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Protocols

High Throughput Screening of Signal Transduction Mutants With Luciferase Imaging

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Abstract. A detailed procedure for high throughput genetic screening of hormone and environmental stress signal transduction mutants of *Arabidopsis thaliana* is described. The screen was carried out with mutagenized plants expressing the firefly luciferase reporter under control of a cold, osmotic stress, and absciscic acid responsive promoter. A thermoelectrically cooled CCD camera was used to detect luminescence emitted by the plants in response to stresses or ABA. Advantages of the screening procedure include high throughput, capability to identify low as well as high expression mutants and employment of a highly sensitive but affordable imaging system and software. This procedure can be used to study complex signal transduction networks in higher plants.

Key words: CCD camera, imaging, luciferase, mutant screening, signal transduction

Introduction

The molecular genetic approach is becoming indispensable for dissecting complex signal transduction networks in higher plants. This approach involves the isolation of mutations based on reporter gene expression driven by an inducible promoter. Many reporters have been employed for this purpose (Suter-Crazzolara et al., 1995). To date, the most commonly used one in plants is β-glucuronidase (GUS). Although the GUS reporter has many advantages, a major drawback for mutant screening purposes is that its detection requires relatively time-consuming fluorometric determination or tissue-staining. Moreover, the relatively high stability of the GUS protein complicates the detection of real time signaling events. Other reporters such as green fluorescence protein (GFP) from jellyfish and luciferase from firefly

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that do not have these limitations have received increasing applications in plant biology. Firefly luciferase (LUC) has a number of advantages over other reporters and its application in the study of light-regulated gene expression has been reported (Millar et al., 1992b; Michelet and Chua, 1996). Because of the non-invasiveness of the LUC imaging and the relatively short half-life of the luciferase enzyme, LUC-expressing plants can be subjected to successive experimental treatments and imaged repeatedly. Due to technological advancements and increasing application to many fields of biology, the instrumentation required for detecting *LUC* expression has become affordable for many laboratories. Here we present an example of luciferase used as a reporter to dissect environmental stress signal transduction in Arabidopsis. Detailed procedures for imaging and identification of plants as well as *Agrobacterium tumefaciens* lines are described.

Materials and Methods

RD29A-LUC transgenic plants

The -650 to -1 fragment of the *RD29A* (Yamaguchi-Shinozaki and Shinozaki, 1994) promoter was obtained by polymerase chain reaction using the following pair of primers: 5′-TCGGGATCCGGTGAATTAAGAGGAGAGA-GGAGG-3′ and 5′-GACAAGCTTTGAGTAAAACAGAGGAGGGTCTCAC-3′. The fragment was inserted into a binary vector containing the firefly *LUC* coding sequence (Millar et al., 1992a). *Arabidopsis thaliana* plants (ecotype C24) were transformed with this *RD29A -LUC* construct via *Agrobacterium tumefaciens* infection of roots (Valvekens et al., 1988). Plants homozygous for the *RD29A -LUC* chimeric gene were selected from T_2 seeds. One line (referred to here as wild type) with a single insertion that showed strong transgene up-regulation was chosen for subsequent experiments. The bulk of the seeds were mutagenized with ethyl methanesulfonate (EMS) and the resultant M_2 seeds were used for mutant screening.

Culture media and solutions

Plant growth medium
Seedlings for luciferase imaging were grown on agar plates. The medium was made with full strength MS salts (Murashige and Skoog salt base, JRH Biosciences, Lenexa, KS) (pH adjusted to 5.7 with 0.1 M KOH), 0.6% agar (A-1296, Sigma) and 3% sucrose. After autoclaving, 30 mg/L kanamycin (passed through a 0.2 μm filter) was added and the medium was poured into sterile disposable plastic petri dishes (150 mM $\times$ 15 mM, Falcon 5038).

Soil medium
Metro-mix 360 growing medium (Scotts-Sierra Horticultural Products Co., Marysville, OH) was used.

Fertilizer (for soil grown plants)
Water soluble fertilizer 20–20–20 (Scotts-Sierra Horticultural Products Co., Marysville, OH).

