Video Article

Measuring Gene Expression in Bombarded Barley Aleurone Layers with Increased Throughput

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Abstract

The aleurone layer of barley grains is an important model system for hormone-regulated gene expression in plants. In aleurone cells, genes required for germination or early seedling development are activated by gibberellin (GA), while genes associated with stress responses are activated by abscisic acid (ABA). The mechanisms of GA and ABA signaling can be interrogated by introducing reporter gene constructs into aleurone cells via particle bombardment, with the resulting transient expression measured using enzyme assays. An improved protocol is reported that partially automates and streamlines the grain homogenization step and the enzyme assays, allowing significantly more throughput than existing methods. Homogenization of the grain samples is carried out using an automated tissue homogenizer, and GUS (β -glucuronidase) assays are carried out using a 96-well plate system. Representative results using the protocol suggest that phospholipase D activity may play an important role in the activation of *HVA1* gene expression by ABA, through the transcription factor TaABF1.

Video Link

The video component of this article can be found at <https://www.jove.com/video/56728/>

Introduction

The barley aleurone layer is a well-established model system for the study of hormone-regulated gene expression in plants¹. In particular, a number of genes required for germination or early seedling development are activated by gibberellin (GA), while genes associated with stress responses are activated by abscisic acid (ABA). The GA and ABA signaling pathways are intertwined, as the expression of some GA-activated genes is inhibited by ABA, and vice-versa¹.

A valuable strategy for understanding the role of particular actors in GA/ABA signaling has been the introduction of effector gene constructs via particle bombardment, followed by transient expression of reporter constructs that allow the resulting effect on downstream gene expression to be determined. The use of reporter genes such as *GUS* (β -glucuronidase) or *Luciferase* allows the sensitive and quantitative measurement of gene expression specifically within the cells that have received the effector construct. For example, the introduction of an effector construct encoding the transcription factor TaABF1^{5,6} established that ABA-induced genes such as *HVA1* are induced by TaABF1, while GA-induced genes such as *Amy32b* are repressed. Particle bombardment as an experimental strategy has been used by multiple laboratories to investigate diverse aspects of GA/ABA signaling. Such work has led to the identification of promoter elements important for the activation of both GA-induced² and ABA-induced genes³, and to the discovery of protein kinases⁴ and transcription factors⁵ that regulate the expression of these genes.

The existing protocols^{2,3,4,5,6} for particle bombardment and subsequent measurement of transient gene expression are quite labor intensive, as each set of bombarded barley grains is homogenized by hand in a mortar and pestle and the enzyme assays are carried out individually. This manuscript reports an improved protocol that partially automates and streamlines the homogenization step and the GUS assays to allow significantly more throughput, permitting a larger number of treatments to be tested in the same experiment, and/or the inclusion of more replicates for each treatment to obtain more statistically robust results. Representative results are shown for the expression of *HVA1* and *Amy32b* reporter constructs, regulated by the transcription factor TaABF1 as well as by GA, ABA, and other regulatory molecules.

Protocol

1. Preparation of Effector and Reporter Gene Constructs

1. Build effector constructs that employ a constitutive promoter (e.g. maize ubiquitin, *UBI*) to drive expression from a desired open reading frame. For example, construct a plasmid containing *UBI::TaABF1* to drive strong constitutive expression of TaABF1⁵.
2. Build reporter constructs that include the promoter whose activity is to be measured, placed upstream of an open reading frame, such as *GUS*. For example, construct a plasmid containing *HVA1::GUS* to measure expression from the *HVA1* promoter^{2,5}.
3. Build an internal control construct (e.g. *UBI::luciferase*).

- Using a silica binding column protocol (see **Table of Materials**), prepare high-quality plasmid for each of the required gene constructs. Using ultraviolet spectroscopy (see **Table of Materials**), carefully measure the concentration of each plasmid preparation.

