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# Perception of Gibberellin and Abscisic Acid at the External Face of the Plasma Membrane of Barley (*Hordeum vulgare* L.) Aleurone Protoplasts[1]

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**The response of protoplasts isolated from aleurone layers of barley (*Hordeum vulgare* L. cv Himalaya) to internally and externally applied hormone was analyzed to localize the site of perception of the hormonal signal. Protoplasts responded to externally applied gibberellic acid ($GA_3$) with increased synthesis and secretion of α-amylase, transient expression of the glucuronidase reporter gene fused to the hormone-responsive elements of the α-amylase promoter, and the vacuolation typical of $GA_3$-treated aleurone cells. When up to 250 μM $GA_3$ was microinjected into the protoplast cytoplasm, none of these responses were observed. This did not reflect damage to the protoplasts during the microinjection procedure, since microinjected protoplasts remained responsive to externally applied hormone. Nor did it reflect loss of microinjected $GA_3$ from the protoplast, since 50% of microinjected [$^3$H]$GA_{20}$ was retained by protoplasts for at least 24 h. Externally applied abscisic acid (ABA) could reverse the stimulation of α-amylase synthesis and secretion, whereas microinjecting up to 250 μM ABA was ineffective at antagonizing the stimulatory effect of $GA_3$. These results suggest that the site of perception of $GA_3$ and ABA in the barley aleurone protoplast is on the external face of the plasma membrane.**

An understanding of where and how plant hormones are perceived at the cellular level is essential to the development of approaches to isolate their receptors, to the characterization of their transduction, and to an understanding of how these hormones could regulate physiological processes of the plant. The identification of the cellular site of plant hormone perception has proved problematical, however. Plant growth regulators such as GA, ABA, and auxin are small organic acids that can relatively easily cross biological membranes in their uncharged forms. When added to the apoplast these hormones can readily enter the cell and could be perceived by internal receptors, as are animal steroid hormones. They could also interact with receptors on the external face of the plasma membrane.

The evidence from experiments using chemically synthesized, impermeant forms of auxin (Venis et al., 1990) and antibodies to auxin-binding proteins (Barbier-Brygoo et al., 1989, 1991; Martin et al., 1991; Venis et al., 1992) suggests that auxin is perceived at the plasma membrane. Electrophysiological studies have also indicated that auxin may directly interact with channels in the plasma membrane (Martin et al., 1991) or through modulation of $H^+$-ATPase activity (Barbier-Brygoo et al., 1989, 1991). An auxin-binding protein that may be an auxin receptor has been identified (Feldwisch et al., 1992; Venis et al., 1992). This protein has been found in the ER, plasma membrane, and extracellular space of maize (Feldwisch et al., 1992; Jones and Herman, 1993), and plasma membrane auxin-binding proteins are found in the plasma membrane of *Zucchini* (Lomax and Hicks, 1992; Hicks et al., 1993). However, definitive evidence for the role(s) of these proteins is lacking. The identification of GA and ABA receptors is equally equivocal.

The biological activity of impermeant GAs (Hooley et al., 1991; Smith et al., 1993) and anti-idiotypic antibodies and the lack of pH dependency of GA action in aleurone cells have been taken as evidence that a surface receptor may be important for GA action (Hooley et al., 1992). Although photoaffinity labeling of GA-binding proteins in oat aleurone has identified several proteins that bind GA (Hooley et al., 1992), these may include not only GA receptors but also GA metabolic enzymes. For example, photoaffinity labeling of *Phaseolus vulgaris* cotyledon preparations has led to the partial purification of a putative GA 2β-hydroxylase (Hooley et al., 1992), an enzyme involved in GA metabolism.

ABA has been shown to be active on guard cells at high extracellular pH where uptake would be expected to be negligible (Hartung, 1983; Baier and Hartung, 1991). However, other studies have reported pH dependency of ABA action, suggesting that uptake is required for ABA action (Ogunkami et al., 1973). A plasma membrane protein as a convincing candidate for an ABA receptor has yet to be reported. These studies highlight the difficulty of designing experiments to directly investigate hormonal receptors in plants when the subcellular site to be probed is unknown.