Bleach solution
Commercial bleach solution with 0.01% Triton X-100.

ABA solution
100 mM ABA stock solution was prepared by dissolving (±)-cis, trans-abscisic acid (Sigma Chem. Co., St. Louis, MO) in ethanol and was stored at −20 °C. The working solution was made by diluting this stock solution to 100 μm with sterile water and was then kept at 4 °C.

NaCl/PEG solution
The 300 mM NaCl solution was prepared by adding NaCl to the MS solution (full strength MS salt, 3% sucrose, pH 5.7) and autoclaving. The 30% PEG solution was similarly prepared by dissolving polyethylene glycol (molecular weight 6000) in MS salt solution (full strength MS salt, 3% sucrose, pH 5.7), but this solution was not autoclaved. Both solutions were kept at 4 °C to deter microbial growth.

Luciferin
The 100 mM luciferin stock solution was prepared by dissolving luciferin (Promega, Madison, WI) in sterile water, and stored in 100 μl aliquots at −80 °C. We found that the stock solution was stable under these conditions for at least two years. The 1 mM luciferin working solution was made by diluting one aliquot (100 μl) of the stock solution into 10 ml of 0.01% Triton X-100. This solution can be stored at 4 °C in the dark (by wrapping the spray bottle with aluminum foil) and is good for at least two weeks.

Luciferin sprayer
It is important that the luciferin substrate be uniformly sprayed on plant samples. A fine misting spray bottle was constructed (Figure 1) by removing the tip from a fine misting sprayer (a nasal decongestant bottle) cut to fit into the screw on cap of another pump spray bottle (e.g., hair spray pump bottle). The solution-guide tube of the sprayer was curved so that its inlet end was at the top of the bottle. This allows all the solution in the bottle to be sprayed while

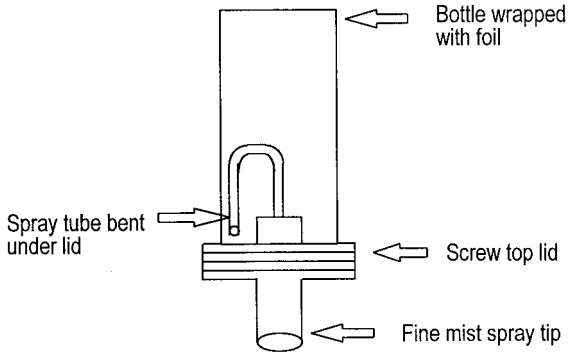

Figure 1. Sketch of the luciferin spray bottle.

