepared for protoplast isolation as described by Bush et al. (1986), except that cell-wall digestion was carried out in solutions exposed to air rather than N_2. Freshly isolated protoplasts were incubated in the dark at 25°C in B5/mannitol and 10 mM $CaCl_2$ in the presence or absence of either 5 μM GA_3 or 5 μM ABA, or both GA_3 and ABA. After incubation, protoplasts were purified on a Nycodenz density gradient (Bush and Jones, 1988), filtered through 88-μm nylon mesh, and diluted with fresh B5/mannitol to approximately 30 protoplasts/μL.

Monitoring α-Amylase Secretion from Individual Protoplasts

Protoplasts were embedded in a GM containing 3% (w/v) low-melting-point agarose (Boehringer-Mannheim, Indianapolis, IN) and 2.75% (w/v) soluble potato starch (Baker Chemical Co., Philadelphia, PA) in B5/mannitol. The agarose and starch were mixed with the B5/mannitol in a closed plastic vial at RT to avoid clumping. The GM was heated to boiling in a microwave oven, and 4 mL were transferred to a 10-mL glass beaker and stirred in a water bath (40°C) on a magnetic stirrer to prevent gelling. A 30-μL drop of warm GM was placed about 1 cm from the frosted end of a microscope slide on a glass plate held at 38°C, the lowest temperature at which the GM remained liquid. Two microliters of a purified protoplast suspension kept at RT were added to the GM and quickly mixed with a plastic micropipet tip for 3 s. A 22-mm coverglass, prewarmed to 38°C, was placed on the GM, which was allowed to spread for 10 s to form an even, 70-μm thick layer of protoplasts in the agarose/starch. The mixing of protoplasts with GM and the formation of a thin layer of GM by the weight of the coverglass was never allowed to exceed 30 s to assure that protoplasts were not heat shocked (Belanger et al., 1986). The slide was removed from the warm glass surface and incubated in a moist chamber at RT for 10 to 45 min. Slides to be stained for α-amylase secretion were transferred to a cool (4°C) metal surface for 2 min to solidify the gel and facilitate removal of the coverglass. The coverglass was removed by sliding it toward the frosted end of the slide, thereby preventing deformation of the gel. Staining was performed with 100 μL of I_2KI solution (6 g of KI, 0.6 g of I_2 in 100 mL of H_2O). After 10 s, excess staining solution was poured off by tilting the slide and blotting the edges with absorbant paper. The slide was then destained in 125 mL of H_2O for 30 s, and a coverglass was applied to prevent the gel from drying. This procedure stains undigested starch dark blue and reveals clear, starch-free halos around protoplasts that have secreted α-amylase (Fig. 1). Halos were counted by viewing the slide with either a dissecting microscope or with a compound microscope with bright-field optics at low magnification (Nikon FL 10× objective).

Testing Viability of Protoplasts in Gels

Protoplasts embedded in the GM were stained with FDA (50 μg mL^{-1} in B5/mannitol prepared from a stock solution of 100 mg mL^{-1} of FDA in DMSO) for 2 min (Huang et al., 1986) prior to examination with an inverted fluorescence microscope (Nikon) equipped with a fluorescein filter set (excitation λ 420–480 nm, dichroic mirror λ 520 nm, emission $\lambda > 520$ nm).

Transient Expression in Protoplasts

Protoplasts were transformed by microinjection with two plasmid constructs, JR249 and pAHC18. JR249 is the amylase promoter fused to GUS (Lanahan et al., 1992). pAHC18 is the ubiquitin promoter fused to LUX (Bruce et al., 1989), which is constitutively expressed in barley aleurone cells (Lanahan et al., 1992). Thus, the activity of GUS indicated the activity of the amylase promoter and LUX indicated the efficiency of transformation. Results are expressed as the ratio of GUS:LUX activity to normalize for variations in transformation efficiency between protoplasts (Bruce et al., 1989; Bruce and Quail 1990; Lanahan et al., 1992). Micropipettes (10 MΩ resistance) were pulled from filament electrode glass (World Precision Instruments, New Haven, CT) using a List model 5A electrode puller (List Electronics, Darmstadt, Germany). Microelectrodes were filled with 50 μM Lucifer Yellow, 12.5 μg μL^{-1} of pAHC18, and 2.5 μg μL^{-1} of JR249.

Protoplasts embedded in GM were impaled and pressure injected using a model SB2 microburet (Micrometric Instruments Co., Cleveland, OH). The injected volume was monitored from the amount of Lucifer Yellow fluorescence injected according to Gilroy et al. (1991).