The barley (*Hordeum vulgare* L.) aleurone cell is well established as a model system for studying hormonal regulation (Fincher, 1989; Jones and Jacobsen, 1991). The aleurone of the barley grain is a digestive tissue that secretes hydrolases that mobilize endosperm reserves during germination. The synthesis and secretion of these hydrolases (principally α-

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Abbreviations: B5/mannitol, modified Gamborg's B5 medium containing 0.6M mannitol; cps, counts per second; GM, gel matrix; GUS, β-glucuronidase; LUX, luciferase.

amylases) are under hormonal regulation. GA stimulates α-amylase synthesis and secretion, whereas ABA reverses this effect (Fincher et al., 1989). The hormonally responsive elements in the promoters of the amylase genes have been identified (Lanahan et al., 1992), and DNA-binding proteins putatively identified as regulators of the gene expression are being characterized (Rushton et al., 1992). Similarly, elements of a $Ca^{2+}$-calmodulin-based signal transduction system for GA and ABA have been reported (Bush and Jones, 1990; Gilroy and Jones, 1992, 1993; Bush et al., 1993). However, the site of perception of these hormones in the aleurone cell remains largely unknown.

We report here that barley aleurone protoplasts respond to GA and ABA applied extracellularly but not to hormone microinjected into the cytosol of the cell. We conclude that GA and ABA are perceived on the external face of the plasma membrane.

## MATERIALS AND METHODS

### Protoplast Isolation

Barley grains (*Hordeum vulgare* L. cv Himalaya; Department of Agronomy, Washington State University, Pullman, WA) were de-embryonated, 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 to $N_2$. 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. After incubation protoplasts were purified on a Nycodenz density gradient (Bush et al., 1988) and filtered through an 88-μm nylon mesh.

### Monitoring α-Amylase Secretion from Individual Protoplasts

Single aleurone protoplasts were embedded in a GM and assayed for secretion of α-amylase essentially according to the method of Hillmer et al. (1993). The GM contained 3% (w/v) ultralow-melting point agarose (Sigma) and 3% (w/v) soluble potato starch (Baker Chemical Co., Philadelphia, PA) in B5/mannitol and was prepared by mixing the agarose and starch with the B5/mannitol, heating in a microwave oven to boiling, and cooling to room temperature, at which the gel remains liquid for 5 to 10 min. A 30-μL drop of GM was placed about 1 cm from the frosted end of a microscope slide, and 2 μL of a purified protoplast suspension were added and quickly mixed with a plastic micropipette tip for 3 s. A 22-mm coverglass was placed on the GM, which was allowed to spread for 10 s to form an even, thin (about 70-μm thick) layer of protoplasts in the agarose/starch. The slide was incubated in a moist chamber at room temperature 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.

Staining with $I_2KI$ solution was performed as described by Hillmer et al. (1993). Briefly, the coverglass was removed by sliding it toward the frosted end of the slide, and 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 absorbent 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, whereas clear, starch-free halos are evident around protoplasts that have secreted α-amylase. Since incubations for longer than 2 h completely cleared the starch from the gel and our microinjection experiments required incubations in the GM for longer than 24 h, a second thin film of GM with starch was poured onto the first after the 24-h incubation. These double films were then incubated for 1 h and stained for starch degradation as described above.

### Transient Expression in Protoplasts

Protoplasts were transformed by microinjection with two plasmid constructs, JR249 and pAHC18. JR249 was a gift from John Rogers (Washington University School of Medicine, St. Louis, MO) and is the amylase promoter fused to *gus* (Lanahan et al., 1992). pAHC18 was a gift from Peter Quail (Plant Gene Expression Center, Albany, CA) and 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, the efficiency of transformation. The ratio of GUS:LUX therefore normalizes for transformation efficiencies (Bruce et al., 1989; Bruce and Quail, 1990; Lanahan et al., 1992).

Microinjection and assays for GUS and LUX activity were performed as described by Hillmer et al. (1993). Briefly, micropipettes (10 MΩ resistance) were pulled from filament electrode glass (World Precision Instruments, New Haven, CT). Microelectrodes were filled with 50 μM lucifer yellow, 12.5 μg $\mu L^{-1}$ pAHC18, and 2.5 μg $\mu L^{-1}$ JR249. Protoplasts embedded in GM were impaled and pressure injected using a model SB2 microburet (Micro-metric Instruments Co., Cleveland, OH) using the amount of lucifer yellow injected to estimate microinjected volume according to the method of Gilroy et al. (1991). Transformed protoplasts contained approximately 5 pg of JR249 plasmid and 25 pg of pAHC18. After 24 h of incubation in GM with 5 μg $mL^{-1}$ nystatin and ampicillin, 25 μM imagene green GUS substrate and 50 μM luciferin were added and incubated for 8 h. The fluorescent product of GUS activity on the imagene substrate (Haugland, 1992) and LUX luminescence were 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 one-eighth 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. Absolute LUX luminescence was in the range of 1000 to 4000 counts $s^{-1}$, GUS fluorescence was approximately 1000 kcounts $s^{-1}$. Protoplasts showing less than 1000 cps of LUX signal were judged to be poorly transformed and were excluded from further analysis. Hillmer et al. (1993) have shown that GA increased the GUS:LUX ratio of responding protoplasts from