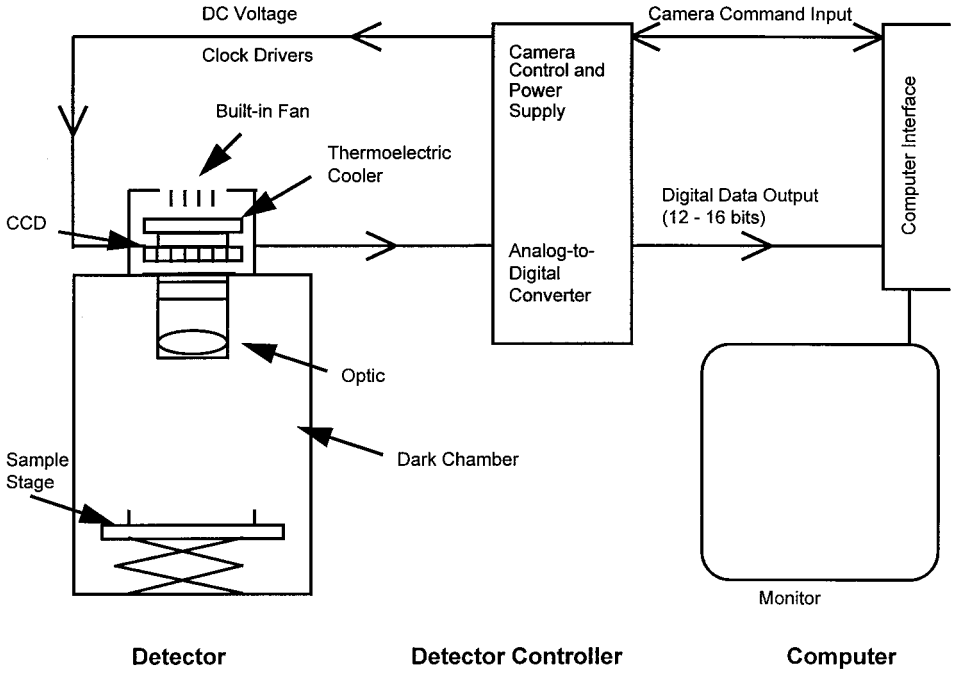

Figure 2. System setup for luciferase imaging.

holding the bottle upside down. The bottle was wrapped with aluminum foil to prevent exposure to light.

Imaging system

The imaging system consists of a detector (CCD camera), a detector controller, and a computer (Figure 2). The detector we use is a thermoelectrically cooled Charge Coupled Device (CCD) camera (Model TE/CCD 512

TKB/VISAR-1, Princeton Instruments, Inc., Trenton, NJ) with 512 × 512 pixels. The lens was a Casina TV lens (F 0.95). The camera is mounted on the topside of a dark chamber measuring 40 × 40 × 55 cm (length × width × height). This dark chamber is placed separately in a small dark room and connected via cable to the detector controller. The controller model is ST-138S (12–16 bits, serial) (Princeton Instruments, Inc., Trenton, NJ). Requirements for computer and monitor are as follows: a minimum of 8 megabytes total memory, a VGA monitor with 256 color graphics and at least 512 kb of memory, two-button Microsoft compatible serial mouse. Because each image file has 528 kb, a large hard drive storage capacity is required. To facilitate mutant screening and isolation, it is better to have a monitor with a wide, flat screen. Supporting software requirements include MS-DOS 3.3 or above, and Windows 3.1 or above. Most new PCs will easily fulfill these requirements. The software for image acquisition and processing (WinView 1.2) is supplied free by the camera manufacturer and is user-friendly.

Protocol

Seed sterilization and planting

Appropriate amounts of *Arabidopsis* seeds were sterilized with bleach solution for 5-10 min and then rinsed 5 times with sterile water. The seeds were then suspended in 0.4% low-melting-point agarose and planted directly on agar plates using a disposable plastic transfer pipette (Corning Samco Corporation, San Fernando, CA). For mutant screening, approximately 500 seeds can be planted in a single 150 mm × 14 mm agar plate. After planting, the plates were sealed with parafilm and kept at 4 °C for 2 d before being transferred to room temperature and supplied with constant illumination for germination and growth.

Plant growth

To avoid water condensation on the lids of the plates, the plates were placed on raised wire mesh or a tube rack to allow for airflow underneath the plates. Seedlings growing under these conditions for one week after germination were ready for treatments and imaging. When needed, the seedlings were transferred to soil, placed in a growth chamber and supplied with 16 h light at 22 °C, and 8 h dark at 18 °C, and 70% relative humidity. Complete fertilizer solution was supplied once a week to soil-growing plants.

Stress treatments for mutant screening

Seedlings were sequentially screened for constitutive expression mutants, low temperature response mutants, ABA response mutants, and osmotic stress response mutants. Treatments were as follows:

Constitutive mutants
Seedlings growing on agar plates without any stress treatments were first screened for mutants that constitutively express the luciferase transgene (termed *cos*, for *c*onstitutive expression of *o*smotic *s*tress-responsive genes).

Low temperature treatment
After being imaged for constitutive *LUC* expression, the plate was placed in a temperature-controlled refrigerator and incubated at 0 °C (±0.1 °C) for 48 h (in the dark). For imaging (see below), the plate was taken out of the refrigerator and immediately sprayed with luciferin (at room temperature). Putative mutants with altered responses to low temperature were marked. The plate was then incubated at room temperature under white light for 24–48 h to allow for decay of the cold-induced *LUC* expression (by checking *LUC* image when needed).