2. Preparation of Barley Grains for Bombardment

- Set up a spreadsheet (see **Figure 1** for an example) indicating the plasmids that will be bombarded, the appropriate hormone treatments, and other relevant information for each treatment that will be part of the experiment.
- Using a cutting board and a sterile razor blade, cut off the embryos from grains of Himalaya barley (40 for each treatment included in the experiment). Exclude grains that are discolored or substantially smaller than average.
- Place all the embryoless grains into a 50 mL tube. Add 40 mL of water and rock for 10 minutes.
- Pour out the water carefully (to avoid losing grains) and add 40 mL of 10% bleach. Rock for 20 minutes.
- Pour out the bleach and add 40 mL of sterile water. Rock for 5 minutes. Repeat this sterile water wash four more times.
- Add 40 mL of Imbibing Solution (20 mM sodium succinate, 20 mM calcium chloride, pH 5.0) and 40 μ L of chloramphenicol stock solution (10 mg/mL).
- Transfer most of the Imbibing Solution (with chloramphenicol) from the tube into a Vermiculite Plate (see **Table of Materials**). Then transfer the grains onto the surface of the wet filter paper in the Vermiculite Plate. Spread out the grains evenly. Pour in enough imbibing solution to keep the grains partially (but not completely) submerged. Place the lid on top of the Vermiculite Plate.
- Incubate the embryoless grains at 24 °C for about 48 h with fluorescent lighting. Add additional Imbibing Solution as necessary.
- Open up a 90 mm plastic petri dish and pour 20 mL of Imbibing Solution into the dish itself and 20 mL into the inverted lid. Add 20 μ L of chloramphenicol (10 mg/mL) to each. Sterilize two pairs of fine point forceps by dipping them in alcohol.
- Place a few of the embryoless grains into the lid of the petri dish. Using the forceps, gently remove the (transparent) seed coat from each grain. While removing the seed coat from the smooth side of the grain, do not damage the underlying (aleurone) cells. Transfer the peeled grain to the petri dish containing Imbibing Solution.
- After peeling the seed coat from all of the grains, return them to the Vermiculite Plate. Incubate the peeled embryoless grains at 24 °C for 16 - 20 hours.
- Just before using the grains for bombardment, remove surface moisture by shaking them between filter papers in a petri dish.

3. Preparation of Plasmid DNA for Bombardment

- Label a 1.5 mL tube for each treatment that will be included in the bombardment experiment. To each tube add 2.5 μ g of *UBI::luciferase* internal control plasmid, 2.5 μ g of a *GUS* reporter plasmid, and the desired amount (typically 0.025 μ g to 2.5 μ g) of an effector plasmid. Add water to bring the total volume to 5 μ L.
- Prepare 1.6 μ m gold microcarriers (see **Table of Materials**) following published protocols⁶.
- For each treatment, add 50 μ L of suspended microcarriers into the 1.5 mL tube containing plasmid DNA. Coat the microcarriers with plasmid DNA according to manufacturer's protocols⁶.
- At the final step, when the plasmid-coated microcarriers are suspended in 48 μ L 100% ethanol, pipet 8 μ L of suspended microcarriers onto each of five macrocarriers and allow them to air dry.

4. Particle Bombardment of Embryoless Barley Grains

- Set up the particle bombardment apparatus (see **Table of Materials**)⁶.
- Place a 1550 psi rupture disk into the retaining cap and mount it on the instrument.
- Place a macrocarrier containing the desired plasmid combination (together with a stopping screen) into the microcarrier launch assembly. Insert the assembly into the top slot of the bombardment chamber. Set the distance between the rupture disc retaining cap and the macrocarrier cover lid at the standard distance (6 mm), and place the stopping screen support at the (standard) middle position, allowing a macrocarrier travel distance of 11 mm (**Figure 2A**).
- Arrange 8 embryoless grains in a tight radial pattern (with the thinner ends pointing inward) on a filter paper circle that is taped down flat to the lid of a 90 mm petri dish (see **Figure 2B**).
- Place the dish with the grains into the bombardment chamber, at a distance of 6 cm from the stopping screen.
- Evacuate the bombardment chamber to 95 kPa and then bombard the sample.
- After releasing the vacuum, transfer the bombarded grains to a labeled 60 mm petri dish containing 4 mL of Imbibing Solution containing 1 mg/mL of chloramphenicol.
- Repeat until all of the bombardments have been completed. Incubate (on a shaking platform at 100 rpm) at 24 °C for 24 hours.