Transformed protoplasts contained approximately 5 pg of JR249 plasmid and 25 pg of pAHC18. After a 24-h incubation in GM with 5 μg mL^{-1} of nystatin and ampicillin, 25 μM Imagene Green GUS substrate and 50 μM luciferin were added and the protoplasts were further incubated for 8 h. The fluorescent product of GUS activity on the Imagene substrate (Haugland, 1992) and LUX luminescence was monitored in single protoplasts using a Nikon Diaphot fluorescence microscope and photon-counting photomultiplier system (Gilroy and Jones, 1992). For Imagene Green, the excitation wavelength was determined using a 485-nm (10-nm half band width) interference filter and 1/8 transmission neutral density filter. Emission filtration consisted of a 510-nm dichroic mirror and 535-nm (10-nm half band width) interference filter. LUX luminescence measurements were made using a 400-nm dichroic mirror and no emission filter.

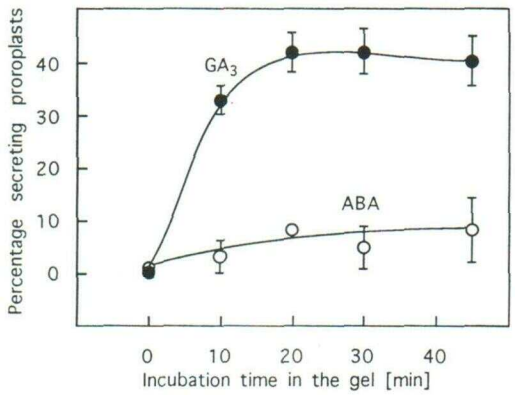

Figure 2. The effect of incubation time in GM on the percentage of secreting protoplasts that can be visualized. Protoplasts were incubated in 10 mM $CaCl_2$ with 5 μM GA_3 or 5 μM ABA for 24 h and embedded in GM. After the specified time of incubation at RT, gels were stained with I_2KI reagent and the percentage of protoplasts showing cleared halos of degraded starch was determined. Results represent mean ± SE; $n \geq 700$ protoplasts monitored in three experiments.

RESULTS AND DISCUSSION

The thin-film method that we have developed permits the visualization of α-amylase secretion in single barley aleurone protoplasts by monitoring the degradation of starch from the agarose matrix in which they are embedded (Figs. 1 and 2). This degradation is visible after 10 min of incubation in the gel (Fig. 2), but in general it is more easily observed after incubations of up to 45 min. Incubations longer than 2 h led to complete clearing of the gel. Although immobilization of protoplasts in low-melting-point agarose has been used to culture cells for callus formation and plant regeneration (Townsend and Adams, 1983), to our knowledge this approach has not been used to monitor parameters such as enzyme secretion. In addition to enabling the study of secretion from single protoplasts, immobilizing these protoplasts in thin-agarose films facilitates other manipulations, such as microinjection, which require their positions to be fixed.

Visualizing α-Amylase Secretion from Single Aleurone Protoplasts

In assessing the usefulness of this thin-film method, it is essential to prove that the characteristics of secretion visualized at the single protoplast level parallel what is known about secretion from aleurone tissue. We have used several approaches to establish that the digestion of starch in the GM is brought about by α-amylase that is synthesized and