ABA treatments
After the cold-induced *LUC* expression subsided, the seedlings were sprayed with 100 μM ABA. To ensure maximum induction, ABA solution must be sprayed heavily so that all the leaves are wet. Usually, it was necessary to spray over 20 times. The plate was then covered (without being sealed with parafilm) and incubated under light at room temperature for 3 h before being imaged to identify ABA response mutants. After luciferase imaging, the plate was sealed with parafilm and incubated at room temperature under white light for 24 h to allow breakdown of the ABA-induced *LUC* expression.

Osmotic stress treatments
After ABA treatment, seedlings on the agar plates were gently pulled out of the agar medium and transferred to a petri dish containing a layer of filter paper soaked with 300 mM NaCl (or 30% PEG). The treatment lasted for 3 h under white light at room temperature. Excess salt solution was drained from the petri dish before imaging. Putative mutants with altered *LUC* expression were removed immediately after imaging and briefly rinsed in MS salt solution before being transferred to soil to grow to maturity. Since the imposed NaCl stress is severe and some mutants may not be able to survive the stress, putative *cos* mutants or mutants with altered responses to cold or ABA were directly transferred to soil and not treated with NaCl. NaCl or PEG treatments may be done later with M_3 seeds of these putative mutants. Progenies of these putative mutants were re-screened and those retaining the mutant phenotypes were kept for further research.

Luciferase imaging

System setup

The imaging system was set up as outlined in Figure 2. The sample stage was about 30 cm away from the camera lens. The camera lens was adjusted to focus on the seedlings so that a sharp luminescence image of seedlings was obtained. This setup was used for screening young seedlings growing in petri plates. Although big sized adult plants can also be imaged, it is relatively difficult to obtain a well-focused image. It is possible to image adult plants by detaching parts (e.g., leaves) and placing them on agar plates or petri dishes with appropriate supporting solutions.

Starting the imaging system

First turn on the power switch and then turn on the cooler switch on the camera controller. To turn off the system, operate in reverse order. Before acquiring images, the camera needs to cool down to a preset temperature (−45 °C). When the designated temperature is achieved, the cooler status light on the controller will change from yellow to the green. Images can then be acquired from the WinView menu on the computer.

Acquiring background

Each time you operate the imaging system, it is necessary to take a background image that reflects random signals generated by cosmic rays and the internal noise of the system. We found, under most circumstances, the background reading stabilized around 200/pixels. The background noise did increase over time. When the background count becomes more than 1,000/pixels (after usually 6–24 months), the camera must be sent back to the manufacturer to be pumped down to maintain the vacuum in the CCD chip chamber. To acquire a background image:

- Place an empty petri dish on the sample stage.
- Close the camera chamber door.
- Run the WinView software; acquire images by activating the menu icon *run as* (for manual background subtraction), or *acquire background* (for automatic subtraction).

The exposure time for the background image should be the same as that used for the luciferase images. We typically use 5 min exposure time for both background and luciferase images.

Spraying luciferin

Hold luciferin spray bottle straight and point downward about 15 cm above the seedlings and spray uniformly on the leaves 5 times (once pointed at the center, the other 4 times pointed to a rectangle corner slightly apart from the center but still well inside the plate). The seedlings were either directly placed

in the camera chamber for a fluorescence image before taking the luciferase image or kept in the dark for 5 min before direct imaging for luciferase expression.

Imaging fluorescence
Green plants that have been exposed to light emit strong fluorescence after they are placed in the dark. The fluorescence emission will disappear after 1–5 min in the dark. A fluorescence image can be acquired immediately after spraying the luciferin. The exposure time is 30 s. Alternatively, it can be acquired after obtaining the luciferase image. In this case, open the camera chamber and expose the plate briefly to light without moving the plate, and then close the camera door quickly and immediately acquire a fluorescence image with a 30 s exposure. The fluorescence image provides a digital position for all green seedlings, which helps to match a luciferase signal to a seedling in a petri plate. The fluorescence image is especially helpful in locating constitutive expression mutants.

Imaging luminescence
After taking a fluorescence image, a luciferase image was taken 5 min after spraying the plants with luciferin. When there was no need to take a fluorescence image, the treated seedlings were sprayed with luciferin and the plate was left in a dark box for 5 min to eliminate interference from chlorophyll fluorescence. All luciferase images were acquired at room temperature and typical exposure time was 5 min. If the luminescence intensity is unusually high or low, a shorter or longer exposure time may be needed and the corresponding backgrounds with the same exposure time subtracted.