1 to 3 × $10^3$ to 7 to 10 × $10^3$. Therefore, protoplasts showing GUS:LUX activity of more than 6 × $10^3$ were scored as responding.

### Microinjection of Protoplasts with GA and ABA

Protoplasts that were embedded in thin films according to the method of Hillmer et al. (1993) were pressure injected with up to 250 μM $GA_3$, $GA_8$ (a GA with low or no activity in the barley aleurone; Crozier and Durley, 1983), ABA, or KCl (as a control) according to the method of Gilroy and Jones (1992). Cytoplasmic concentrations of injected compounds were assessed from the fluorescence intensity of co-injected lucifer yellow (Gilroy and Jones, 1992). The intensity of co-injected lucifer yellow was measured using a Nikon Diaphot microscope and a photon-counting photomultiplier system (C716-01 photomultiplier coupled to a C1230 photon counter; Hamamatsu, Bridgewater, NJ). Cellular lucifer yellow concentrations were obtained from a standard curve of lucifer yellow concentration versus fluorescence intensity. This calibration curve was constructed using protoplast-sized (40 μm diameter) droplets of a range of concentrations of lucifer yellow that had been pressure microinjected into a film of immersion oil (Gilroy et al., 1991; Gilroy and Jones, 1992). For microinjection of [$^3$H]$GA_{20}$ protoplasts were iontophoretically microinjected (model 5-A microelectrode amplifier; Getting, Iowa City, IA) with [$^3$H]$GA_{20}$ (55 Ci $mmol^{-1}$) for 10 min at less than 0.5 nA to accumulate approximately 200 cpm per protoplast. Lucifer yellow (0.005% [w/v]) was co-injected, and injections were calibrated such that 100 cpm of [$^3$H]$GA_{20}$ per protoplast were equivalent to co-injecting 150 kcps of lucifer yellow, monitored in the photon-counting photometry system. For time courses of [$^3$H]$GA_{20}$ retention, the initial amount of [$^3$H]$GA_{20}$ present was calculated from the amount of co-injected lucifer yellow measured directly after microinjection. Radioactivity was measured by liquid scintillation counting of each gel (containing a single injected protoplast) that had been washed into a scintillation vial containing 25% (v/v) Triton X-114, 75% (v/v) Xylene, and 3 g $L^{-1}$ 2,5-diphenyloxazole, using four washes of 100 μL of distilled $H_2O$. We have been unable to perform similar experiments with ABA because of an unavailability of radiolabeled ABA of sufficient specific activity.

## RESULTS

### The Response of Single Aleurone Protoplasts to GA and ABA

We have used three assays to assess the response of single aleurone protoplasts to added GA or ABA. Protoplasts were embedded in thin films of agarose and incubated for 24 h with or without $GA_3$. These protoplasts showed the characteristic vacuolation associated with the response of protoplasts to GA (compare Fig. 1, A and B, and Hillmer et al., 1993; Bush et al., 1986). Protoplasts showing the development of large vacuoles visible at the light microscope level (stage 3–4 in Bush et al., 1986) were scored as having responded to GA. Those with dense cytoplasm and no obvious vacuolation (Fig. 1A) were scored as nonresponsive.

A second functional assay was to visualize amylase secretion from single protoplasts embedded in agarose using the thin-film, starch-iodine staining technique (Hillmer et al., 1993). In this assay protoplasts are embedded in a thin agarose gel impregnated with starch. After a 45-min incubation the gel is incubated with $I_2KI$ to stain starch blue. Protoplasts that were secreting α-amylase were surrounded with an unstained halo of digested starch that could be seen in the light microscope (Fig. 1, C and D).