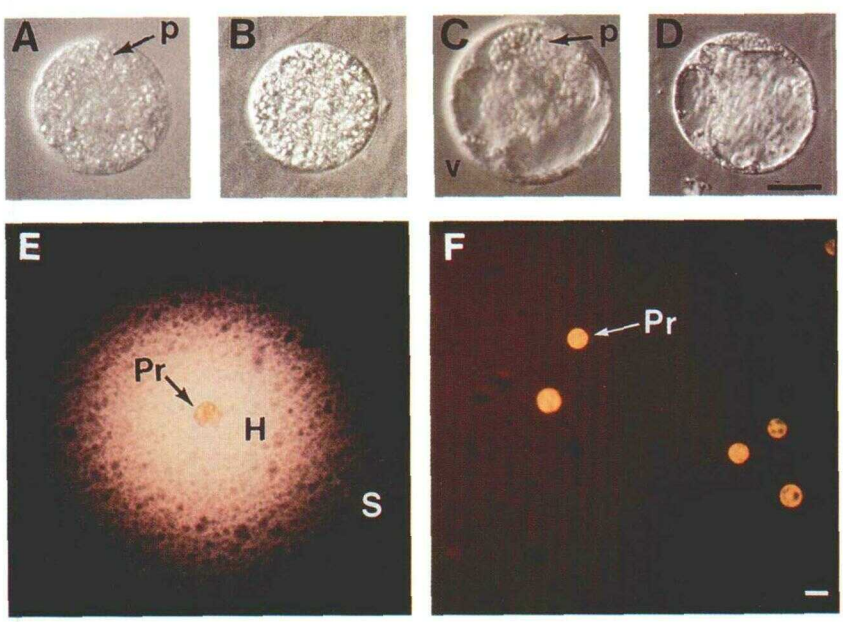

Figure 1. Starch-iodine, bright-field, and FDA staining of protoplasts embedded in starch/agarose thin films. Protoplasts were incubated for 18 h with (C and D) or without (A and B) GA_3. Protoplasts were then applied to slides and photographed before (A and C) and after (B and D) they were embedded in GM, and after incubating the gel for 45 min and staining with I_2KI reagent (E) or FDA (F). S, Starch stained blue by I_2KI; H, unstained halo of degraded starch; Pr, protoplast; p, phytic acid crystal; v, vacuole. Note that protoplasts embedded in the gel matrix (B and D) are morphologically indistinguishable from those in B5/mannitol (A and C) and also show the vacuolation characteristic of the response to GA_3. Scale bar = 20 μm (A–D) and 50 μm (E and F).

secreted from aleurone protoplasts. First, we have shown that secretion of α-amylase by aleurone protoplasts is dependent on sustained protein synthesis by these cells. α-Amylase synthesis and secretion from barley aleurone layers is dependent on the de novo synthesis of the protein (Filner and Varner, 1967), and inhibitors of protein synthesis, such as CH, inhibit the secretion of α-amylase from intact aleurone layers with a lag of several hours (Chrispeels and Varner, 1967; Varner and Mense, 1972). When protoplasts that had been preincubated in GA_3 for 18 h were incubated with as little as 2.5 μg mL^{-1} of CH for 2 h prior to embedding them in GM, the number of α-amylase-secreting protoplasts decreased from 45% in the absence of CH to 10% in its presence (Fig. 3). These data establish that in the thin-film method, as in vivo, the secretion of α-amylase is not sustained in the absence of de novo protein synthesis (Fig. 3). Incubation in CH for 45 min or less did not reduce the number of secreting protoplasts (data not shown), supporting previous observations that the pool of newly synthesized α-amylase in the aleurone cell can support secretion for up to 2 h (Varner and Mense, 1972).

Second, we have established that the synthesis and secretion of α-amylase by protoplasts embedded in thin agarose layers is dependent on aerobic metabolism. Protoplasts preincubated in GA_3 for 18 h under aerobic conditions were incubated in GM under aerobic or anaerobic conditions and secretion was monitored (Fig. 4). When GA-treated protoplasts were embedded in GM that was saturated with N_2 and incubated for 40 min in a N_2-saturated atmosphere, only 18% of the protoplasts were surrounded by halos, whereas 42% of the protoplasts incubated under aerobic conditions had halos (Fig. 4). Anoxia had no effect on the small number of protoplasts that secreted α-amylase in the absence of GA (Fig. 4).

Secretion from aleurone cells is known to be an energy-dependent process that is inhibited by anoxic conditions (Varner and Mense, 1972; Moll and Jones, 1982), although anoxia has a more pronounced effect on α-amylase secretion from aleurone protoplasts than from aleurone layers. Treatment of aleurone layers with inhibitors of oxidative metabolism causes a transient increase in α-amylase release from the layer before an inhibition of secretion becomes apparent (Varner and Mense, 1972; Moll and Jones, 1982). The transient stimulation of α-amylase release from layers was attributed to the release of α-amylase from the cell wall, a process that has confounded studies of secretion from walled tissue (Varner and Mense, 1972; Moll and Jones, 1982). Our experiments on the inhibitory effects of anoxia on α-amylase release from aleurone protoplasts support the interpretation that the initial stimulation of α-amylase release from aleurone layers by inhibitors of oxidative metabolism represents loss of enzyme from the apoplast. Thus, anoxia does not stimulate secretion from wall-less protoplasts; rather, secretion is rapidly inhibited by this treatment (Fig. 4).

