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43 High-Throughput Screening of *Arabidopsis* Mutants with Deregulated Stress-Responsive Luciferase Gene Expression Using a CCD Camera

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43.1 INTRODUCTION

Over the course of the past several years we have developed a high-throughput screening protocol to isolate stress signal transduction mutants in the plant *Arabidopsis thaliana* using luciferase imaging with a charge-coupled device (CCD) camera. This method involves using plants containing the firefly luciferase gene fused with different promoters from genes involved in cold temperature stress, osmotic stress, abscisic acid response, or in general stress perception. The system works by inducing the promoter–luciferase fusions by various environmental perturbations and recording the amount of light from luciferase-catalyzed reactions with a CCD camera. The level of luminescence is an indication of plant responsiveness to the treatment.

Using luciferase in this system has several advantages over other reporters. One is that the screening process is noninvasive to the plants. The fact that the luciferase enzyme is quickly degraded

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within plants allows several experimental treatments of the same plant followed by CCD imaging. Additionally, the equipment required to detect luciferase expression has recently become more affordable, and currently there are several integrated systems available that are designed specifically for luciferase imaging. This makes it quick and easy for even a novice to set up the necessary equipment to start luciferase imaging.

43.2 EQUIPMENT

The CCD camera system as shown in Figure 43.1 is a product of Roper Scientific (Princeton, NJ). It consists of the camera itself, with a lens, a camera controller, a computer interface card, a Cryotiger® cryogenic cooler, a dark box, and a standard IBM-compatible computer running WinView/32 software provided with the camera.

The camera resolution is a function of the number of pixels. This model has 1300 × 1340 pixels. This high resolution makes it possible to image large numbers of small plants and also to

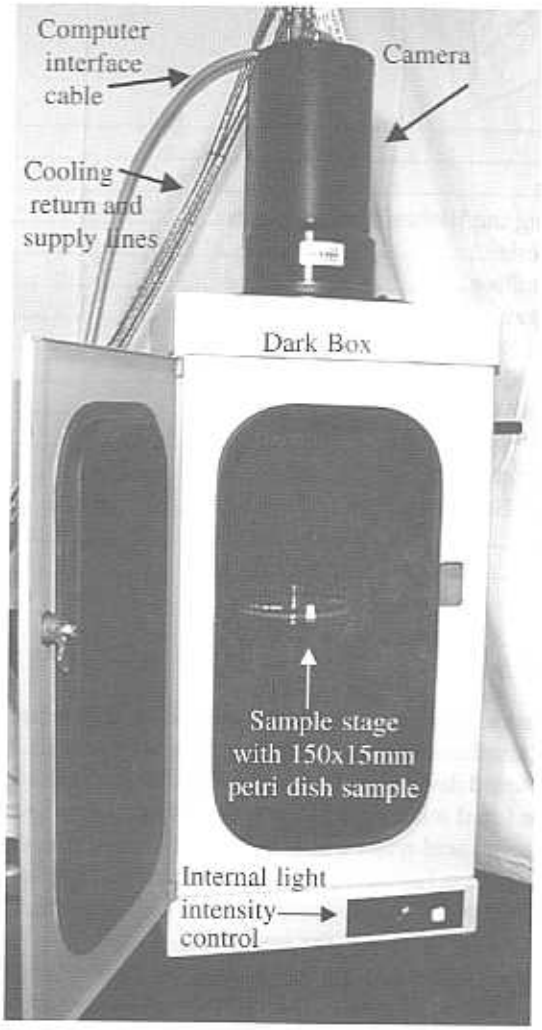

FIGURE 43.1 Camera system with dark box.

identify more clearly which part of the sample the luciferase signal is coming from. The lens on the camera is a standard f-mount Nikon lens. Since the f-mount is one of the most common types for 35 mm cameras, there are many types of lenses that are available, and one can be found that is tailored to each sample and image size. To detect luciferase expression, the CCD chip inside the camera must be cooled. This model uses the Cryotiger cryogenic cooler, a compressed gas system. It is able to cool the CCD chip inside the camera to −100°C. It is important to cool the CCD chip because the warmer the chip is, the higher the noise and the more likely the luciferase signal will be lost in the noise. There are thermoelectrically cooled cameras, which be cooled to approximately −40°C, that are still useful for luciferase detection. These thermoelectrically cooled models are slightly more affordable, although we have found the sensitivity of the cryogenically cooled model to be a benefit when studying genes that are not highly expressed.

The camera is fixed to the top of a dark box into which the sample to be imaged is placed. Inside the box there is a movable stage and a light to be used to take a brightfield image if one is required for sample orientation. The box is designed to be light tight when used in a room with normal light conditions. This eliminates the need for employing the camera in a darkroom.

The camera controller is an ST-133 model. It controls the temperature as well as the shutter of the camera. It also contains the analog-to-digital converter that is required for the computer to be able to interpret the camera data output. The computer interface card is a PCI serial card that is installed in the computer and connected to the camera controller.

The computer requirements for this system are operating system Windows 95 or newer, with 32 megabytes of RAM, a VGA monitor with at least 256 colors and at least 512 kilobytes of memory, and a Microsoft two button compatible mouse. A large hard drive and/or a CD writer are recommended because of the large numbers of image files that can be produced, and the fact that each image is approximately 530 kb. WinView is the software that comes with the camera. This software allows the user to control exposure time, the speed of the analog-to-digital converter, and pixel binning. Pixel binning is basically adding the signal from a group of pixels and treating them as one pixel. This has the effect of increasing the signal while reducing the time of exposure. There is a loss of resolution when pixel binning is used, but with this chip size of 1300 × 1340 the loss of resolution has no effect on this screening method until the range of about 6 × 6 binning.