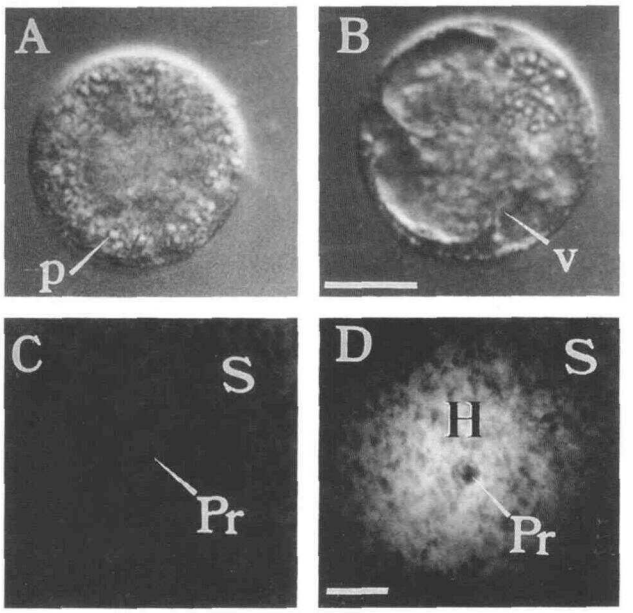


**Figure 1.** Bright-field, starch-iodine staining of amylase secretion in aleurone protoplasts. Freshly isolated protoplasts were embedded in starch-agarose thin films and incubated for 24 h without (A) or with (B) $GA_3$. $GA_3$-treated protoplasts show characteristic vacuolation. For starch-iodine staining (C and D), protoplasts were embedded in starch-agarose thin films and incubated for 24 h with (D) or without (C) incubation in 5 μM extracellular $GA_3$. A second gel was then poured over the first and after 1 h of incubation it was stained with $I_2KI$. Protoplasts secreting amylase show a "halo" of unstained, digested starch. S, Starch stained blue by $I_2KI$; H, unstained halo of degraded starch; Pr, protoplast; p, phytic acid crystal; v, vacuole. Scale bar, 20 μm (A and B); 100 μm (C and D).

The third assay for hormonal response utilizes the transient expression of the α-amylase promoter (including the hormonally responsive regions) linked to the reporter gene *gus*. Protoplasts are transformed by microinjecting the amylase promoter-*gus* construct. The level of response to GA or ABA is then gauged from the activation or supression of GUS production (Hillmer et al., 1993). Using these three assays we demonstrated that single aleurone protoplasts embedded in agarose thin films responded to $GA_3$ or ABA added to the medium as did protoplasts free in solution (Fig. 1; Hillmer et al., 1993).

### Response of Protoplasts to Microinjected and Externally Applied $GA_3$ or ABA

To investigate whether $GA_3$ was being perceived on the external face of the plasma membrane or by internal recep-

tors, we microinjected $GA_3$ into freshly isolated aleurone protoplasts, incubated these for 24 h, and assayed for $GA_3$ response at the morphological, secretory, and gene expression levels. Protoplasts injected with 1 to 250 $\mu$M GA ($n = 67$) did not respond to the hormone as assessed by these assays (Fig. 2). This failure to respond was not due to disruption of protoplast function by the microinjection. Protoplasts microinjected with $GA_3$, KCl, or $GA_8$ and then incubated in externally applied $GA_3$ responded to externally applied $GA_3$ (Fig. 2).

Similar experiments were performed to test the site of perception of ABA. Protoplasts were embedded in agarose layers after treatment with $GA_3$ for 24 h to induce high levels of $\alpha$-amylase synthesis and secretion (Hillmer et al., 1993; Fig. 3). Protoplasts were then either incubated in or microinjected with 1 to 250 $\mu$M ABA ($n = 63$), and the effect of the ABA on inhibiting the $GA_3$-induced $\alpha$-amylase production was observed. Protoplasts incubated in $GA_3$ for 24 h and then in 5 $\mu$M extracellular ABA for 24 h ceased to secrete amylase. However, if ABA was microinjected into the protoplasts rather than added on the outside, no inhibitory effect on secretion was observed (Fig. 3). ABA microinjection was not disruptive of cellular responses to ABA, since protoplasts microinjected with ABA or KCl and then incubated in externally added ABA showed the expected inhibition of GA-induced $\alpha$-amylase synthesis and secretion (Fig. 3). Morphology was not a good measure of ABA responsiveness in this case because $GA_3$-induced protoplast vacuolation is irreversible.