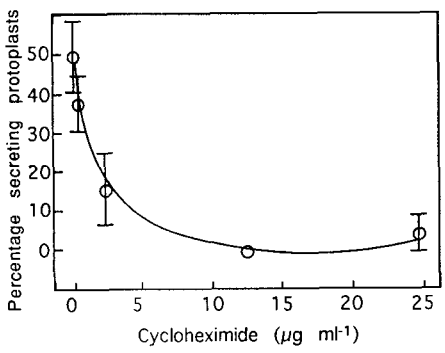

Figure 3. The effect of CH on secretion of α-amylase from single aleurone protoplasts. Protoplasts were treated for 18 h in 10 mM $CaCl_2$ and 5 μM GA_3 and then for 2 h with the indicated concentrations of CH and embedded in GM. After 45 min, the gels were stained for starch with I_2KI and the percentage of protoplasts surrounded by a clear halo determined. Results represent mean ± SE, $n = 3$.

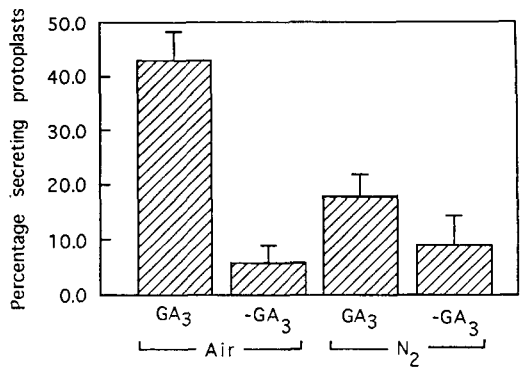

Figure 4. The effect of anoxia on percentage of secreting protoplasts. Protoplasts that had been treated for 18 h with 10 mM $CaCl_2$ and with or without 5 μM GA were incubated for 2 h under a stream of N_2 gas. All solutions were degassed before use by bubbling for 10 min with N_2. The protoplasts were purified on a Nycodenz gradient into fresh B5/mannitol, embedded in GM, and stained with I_2KI after 45 min of incubation under a stream of N_2. Control protoplasts were treated identically except that all incubations were performed in air and solutions were not degassed prior to use. Results represent mean ± SE, $n = 3$.

Third, we have shown that the digestion of starch by protoplasts embedded in GM is not caused by the carry-over of extracellular α-amylase from the medium in which protoplasts were preincubated. To establish that starch digestion did not result from adherence of α-amylase to protoplasts, GA_3-treated protoplasts, as well as protoplasts preincubated under conditions that do not induce high amounts of α-amylase secretion, i.e. the absence of GA_3 or the presence of ABA, were incubated in a medium containing high levels of secreted barley α-amylase for 2 h prior to embedding them in GM. Control protoplasts were incubated for 2 h in a medium containing only B5/mannitol. After incubation for 2 h in the presence or absence of α-amylase, protoplasts were washed briefly with B5/mannitol and incubated in the GM for 45 min (Fig. 5). When compared with controls, incubation of protoplasts in high concentrations of α-amylase did not increase the number that had halos (Fig. 5). Thus, there is no evidence that carry-over of α-amylase from the incubation

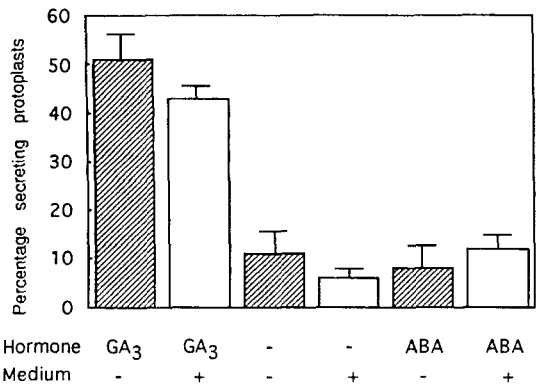

Figure 5. The effect of carry-over of α-amylase on the percentage of protoplasts secreting α-amylase visualized by thin films. Protoplasts were incubated for 18 h with or without GA_3 or ABA, then purified by flotation through a Nycodenz gradient into fresh medium or into the medium in which protoplasts had been incubated for 18 h with GA_3 and which, therefore, contained high levels of α-amylase (7.1 ± 0.6 units mL^{-1}). All media contained 10 mM $CaCl_2$. Protoplasts were then incubated for 2 h, repurified, and embedded in GM. The thin films were then incubated for 45 min, stained with I_2KI, and the percentage of protoplasts surrounded by a halo of degraded starch was determined. Results represent mean ± SE.

medium is responsible for the digestion of starch in the GM surrounding the protoplasts.