Image processing and identification of putative mutants
The background was subtracted either automatically or manually from the sample image using the WinView software. The image contrast could be adjusted for easy identification of putative mutants. When it was difficult to identify the position of a particular putative mutant in a plate of many seedlings, comparison of the luciferase image against the fluorescence image helped to precisely locate a particular mutant.

The luminescence intensity of any single seedling could be quantified by examining the intensity of each pixel or by quantifying the total counts of the whole seedling using the statistics feature of the software. When characterizing individual mutants, an equal number of pixels of the mutant or the wild type seedlings were highlighted and 20 individual seedlings were quantified for statistical analysis. The averages and standard deviations between mutants and wild type could then be compared.

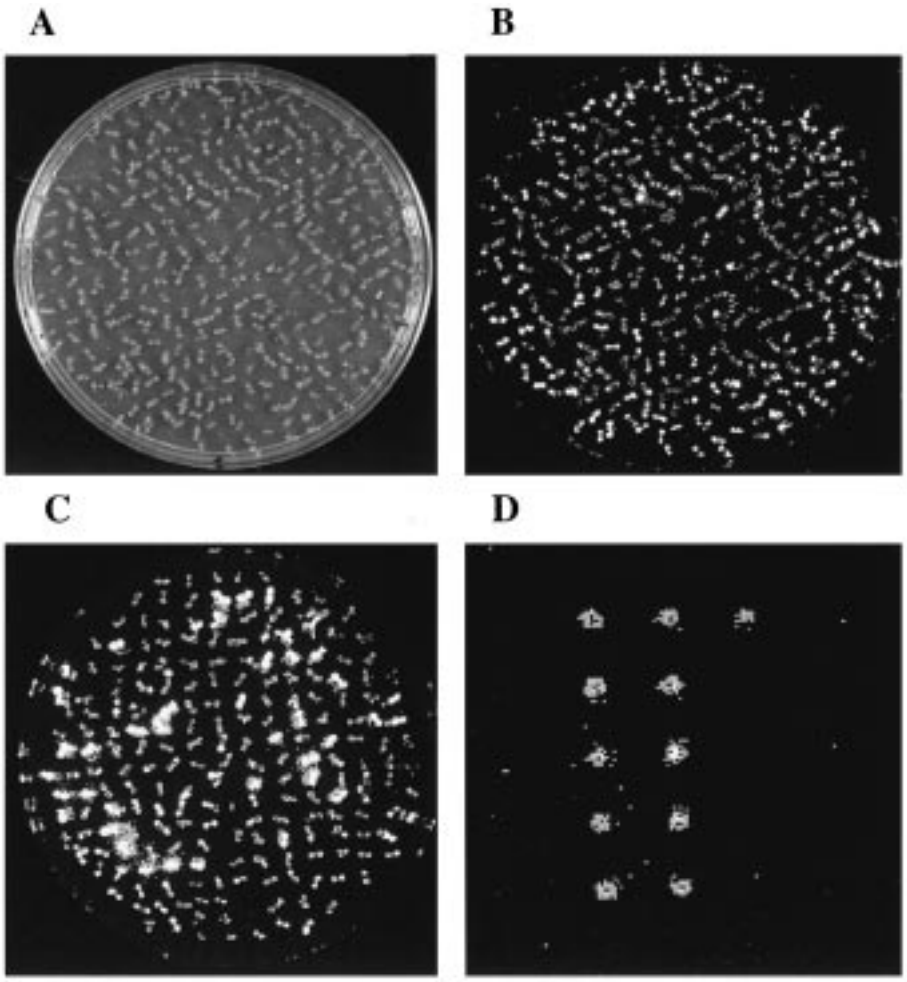

Figure 3. Examples of luciferase images. A. Picture of *RD29A-LUC* seedlings (M_2) in an agar petri dish ready for mutant screening. B. A luciferase image of the plants in Figure 3A after a 2-day cold treatment. C. A luciferase image of a segregated F_2 population generated from selfed F_1 plants. The F_1 seeds were obtained from a cross between an ABA/osmotic stress- responsive mutant and the wild type *RD29A-LUC* plant. Before imaging, seedlings were sprayed with 100 μm ABA and incubated under light for 3 h. The brighter ones are the recessive mutants and others are with wild-type phenotype. D. Luciferase image of *Agrobacteria* expressing *Osmotin-LUC* transgene. Five μl each of *Agrobacterium* cultures and luciferin were placed in microtiter plate and imaged. Bright dots represented individual transformants. Four untransformed *Agrobacterium* controls were in the third column.

Screening of agrobacteria for positive transformants

When constructing a *LUC* expressing plant, it is important to check whether the *Agrobacterium* used for plant transformation contains the appropriate *LUC* construct. This can be done conveniently by luciferase imaging (Figure 3).

- Transform an appropriate *Agrobacterium* strain with the *LUC* reporter gene fused with a promoter of interest.
- Grow the *Agrobacteria* in liquid LB medium with appropriate antibiotics at 30 °C overnight.
- Pipette an aliquot (5 μl) of *Agrobacteria* liquid culture into a well in a microtiter plate, add 5 μl of 1 mM luciferin solution. Use ten μl of *Agrobacterium* culture without luciferin for control.
- Place the plate on the sample stage and take the luciferase image using the same procedure as for imaging plants.

Results and Discussion

Luminescence of transgenic plants expressing firefly luciferase