5. Extraction of Soluble Protein from the Bombarded Grains

- Using forceps, remove the 8 bombarded grains from a 60 mm petri dish and blot them dry on a paper towel. Divide the grains into two labeled 2.0 mL tubes (4 grains per tube) each containing 800 μ L Grinding Buffer (100 mM Na phosphate pH 7.2, 5 mM DTT, 10 μ g/mL leupeptin, 20% glycerol) and a 5 mm stainless steel bead. Repeat this process for all of bombarded grains.
- Place (up to 48) 2.0 mL tubes into the two racks of the bead homogenizer (see **Table of Materials**). Mount the racks on the homogenizer.
- Shake the samples at 30 Hz for 3 minutes.
- Remove the homogenizer racks from the homogenizer and place them on ice (5 min) to re-cool the samples.
- Mount the racks back on the homogenizer - in the opposite orientation. Shake the samples again at 30 Hz for 3 minutes.
- Cool the racks on ice, then repeat Steps 5.3 to 5.5. This will add up to a total of 12 minutes of shaking.
- Centrifuge the grain extracts at 16,000 x g at 4 °C for 10 minutes.
- Immediately** after the centrifugation, decant the clear supernatants into a second set of microcentrifuge tubes. Give each tube a quick vortex. Store the tubes on ice.

- After transferring the supernatant, recover the steel beads from the pellets so they can be washed and reused.

6. Measurement of Luciferase Activity from Grain Extracts

- For each tube of grain extract to be assayed, prepare a 12 mm x 75 mm glass test tube containing 200 μ L of Luciferase Assay Mixture (45 mM Tris sulfate pH 7.7, 15 mM $MgCl_2$, 20 mM DTT, 1.5 mM EDTA, 1 mM luciferin, 1 mM ATP).
- Add 100 μ L of the first grain extract to the first assay tube. Vortex quickly to mix well. **Immediately** place the tube into the tube holder of the luminometer and close the door. When the measurement is finished, remove the tube from the luminometer – leaving the door open for placement of the next tube.
- Repeat this process until all the samples have been measured.

7. Measurement of GUS Activity from Grain Extracts

- Add 200 μ L of GUS Assay Buffer (50 mM Na phosphate pH 7.2, 2.5 mM 4-methylumbelliferyl--D-glucuronide, 10 mM DTT, 2 mM EDTA, 10 μ g/mL leupeptin, 20% methanol, 0.02% sodium azide) to each well of a 96 well plate.
- Place 50 μ L of each grain extract into the corresponding well. Mix with the tip after adding. Seal the top with sealing film.
- Incubate the 96 well plate at 37 $^{\circ}$ C in the dark for 20 hours.
- Centrifuge the 96 well plate at 4,000 x g at 4 $^{\circ}$ C for 10 minutes and place it on ice.
- Add 250 μ L of 0.2 M Na_2CO_3 to each well of a black 96 well plate.
- Add 6.25 μ L of each centrifuged GUS assay mixture into the corresponding well (containing 0.2 M Na_2CO_3) of the black 96 well plate. Mix with the tip after adding. Include wells with 6.25 μ L of 10 μ M, 40 μ M, and 100 μ M 4-methylumbelliferone as standards.
- Place the black plate into a plate fluorometer and read the fluorescence of methylumbelliferone. Take the readings at an excitation wavelength of 360 nm, and an emission wavelength of 460 nm. Set the gain of the instrument such that a well containing 6.25 μ L of 40 μ M 4-methylumbelliferone and 250 μ L of 0.2 M Na_2CO_3 gives a reading of 1000 fluorescence units.