### Evaluation of the Nonresponsiveness of Microinjected Protoplasts

The failure of microinjected $GA_3$ and ABA to induce protoplast responses could have been due to the injected hormone being lost from the protoplast during the incubation period. This is unlikely because when the fluorescent dyes lucifer yellow and Fluo-3, whose acidic $pK_a$ and $M_r$ are similar to GA and ABA, were co-injected with the hormones, 55 to 60% of the dyes were retained after 24 h (Fig. 4). Also, if high specific activity [$^3$H]$GA_{20}$ (55 Ci mmol$^{-1}$), a GA with biological activity in aleurone cells (Crozier and Durley, 1983), was iontophoretically injected into protoplasts, 50% of the radiolabel introduced into a single protoplast was recovered after 24 h of incubation (Table I).

To test the possibility that the [$^3$H]GA was bound to the GM and thus not reflecting GA retained by the protoplast, [$^3$H]$GA_{20}$ was microinjected into the GM and incubated for 24 h. The gel was then washed by perfusion, and residual radioactivity was measured. In another control, potential binding of GA to cell debris was assessed. Radioactivity retained by protoplasts that were microinjected with [$^3$H]-$GA_{20}$ but died during incubation was measured. Dead protoplasts were easily distinguished microscopically because of their loss of turgor and the development of granularity in the cytoplasm during the 24-h incubation. These two experiments showed no radioactivity above background associated with the gel (Table I). Since neither the gel nor protoplast debris bound detectable levels of labeled GA, we conclude

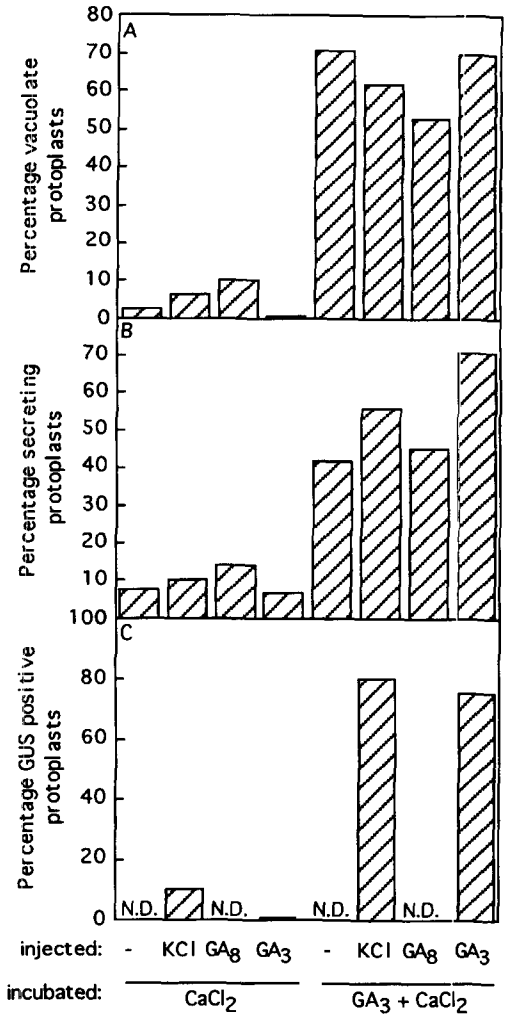


**Figure 2.** The response of aleurone protoplasts to microinjection with $GA_3$. Protoplasts were embedded in starch-agarose thin films, microinjected with $GA_3$, $GA_8$, or KCl (control), and incubated for 24 h with or without extracellular $GA_3$. Morphology, secretion, and amylase-GUS reporter gene activity were then assayed for modulation by GA. For amylase-GUS assays protoplasts were also microinjected with approximately 5 pg of JR249 plasmid (barley $\alpha$-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 $\mu$M imagene green GUS substrate and 50 $\mu$M 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. Protoplasts showing a more than $6 \times 10^3$ GUS:LUX ratio were considered "GUS positive", i.e. responding to $GA_3$. Results represent data from 67 individual protoplasts assayed in nine separate experiments. The internal concentration of $GA_3$ microinjected was 1 to 10 $\mu$M, $n = 19$; 10 to 100 $\mu$M, $n = 17$; 100 to 250 $\mu$M, $n = 31$. N.D., Not determined. Because transformation required microinjection, noninjected controls were not obtainable for this assay.