Our experiments establish that digestion of starch in the GM is a result of the activity of newly synthesized α-amylase that is secreted from the cell along a pathway that is dependent on aerobic metabolism (Figs. 3–5).

Measuring α-Amylase Secretion from Single Aleurone Protoplasts

We used two approaches to show that the thin-film method can be used to measure α-amylase secretion from single barley aleurone protoplasts. We showed that the size of the starch-free halo in the GM can be correlated with the amount of α-amylase diffusing from a spherical source. We loaded α-amylase affinity beads (CHA-Sepharose) with diameters similar to aleurone protoplasts (50 μm for CHA-Sepharose 4B beads, 40 μm for aleurone protoplasts) with secreted barley α-amylase and embedded them in GM containing 10 mg mL^{-1} of CHA, which displaces α-amylase from the affinity matrix (Silvanovich and Hill, 1976). The beads were incubated in increasing concentrations of barley α-amylase from 0 to 1 mg mL^{-1} for 15 min, washed free of unbound enzyme, and embedded in GM. The beads released their bound amylase in the GM in the presence of CHA, mimicking amylase secretion from protoplasts.

After a 15-min incubation in GM, the diameter of the halo surrounding the resin bead increased with the amount of α-amylase loaded onto the bead (Fig. 6A). The relationship between halo diameter and α-amylase concentration was not linear, however (Fig. 6A). Because the volume of the GM into which α-amylase diffuses increases with approximately the cube of the distance from the surface (volume of sphere = $4/3\pi r^3$; volume of hemisphere = $2/3\pi r^3$) of the resin bead, the size of the starch-free halo did not increase linearly as the concentration of α-amylase increased (Fig. 6A). We also measured halo diameter with time of incubation of protoplasts in GM and observed a similar nonlinear relationship (Fig. 6B). Although the diameter of the halo surrounding secreting protoplasts increased with time of incubation, the rate at which it increased declined with time (Fig. 6B).

The dependence of halo size on α-amylase concentration (Fig. 6A) and incubation time (Fig. 6B) indicates that the thin-film technique can provide a semi-quantitative measure of enzyme secretion from single cells. In practical terms, however, the nonlinear relationship of α-amylase concentration to halo diameter and the difficulty in precisely identifying the edge of the starch-free halo (see Fig. 1E) make quantitation of α-amylase secretion by measurement of halo diameter impractical. Therefore, we have used this method primarily to distinguish secreting and nonsecreting protoplasts in samples incubated in GM for short, fixed time periods (Figs. 2, 7). Forty-five-minute incubations in GM were chosen as

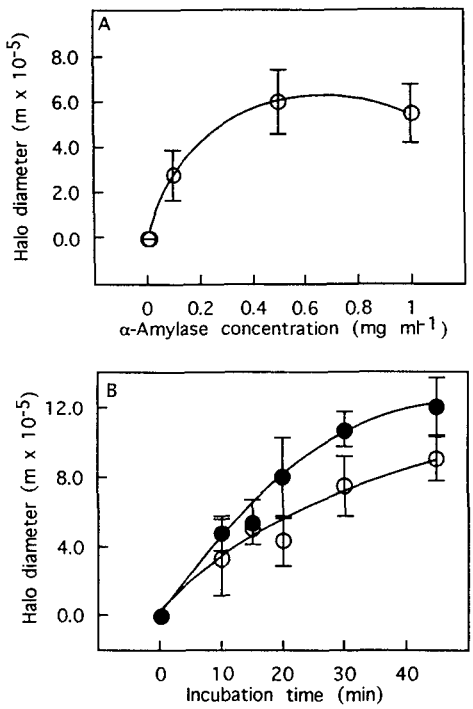

Figure 6. The effect of α-amylase concentration (A) and time of incubation (B) on halo size. A, α-Amylase affinity beads (CHA-Sepharose, bead size 20–50 μm) were incubated for 15 min in various concentrations of barley α-amylase (Sigma, type VIII-A from barley malt), washed twice by sedimentation and resuspension in fresh B5/mannitol, and embedded in GM containing 10 mg mL^{-1} of CHA. Halos produced by diffusion of α-amylase from the beads were measured from photographs of the gels taken after 30 min of incubation at RT and staining with I_2KI. B, Protoplasts were incubated in 10 mM $CaCl_2$ with (●) or without (○) 5 μM GA_3 for 18 h and embedded in GM. After the appropriate time of incubation at RT, starch-free halos were visualized by I_2KI staining and halo diameters were measured from photographs. Protoplasts that did not develop halos were excluded from the analysis. Results represent mean ± SE, $n = 3$.