8. Data Analysis

- Enter the values for the luciferase and GUS assays into a spreadsheet. Exclude from consideration any sample with a luciferase reading of lower than 20,000 relative luminescence units s^{-1} , as this indicates that the gene constructs were not successfully delivered to the aleurone cells.
- For each successfully bombarded grain extract (representing a group of four embryoless grains), calculate the normalized GUS expression from the raw GUS and luciferase data as follows: normalized GUS = [(GUS expression - GUS blank) x (2,000,000)] / (luciferase expression - luciferase blank).
NOTE: This normalizes the amount of GUS activity to what would have been observed in a bombardment that gave a luciferase reading of 2,000,000.
- Report the relative level of expression of a particular reporter construct in the presence of effector constructs or hormone treatments being tested by comparing to the level of expression in the absence of effectors or hormones.

Representative Results

The technique described here can be used to introduce any test gene construct into aleurone cells of barley grains. The level of expression from the test gene may then be conveniently measured (**Figure 2**). The higher throughput protocol described here greatly increases the efficiency of the seed grinding and enzyme assay steps. This method has been used to assess the ability of the transcription factor TaABF1^{5,6} to activate expression of the ABA-induced gene *HVA1'*. In the representative results presented here, an *HVA1::GUS* reporter construct was introduced into aleurone cells together with a *UBI::TaABF1* effector construct (**Figure 3A**). While a *TaABF1/HVA1* ratio of just 0.01 was sufficient to cause a 3-fold induction of the *HVA1* reporter gene in the absence of exogenous ABA, higher levels of the *TaABF1* construct resulted in progressively greater *HVA1* expression, in a dose-dependent manner (**Figure 3B,C**).

This experimental protocol may also incorporate the use of hormones or other compounds during the post-bombardment incubation period to assess the role of those molecules in the regulation of gene expression. Previous work by other investigators has suggested the importance of phospholipase D in some aspects of ABA signaling. Specifically, the product of phospholipase D activity, phosphatidic acid, has been implicated as an intermediate in some responses to ABA^{8,9}. The fact that 1-butanol, an inhibitor of phospholipase D (PLD), has been shown to prevent ABA-induced gene expression in aleurone cells suggests that PLD might be an integral part of ABA signaling in this tissue. The bombardment protocol was used to test whether 1-butanol could inhibit either ABA-induced or TaABF1-induced *HVA1* expression. As expected, either exogenous ABA or TaABF1 could strongly induce expression of the *HVA1::GUS* reporter construct (**Figure 3C**). In either case, the presence of 1-butanol strongly inhibited the *HVA1* induction. This suggests that PLD could play an important role in the activation of *HVA1* gene expression by ABA, through TaABF1.

In addition to its role in developing and germinating grains, ABA is also critical for regulating stomatal aperture in leaves. In the guard cells of stomata, the production of phosphatidic acid is stimulated by nitric oxide (NO), which is in turn induced by hydrogen peroxide (H_2O_2). For some responses, NO and H_2O_2 can substitute for ABA⁹. To determine whether this is also the case in aleurone cells, the bombardment protocol was used to test whether the NO donor sodium nitroprusside (SNP) or H_2O_2 could substitute for ABA in inhibiting the expression of the GA-induced *Amy32b* gene. While ABA could strongly inhibit GA-induced expression for the *Amy32b::GUS reporter construct*, neither H_2O_2 nor SNP caused any significant reduction (**Figure 3D**). These results, therefore, do not support a role for NO and H_2O_2 in aleurone cell ABA responses.