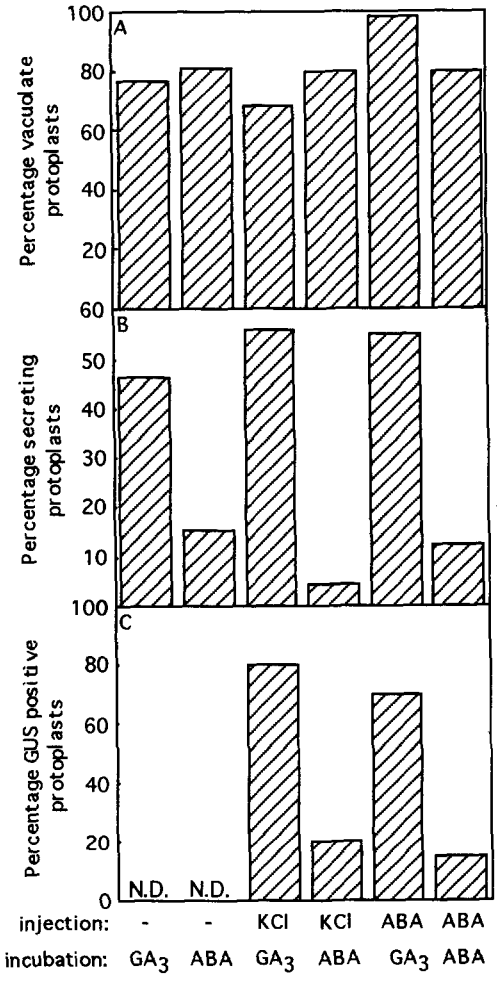


**Figure 3.** The response of aleurone protoplasts to microinjection with ABA. Protoplasts were incubated for 18 h with $GA_3$ to induce high levels of amylase synthesis and secretion and then embedded in starch-agarose thin films, microinjected with ABA or KCl (control) plus the plasmid for transient expression studies, and incubated for 18 h with or without extracellular ABA. ABA inhibition of the $GA_3$-induced secretion and amylase-GUS reporter gene activity were assayed. Protoplasts showing a more than $6 \times 10^3$ GUS:LUX ratio were considered "GUS positive", i.e. responding to $GA_3$. Results represent data from 63 individual protoplasts assayed in eight separate experiments. The internal concentration of microinjected ABA was 1 to 10 $\mu$M, $n = 17$; 10 to 100 $\mu$M, $n = 26$; 100 to 250 $\mu$M, $n = 20$. N.D., Not determined. Because transformation required microinjection, noninjected controls were not obtainable for this assay.

that radioactivity associated with the microinjected protoplasts after 24 h of incubation reflected retained GA.

## Uptake of [$^3$H]$GA_3$ by Aleurone Protoplasts

We also attempted to assess the pool size of GA in GA-treated aleurone cells to exclude the possibility that the lack of response to microinjected $GA_3$ was due to introducing too little hormone into the cytoplasm. Protoplasts ($5 \times 10^5$ mL$^{-1}$) were incubated with 5 $\mu$M $GA_3$ and 100,000 cpm of [$^3$H]$GA_{20}$

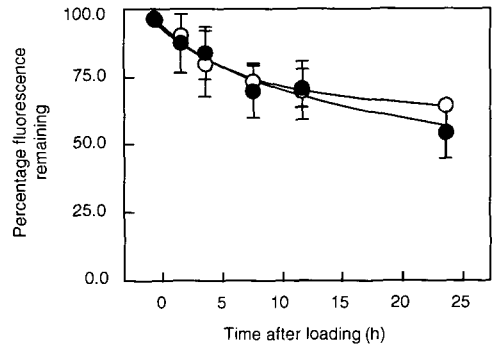


**Figure 4.** Retention of fluorescent dyes by barley aleurone protoplasts. Aleurone protoplasts were embedded in thin films and microinjected with the fluorescent dyes lucifer yellow (○) or fluo-3 (●). The amount of dye retained was monitored at the indicated times using a fluorescence-microscope and photomultiplier system (Gilroy and Jones, 1992). Results represent means ± SE, $n \geq 35$ cells.