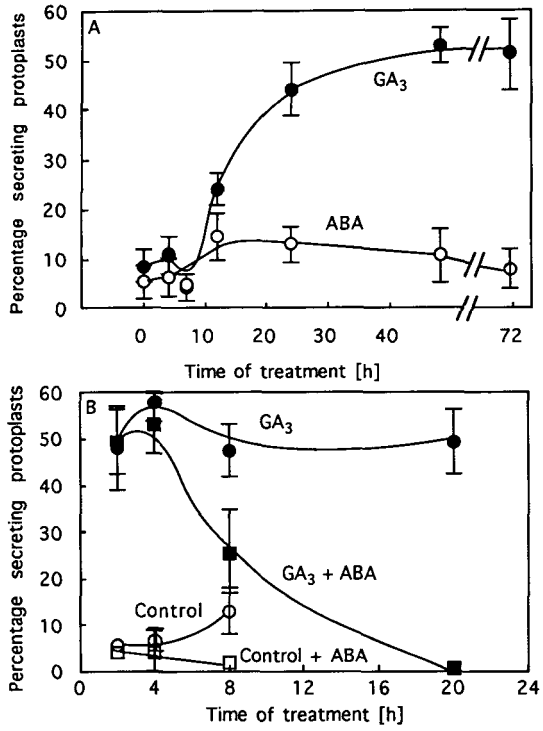

Figure 7. The effect of time of treatment with GA_3 and ABA on percentage of secreting protoplasts. A, Protoplasts were treated with 10 mM $CaCl_2$ with 5 μM GA_3 or 5 μM ABA. At the specified times, samples were removed, embedded in GM, and incubated for 45 min at RT. The thin films were then stained with I_2KI and the percentage of protoplasts surrounded by a clear halo of degraded starch was determined. B, Protoplasts were treated for 24 h with 10 mM $CaCl_2$ without (control) or with 5 μM GA_3. ABA (5 μM) was then added to these protoplasts, and at appropriate time points samples were removed, embedded in GM, and incubated for 45 min at RT. The thin films were then stained with I_2KI and the percentage of protoplasts secreting α-amylase was determined. Results represent mean ± SE.

standard because it was enough time to form distinct clear halos of ≥100 μm diameter around the secreting protoplasts. When the response of protoplasts to GA is expressed as a percentage of responding protoplasts, the results are comparable to those obtained by measuring halo diameter (Figs. 2 and 6B) or by measuring α-amylase secreted from populations of protoplasts (Bush et al., 1986, and data not shown).

Measuring α-amylase secretion by counting the percentage of secreting protoplasts also correlates positively with treatments that are known to affect α-amylase synthesis and secretion by aleurone protoplasts (Fig. 7). Thus, GA_3 stimulates a 5- to 10-fold increase in α-amylase synthesis and secretion and also in the percentage of protoplasts that secrete α-amylase compared with protoplasts incubated in ABA or in the absence of hormone (Fig. 7). Similarly, when protoplasts that had been incubated in GA_3 for 24 h were treated with ABA, the percentage of secreting protoplasts decreased within 20 h from 50 to 2% (Fig. 7B), directly paralleling the effect of ABA on amylase synthesis and secretion measured at the population level (Bush et al., 1993).

Not All Aleurone Protoplasts Respond to GA_3

At any one time, not all barley aleurone protoplasts respond to GA_3 by secreting α-amylase. In all of our thin-film experiments, only 50 to 60% of protoplasts preincubated in GA_3 for as long as 72 h responded to the hormone by secreting α-amylase (Fig. 7). Similarly, when the effect of GA_3 was monitored at the level of amylase gene expression using transient expression of the amylase promoter-GUS reporter gene, approximately 50% of protoplasts responded to the hormone by inducing GUS production (Fig. 8). This lack of response to GA_3 by as many as half of the protoplasts in a population cannot be explained on the basis of cell viability, since FDA staining showed that more than 90% of protoplasts were still alive following incubation in GM for 45 min (Fig. 1, Table I). Indeed, viability was maintained in the gel for at least 32 h (FDA staining: initial viability = 77 ± 9%; viability at 32 h = 73 ± 12%). Protoplasts incubated in GM also showed similar morphology to those not embedded in GM (Fig. 1).