	A	B	C	D	E	F	G	H
1	Particle Bombardment #100							
2	tube name	A	B	C	D	E	F	
3								
4	number of seeds	8	32	32	32	32	32	
5	number of shots	1	4	4	4	4	4	
6								
7	H ₂ O (μL)	5	1.25	1.13	1.03	0.69	0.13	
8								
9	<i>UBI::Luciferase</i> plasmid (2.0 μg/μL)	0	1.25	1.25	1.25	1.25	1.25	
10	<i>HVA1::GUS</i> reporter plasmid (2.0 μg/μL)	0	1.25	1.25	1.25	1.25	1.25	
11								
12	<i>UBI::TaABF1</i> effector plasmid (0.2 μg/μL)			0.13	0.25	0.62	1.25	
13								
14	dummy effector plasmid (2.0 μg/μL)	0	1.25	1.24	1.22	1.19	1.12	
15								
16		total # of seeds			total # of shots			
17		168				21		

Figure 1: Spreadsheet for a typical bombardment experiment. The amount of each plasmid preparation to be placed in the tube for each bombardment is indicated. In this experiment, four shots (8 grains each) were used for each treatment to give a total of eight possible samples (4 grains each) for each treatment. In order to maintain the same total DNA concentration in each bombardment, a "dummy" effector plasmid was used to substitute for the *UBI::TaABF1* effector plasmid in some cases. Treatment A is a "blank" bombardment to establish the background level of GUS and luciferase activity in aleurone cells. The results of the bombardment experiment outlined in this spreadsheet are shown in **Figure 3B**. [Please click here to view a larger version of this figure.](#)

Figure 2: Diagram of the protocol. (A) Schematic of the bombardment device. (B) Gold particles coated with a reporter construct carrying the promoter to be tested, an effector (if needed), and an internal control (*UBI::luciferase*) are introduced into the barley aleurone cells by particle bombardment. After incubation (typically for 24 h), the grains are ground and assayed for both GUS activity and luciferase activity. [Please click here to view a larger version of this figure.](#)

Figure 3: Regulation of *HVA1* and *Amy32b* promoters by TaABF1, ABA, and potential mediators of ABA signaling. (A) Reporter and effector constructs used in the experiments. (B) The effector construct *UBI::TaABF1* was co-bombarded into aleurone cells with the *HVA1::GUS* reporter and the internal control construct (*UBI::luciferase*). The amount of *UBI::TaABF1* effector (relative to the *HVA1::GUS* reporter) varied from 0 to 0.1, as indicated below the bars. GUS and luciferase activities were measured after 24 h of incubation. Data shown are the means ($\pm$ SE) of 6 - 8 biological replicates. (C) The *HVA1::GUS* reporter was co-bombarded into aleurone cells with the internal control construct (*UBI::luciferase*) in the presence or absence of the effector construct *UBI::TaABF1*. The amount of TaABF1 effector (relative to the reporter) was 1.0. GUS and luciferase activities were measured after 24 h of incubation with or without 20 μ M ABA and 0.4% 1-butanol. (D) The *Amy32b::GUS* reporter was co-bombarded into aleurone cells with the internal control construct (*UBI::luciferase*). GUS and luciferase activities were measured after 24 h of incubation in the presence of 1 mM GA with or without 20 μ M ABA, 1 mM H₂O₂, or 100 μ M sodium nitroprusside (SNP). Values for GUS activity are relative to the expression of the *Amy32b::GUS* reporter in the absence of GA. [Please click here to view a larger version of this figure.](#)

Discussion

The introduction of effector gene constructs via particle bombardment, followed by transient expression of reporter constructs is a valuable strategy for dissecting the role of particular actors in GA/ABA signaling and in the resulting hormone-regulated gene expression. However, existing protocols for carrying out such experiments in barley aleurone cells^{2,3,4,5,6} are very labor intensive. Each group of bombarded barley grains must be hand homogenized in a mortar and pestle, and the resulting extracts are assayed individually for GUS and luciferase activity. The current protocols therefore limit the number of separate treatments that can be included in the same experiment, and restrict the number of biological replicates that can reasonably be included for each treatment. These limitations make it more difficult to carry out comprehensive and simultaneous comparisons between multiple treatments. Such comparisons can be especially difficult if the difference in gene expression between the treatments is modest and a relatively large number of biological replicates is needed to establish statistical significance.