(used as a marker for GA uptake). After 24 h of incubation protoplasts were purified on N3/N5 gradients (Bush et al., 1986), and the amount of radioactivity taken up by the protoplasts was measured by liquid scintillation counting. Carryover of [$^3$H]$GA_{20}$ was assessed by addition of 1 $\mu$M lucifer yellow-labeled dextran ($M_r$ 10,000; Molecular Probes, Eugene, OR) to the incubation medium immediately prior to purification of the protoplasts. Lucifer yellow dextran will not readily cross the plasma membrane; therefore, lucifer yellow present in the purified protoplast preparation represented carryover of medium in the purification process. Lucifer yellow fluorescence was measured in 50-$\mu$L droplets using the photon-counting photometry system. In a second set of experiments to assess carryover, [$^3$H]$GA_{20}$ (5000 cpm) was added to protoplasts, which were immediately purified on N3/N5 gradients, and the purified preparation was counted by liquid scintillation. Carryover of [$^3$H]$GA_{20}$ was 3 ± 0.5% in the lucifer yellow and 5 ± 0.9% in the [$^3$H]$GA_{20}$ experiment. Therefore, 4% (4000 cpm) of the total radioactivity added was subtracted from the counts associated with the

**Table I.** *Retention of [$^3$H]$GA_{20}$ by barley aleurone protoplasts*

Protoplasts were iontophoretically microinjected with approximately 200 cpm of [$^3$H]$GA_{20}$. The actual amount initially injected was determined from the fluorescence from co-injected lucifer yellow. After 18 h the gel was perfused with 3 mL of B5/mannitol to remove leaked GA, and the radioactivity remaining in the protoplast was determined by liquid scintillation of the gel. Background was 9 ± 5 cpm in uninjected gels or in gels in which approximately 500 cpm of [$^3$H]$GA_{20}$ were microinjected into the GM, incubated for 18 h, and then washed and counted. Results represent means ± SE, $n \geq 19$.

| Time of Incubation | Percentage of Remaining [$^3$H]$GA_{20}$ | | |
|---|---|---|---|
| | Microinjected protoplasts | Gel medium | Dead protoplasts |
| *h* | | | |
| 0 | 100 | 100 | 100 |
| 24 | 56 ± 12 | 1.8 | 3.6 |

protoplasts before calculation of intracellular GA concentration. Protoplasts ($5 \times 10^5$) accumulated 2030 cpm (corrected for carryover). Since uptake was equivalent to the carryover correction, absolute internal [GA] was difficult to reliably determine. However, an upper limit of GA level was estimated; assuming protoplast cytoplasmic volume of 15 pL (Gilroy and Jones, 1992), internal [GA] was less than 5 $\mu$M.

## DISCUSSION

Our results show that if GA or ABA is microinjected into a barley aleurone protoplast, no effects on $\alpha$-amylase gene expression, secretory activity, or protoplast development are observed (Figs. 2 and 3). If the protoplasts are treated with externally applied hormone, however, GA-induced $\alpha$-amylase synthesis and secretion and ABA reversal of this stimulation are observed (Figs. 2 and 3). These results suggest that the sites of perception of GA and ABA are on the external face of the plasma membrane in the aleurone cell. This conclusion is supported by recent work showing that $GA_4$ can induce $\alpha$-amylase synthesis and secretion in oat aleurone protoplasts even when covalently linked to Sepharose beads (Hooley et al., 1991). Also, anti-idiotypic antibodies to $GA_4$ can mimic $GA_4$ activity in oat aleurone and would be expected to act on the external face of the plasma membrane (Hooley et al., 1992). Thus, there seems to be no correlation between rate of GA uptake and induction of the secretory response. Our data indicate that external receptors perceive the GA signal that triggers the secretory response of the aleurone cell.

No data are available about the site of ABA perception in aleurone cells. In stomatal guard cells ABA induces stomatal closure at alkaline external pH when uptake of the hormone is low (Hartung, 1983; Baier and Hartung, 1991). Anderson et al. (1994) have also shown that ABA will only inhibit stomatal opening when added to the outside of the cell. It is inactive when microinjected into the cell, indicating that an ABA receptor is also located on the external face of the plasma membrane. However, with all of these cell types there may be multiple hormone receptors in several subcellular compartments. These receptors could modulate different cell activities or be active under different environmental conditions. For example, further work will be required to test whether the regulation of ABA-responsive genes and processes is mediated by an externally facing receptor, as seems to be the case for ABA antagonism of GA-induced amylase production.