It is well established that one of the responses of barley aleurone cells to GA_3 is an increase in the level of transcription of α-amylase genes (reviewed in Fincher, 1989). To determine the effect of GA_3 on gene expression in individual protoplasts, we microinjected protoplasts with a plasmid (JR249) containing the hormone-response elements of the α-amylase gene fused to the GUS reporter gene (Gopalakrishnan, 1991; Jacobsen and Close, 1991; Lanahan et al., 1992). We used the fluorescent GUS substrate Imagene Green to

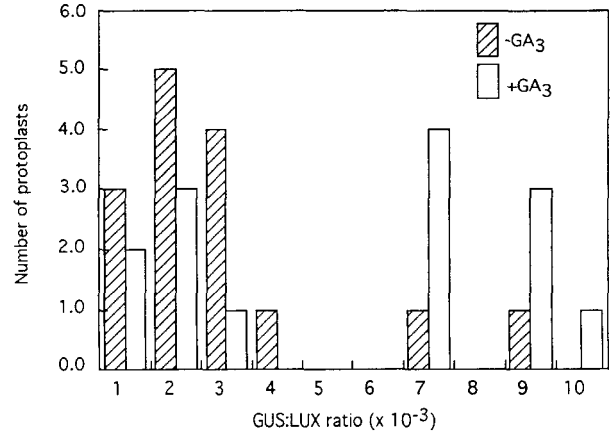

Figure 8. The effect of GA_3 on the expression of α-amylase promoter-GUS reporter gene. Freshly isolated protoplasts were embedded in GM and microinjected with approximately 5 pg of JR249 plasmid (barley α-amylase promoter linked to GUS) and 25 pg of pAHC18 (maize ubiquitin promoter linked to LUX). After 24 h of incubation with or without GA_3, 25 μM Imagene Green GUS substrate and 50 μM luciferin were added and the sample was further incubated for 8 h. GUS and LUX activity in single protoplasts were monitored using a fluorescence microscope and photon-counting photomultiplier system. Results are expressed as the ratio of GUS:LUX activity to normalize for variations in transformation efficiency between protoplasts. Absolute LUX luminescence was in the range 1000 to 4000 counts s^{-1}; GUS fluorescence was approximately 1000 kcounts s^{-1}. Results represent data from 29 individual protoplasts assayed in five separate experiments.

Table I. *Viability of protoplasts embedded in starch-agarose thin films compared with unembedded controls*

Protoplasts were incubated for 24 h in B5/mannitol with 10 mM $CaCl_2$ with or without 5 μM GA_3 or 5 μM ABA. The protoplasts were then embedded in GM, incubated for 45 min at RT, stained for viability using FDA, and viewed using a Nikon Diaphot fluorescence microscope and fluorescein filters. Control protoplasts were treated identically except that they were not embedded in GM prior to staining with FDA. The viability of control protoplasts was 76 ± 9% irrespective of hormonal treatment. This value was used as 100% relative viability in the table. Results represent mean ± SE, $n = 3$.

Treatment	Viability
	%
Ca^{2+}	94 ± 4
Ca^{2+} + GA_3	90 ± 6
Ca^{2+} + ABA	93 ± 3

visualize the expression of the α-amylase promoter. To indicate the efficiency of transformation, we simultaneously injected protoplasts with a plasmid containing the ubiquitin promoter fused to LUX (pAHC18; Bruce et al., 1989), a construct that is constitutively expressed in the aleurone cell (Lanahan et al., 1992), and visualized the activity of LUX with luciferin. GUS:LUX ratio (Fig. 8) indicated that only 50% of the protoplasts responded to GA_3 by increasing amylase gene transcription, even though all protoplasts expressed the ubiquitin promoter-LUX construct, confirming that all were viable.

Thus, whether assayed at the level of gene transcription or at the level of protein secretion, only about half of the protoplasts in a population responded to GA_3 at any one time. Although we cannot completely exclude the possibility, failure of all protoplasts to respond to GA_3 is unlikely to reflect damage resulting from protoplast isolation. In other respects isolated protoplasts respond as intact aleurone layers (Hillmer et al., 1990), and other workers have reported that not all cells in the intact barley aleurone layer respond to GA_3 (Ashford and Jacobsen, 1974) or show evidence of vacuolar protein mobilization following malting (B. Bakhuizen, personal communication).