A streamlined protocol is presented here that partially automates the grain extract preparation and the GUS assays to allow substantially higher throughput. This new version of the protocol makes it feasible to include a much larger total number of samples in an experiment, allowing for both the inclusion of more different treatments per experiment, and more replicates for each treatment. For the preparation of grain extracts after bombardment and incubation, we have substituted a largely automated grinding protocol that utilizes a bead homogenizer that can process 48 samples simultaneously (in about 20 minutes). Thus, a single investigator can prepare soluble protein extracts from nearly 200 samples in less time that it would take a team of four investigators to hand grind only 100 samples. The modified GUS assay, carried out in 96 well plates, also requires much less labor than the original protocol that employed individual tube assays.

In preparing the plasmid mixtures for bombardment (Step 3.1), it is possible to use an effector to reporter ratio of 1.0, especially for preliminary tests to determine if a particular effector has any effect at all. However, because of the strength of the maize ubiquitin (UBI) promoter, a strong effect can often be observed with much lower ratios, as seen in **Figure 2B** and in previously published reports^{4,5,6}. Other critical steps in this protocol include peeling of the seed coat from the barley grains and the use of carefully defined settings in the bombardment apparatus. If the seed coats are not fully removed, the gold microcarriers will be prevented from entering the subtending aleurone cells. Successful delivery of the gold microcarriers into the aleurone cells is also quite dependent on the specific type of rupture disc used, the position of the microcarrier cover lid, stopping screen, and tissue to be bombarded. The conditions reported here typically result in successful delivery of the gene constructs (see Step 8.1) to a high percentage ($\geq 70\%$) of bombarded barley grain samples. It should be expected that other biological materials would require different conditions for optimal gene delivery.

Although it is not described here, it would be possible to further streamline the procedure by carrying out the flash luciferase assays in 96 well plates. However, as the light production from luciferase must be measured in a luminometer immediately after mixing the protein extract with the substrate (luciferin), this would require the use of a plate flash luminometer with injectors, an instrument that is much more expensive than a single tube luminometer.

The representative results reported here confirm that the transcription factor TaABF1 can activate *HVA1* expression in the absence of exogenous ABA^{5,6}. Activation of *HVA1* by either TaABF1 or exogenous ABA was prevented by the PLD inhibitor 1-butanol, suggesting that production of phosphatidic acid by PLD may be required for *HVA1* activation. These results for *HVA1* regulation by TaABF1 and ABA are similar to those obtained by Zou *et al.*¹⁰ for the activation of *HVA22* by the transcription factor LtWRKY21 and by ABA. The finding that H₂O₂ or SNP do not substitute for exogenous ABA in suppressing aleurone expression of *Amy32b*, confirms that some aspects of ABA signaling in cereal grains are distinct from what occurs in stomatal guard cells. The finding that H₂O₂ does not downregulate *Amy32b* expression is consistent with the results observed by Ishibashi *et al.*¹¹ for amylase enzyme activity.

The increased throughput protocol is now being used to carry out a comprehensive study of the effects of TaABF1 phosphorylation on its ability to regulate downstream gene expression. As other members of the ABF family of transcription factors can be phosphorylated at a number of different sites^{12,13,14,15,16}, multiple putative phosphorylation sites on TaABF1 need to be tested. The higher throughput allowed by this protocol will permit a large number of mutant versions of TaABF1 (with potentially phosphorylatable serine and threonine residues modified) to be interrogated, each with a large number of biological replicates.

Disclosures

The authors have nothing to disclose.

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