Untransformed plants will not yield a signal after being kept in the dark for a few minutes to eliminate chlorophyll fluorescence. There is no background interference with plants expressing firefly luciferase. This is an advantage over the fluorometric GUS assay since there are many compounds in stressed plants that interfere with the GUS assay. Transgenic plants containing the *RD29A-LUC* transgene display no luminescence in the absence of stress. However, when the plants are treated with either cold, ABA or osmotic stress, significant expression of luciferase is induced (Ishitani et al., 1997) (Figures 3B and 3C). This property not only allows the isolation of mutants that have enhanced induction of the transgene but also mutants that show reduced induction of the transgene by stress signals.

A high throughput mutant screen

The current system is very efficient for the isolation of mutants defective in environmental signal transduction. In a single Petri plate, one can plant up to 1000 mutagenized *Arabidopsis* seeds (Figure 3A). The image process takes just 5 min. With this system, one can screen over 30,000 seedlings in one day. The limiting factor is the speed at which seeds can be planted on the agar plates. Moreover, it is very easy to identify mutants that have altered expression of luciferase (with altered intensity of luminescence relative to the wild type plants). This greatly reduces the chance of recovering false mutants.

With our screening of *RD29A -LUC* plants, most of the putative mutants we identified in the first screening were true mutants.

Multiple stress treatments of the plants

Luciferase is relatively unstable compared to the GUS protein. The half life of the protein is around 3 hr. This allows accurate measurement of the induction and it offers the possibility to detect *LUC* responses to multiple stresses for the same plant over a short period of time. With the transgene *RD29A-LUC*, we found that under the conditions described here, there was no obvious interference of a previous treatment on subsequent stress treatments in the regulation of the transgene expression. By contrast, with a more stable reporter like GUS, it is difficult to overcome the residual GUS activity left over by previous stress treatments.

Non-invasive imaging for mutant isolation

This video imaging system has the additional advantage that the plants are not affected by spraying luciferin, the only treatment required for *LUC* imaging. This nondestructive *in vivo* detection of luciferase activity allows the recovery of mutants too sick to endure the severe treatments required for detection of other reporters. For instance, we recovered over a dozen *cos* mutants, which are probably too small and weak to withstand the cutting of the plants for assaying the activity of other reporters.

The usefulness of the luciferase reporter in screening signal transduction mutants depends largely on the strength of the promoter used. Although the CCD camera we used here is very sensitive, some weak promoters may not be strong enough to activate *LUC* to a level easily detectable by the camera. Prolonged exposure may increase the detection limit, but recovery of low expression mutations may be difficult.

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