In our experiments we have tried to eliminate the possibility that damage of protoplasts during the microinjection procedure could account for the failure of protoplasts microinjected with GA or ABA to respond. Similarly, 50% of injected hormone is retained by the protoplast during the 24-h incubation, making it unlikely that leakage to below a critical stimulatory level could account for the failure of the protoplast response. If we assume that this 50% leakage of hormone is at a constant rate throughout the 24 h (up to 80 nM $min^{-1}$ from protoplasts injected with 250 $\mu$M GA), the local GA concentrations, just outside the plasma membrane, could approach stimulatory ($\mu$M) levels. However, this does not appear to be the case. The volume of the gel is very large compared to the protoplast volume and, if we assume a diffusion constant for GA of $10^{-9}$ $m^2$ $s^{-1}$, typical for small molecules in aqueous solutions (Nobel 1991), then the mean concentration of GA in the gel volume reached by diffusion during 24 h should increase by less than 1 nM. It would also take only 2 s for 50% of the leaked GA to diffuse 50 $\mu$m from the protoplast surface (following the assumptions of Ficks second law; Nobel, 1991). Induction of amylase gene expression is typically seen after 4 to 8 h of continuous incubation of aleurone tissue in $10^{-6}$ M $GA_3$ (Jones and Jacobsen, 1991). We therefore conclude that rapid diffusion of leaked GA away from the protoplast prevents a sustained accumulation in the gel to levels that stimulate amylase production. Similarly, we have never observed noninjected protoplasts that were near (150–200 $\mu$m away) a protoplast microinjected with GA to show induction of amylase synthesis and secretion when assayed by the thin layer method or to show morphological evidence of a GA response, reinforcing the view that leaked GA does not reach a stimulatory level in the gel.

We cannot exclude the possibility that, after microinjected into the cytoplasm, the active hormone is rapidly metabolized into an inactive form. However, it is thought that the internal concentration of GA is much less than the 5 $\mu$M added to the incubation medium (Hooley et al., 1992). Our estimation of less than 5 $\mu$M internal GA concentrations would agree with this assumption and is much less than the 250 $\mu$M GA we have microinjected. Thus, even assuming metabolism to inactive forms over 24 h, the amount of GA microinjected should have been present at high levels for most of the incubation and should have been physiologically active.

Internal ABA concentrations have been estimated to be 3.5 to 9 $\mu$M in barley aleurone cells incubated for 2 to 24 h in 10 $\mu$M ABA (Uknes and Ho, 1984). This is in agreement with internal ABA concentrations found in other cell types, for example, 1 to 5 $\mu$M in guard cells (Harris and Outlaw, 1991). These in vivo ABA levels are well below the amount we have microinjected into the aleurone cell cytoplasm. There is still 30% unmetabolized ABA in aleurone tissue after 12 h of incubation, at which point the rate of ABA degradation is minimal (Uknes and Ho, 1984). The effects of ABA on amylase synthesis and secretion can be observed as early as 4 h after addition (Gilroy and Jones, 1992), when 60% of ABA in the aleurone cell is unmetabolized (Uknes and Ho, 1984). Thus, an initial injected internal concentration of 1 to 250 $\mu$M ABA would be expected to cover the physiologically active ABA levels found in aleurone cells during the period of our experiments. Therefore, it is unlikely that we fail to see a response to microinjected hormone due to decreasing outside the critical stimulatory range.

The possibility that hormones are perceived at the plasma membrane is especially interesting in aleurone cells because GA and ABA induce changes in cytosolic $Ca^{2+}$ levels in the peripheral cytoplasm of the aleurone protoplast (Bush and Jones, 1990; Gilroy and Jones, 1992). These changes in $Ca^{2+}$ are thought to play a role in signal transduction in these cells. The increase in cytosolic $Ca^{2+}$ may occur via $Ca^{2+}$ influx at the plasma membrane (Gilroy and Jones, 1992). Similarly, in guard cells of *Vicia faba* ABA has been shown to cause opening of $Ca^{2+}$-permeable channels in the plasma mem-

brane (Schroeder and Hagiwara, 1990). Thus, GA and ABA may interact with $Ca^{2+}$ channels and pumps in the plasma membrane to bring about cytosolic change. However, whether this modulation is direct or through a series of intermediate steps such as G-protein action (Li and Assmann, 1993) or modulation of plasma membrane protein phosphorylation is unknown.

Although GA- and ABA-binding proteins have been identified in plant tissues (Hornberg and Weiler, 1984; Hooley et al., 1992), molecular candidates for a receptor are not well characterized. Part of the problem in identifying potential receptors has been that we do not know where to look. This problem is exemplified by the putative auxin receptor, which, although bearing the characteristics of a plasma-membrane auxin receptor, also appears in the ER and cell wall (Feldwisch et al., 1992; Jones and Herman, 1993). Our results suggest that barley aleurone plasma membrane may be a fruitful place to search for a GA or ABA receptor.

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