Taken together, these observations indicate that the ability of aleurone cells to respond to GA_3 at any one time may be limited to a subpopulation of cells in the layer. Although the reasons why some viable aleurone cells do not respond to GA_3 are not known, it may reflect developmental differences within the layer with respect to, for example, the position of the aleurone cell relative to the embryo. Developmental differences may be temporally expressed, so that not all cells in a population of protoplasts are capable of responding to GA_3 at the same time. Despite these possibilities, the response of single protoplasts was limited to approximately 50% even after incubations as long as 72 h (Fig. 7A). It is interesting that 5 to 10% of non-GA_3-treated or ABA-treated protoplasts secrete α-amylase (Fig. 7). This could represent a population of cells that are responding to some factor other than GA_3 and may account for the small but highly reproducible GA_3-independent secretion of α-amylase seen in barley aleurone tissue.

Whatever its origin, the fact that not all cells in the aleurone layer respond to GA_3 with increased α-amylase secretion has important implications for the malting industry, where high α-amylase production is required to produce high-quality malt for brewing.

GA_3 Recruits Protoplasts from a Nonsecreting Population

Perhaps the most interesting aspect of the response of aleurone cells to GA_3 is the way that members of a population of protoplasts respond to the hormone. The effect of GA_3 treatment is to increase the proportion of protoplasts in the population that secrete α-amylase rather than gradually increasing secretion from all responding protoplasts. This phenomenon is most clearly demonstrated in a time-course experiment (Fig. 7A). Thus, with increasing time of exposure to GA_3, the proportion of protoplasts in the population that respond to the hormone by synthesizing and secreting α-amylase increases gradually to a maximum of 50 to 60% after about 24 h (Fig. 7A). That GA_3 recruits more protoplasts in the population to synthesize and secrete α-amylase is also shown by our experiments comparing the effects of GA_3 and ABA on enzyme secretion. In a population of protoplasts preincubated for up to 24 h in the absence of GA_3 or in the presence of ABA, the number of α-amylase-secreting protoplasts with starch-free halos is generally less than 10% of all viable protoplasts, compared with more than 50% of protoplasts following GA_3 treatment (Fig. 7A).

The response of aleurone protoplasts to GA_3 and ABA has also served to validate the thin-film method as an indicator of secretory activity from individual aleurone cells. The magnitude of the response to GA_3 and ABA indicated by the proportion of protoplasts surrounded by halos is almost identical to that obtained by measuring the activity of the enzyme released into the medium from a population of aleurone cells (Fig. 7). In addition to showing that GA_3 stimulates the synthesis and secretion of α-amylase 8- to 12-fold above cells incubated in ABA or in the absence of GA_3, we have demonstrated the reversal of the effects of GA_3 by ABA (Fig. 7B). When protoplasts preincubated for 24 h in the presence or absence of GA_3 were transferred to media containing ABA, fewer protoplasts secreted α-amylase (Fig. 7B). The time course of the effect of ABA measured by the thin-film method is almost identical to that observed in isolated aleurone layers or batches of aleurone protoplasts (Bush et al., 1993).

CONCLUSIONS

We have developed a method that permits the visualization of α-amylase secretion from single barley aleurone protoplasts by monitoring the digestion of starch from the agarose matrix in which the protoplasts are embedded. Protoplasts embedded in GM are viable and continue to secrete α-amylase. Using this method, we have shown that GA_3 increases the number of protoplasts in a population that synthesize and secrete α-amylase. Thus, with increased duration of exposure to GA_3, a greater number of protoplasts are recruited to respond by secreting α-amylase. We have also shown that not all protoplasts in a population of protoplasts

are recruited to secrete α-amylase by GA_3, since only a subpopulation of protoplasts respond to the hormone at both the level of enzyme secretion and the level of gene transcription. Such heterogeneity is becoming a well-known, if not well-understood, phenomenon of many cellular responses when assayed at the single-cell level. Thus, data from single cells reveal complexities of the GA_3 response in aleurone cells that are not apparent when measuring the response of populations of cells. We have chosen to use α-amylase as a well-characterized marker of secretion from aleurone cells. However, the thin-film approach has great potential for investigations at the single-cell level of the regulation of secretion of other enzymes, for example proteases, for which a suitable substrate can be incorporated into the agarose GM. We anticipate that the combination of this thin-film technology and the microinjection of putative regulatory elements such as Ca^{2+} and calmodulin will provide exciting insights into the events between hormonal perception and modulation of secretory activity in the aleurone cell.

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