43.3 SCREENING PROCESS

43.3.1 Generating the Bioluminescent Plants

We employed the luciferase reporter system to study stress signal transduction in plants. Because of a scarcity of morphological phenotypes for stress mutants, altered expression of stress-responsive genes can be used in screening for stress mutants. Luciferase was chosen in this case because it has several advantages over other frequently used reporters in plant biology such as β-glucuronidase (GUS) and green fluorescent protein (GFP).

A stress-responsive promoter fused with luciferase can be introduced into a plant to produce a bioluminescent plant. Several stress-responsive promoters are well characterized such as *RD29A* (also known as *COR78* or *LTI78*), *DREB1A* (*CBF3*), *DREB1B* (*CBF1*), *DREB1C* (*CBF2*), and *DREB2A*.[1,2] Here the application of *RD29A::LUC* lines will be described in detail.

Expression of the *RD29A* gene is induced by cold, abscicic acid (ABA, a plant hormone), osmotic stress, and drought.[1] The promoter of *RD29A* spanning from −650 to −1 was obtained by polymerase chain reaction (PCR) with *Arabidopsis* genomic DNA and two primers: 5′-TCGGGATCCGGTGAATTAAGAGGAGAGAGGAGG-3′ and 5′-GACAAGCTTTGAGTAAAA-CAGAGGAGGGTCTCAC-3′. This promoter fragment was inserted into a plant transformation vector containing the firefly luciferase coding region to produce the *RD29A::LUC* vector.[3] The vector was introduced into *Arabidopsis* (ecotype C24) via the root transformation method with

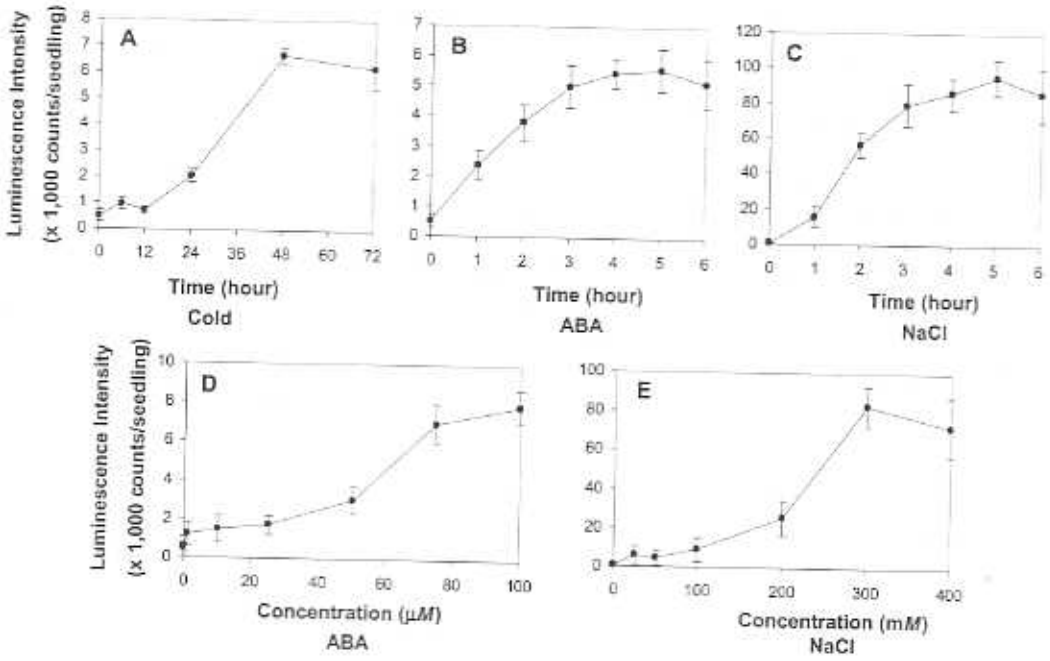

FIGURE 43.2 *RD29A::LUC* expression in response to cold, ABA, and NaCl treatment. (A) Time course of *RD29A::LUC* expression at 0°C; (B) time course of *RD29A::LUC* expression after 100 μ*M* ABA treatment; (C) time course of *RD29A::LUC* expression after 300 m*M* NaCl treatment; (D) *RD29A::LUC* expression 3 h after ABA treatment; (E) *RD29A::LUC* expression 5 h after NaCl treatment.

Agrobacterium tumefaciens.[4] The plant line homozygous for the *RD29A::LUC* transgene was selected in the T2 generation (the second generation after transformation).

Bioluminescence of selected *RD29A::LUC* lines was tested and characterized under various stress conditions and at different time points, as shown in Figure 43.2.

43.3.2 Mutagenesis

We took a genetic approach to dissect stress-signaling pathways in plant by generating mutants showing altered stress gene expression (*RD29A* in this case) and searching for the mutated genes. There are several mutagens to induce mutations in plants; EMS (ethyl methanesulfonate), fast neutron, foreign DNA such as T-DNA and transposon, and so on. Mutagensis methods can be found elsewhere.[5–7] We used EMS and T-DNA as mutagens to mutagenize *RD29A::LUC Arabidopsis*. We generated about 300,000 EMS-mutagenized mutants and 50,000 T-DNA insertion lines. Recently, our T-DNA-mutagenized plants are publicly released and available at ABRC (*Arabidopsis* Biological Resource Center, Ohio State University). T-DNA mutants have advantages over EMS mutants in that it is easier to clone the genes responsible for the mutation. Because the DNA sequence is known for the T-DNA insert, the T-DNA can be used as a "tag." TAIL-PCR,[8] inverse PCR,[9] PCR-walking,[10] plasmid rescue,[11] and even genomic library screening with a probe from T-DNA have been successfully applied to clone the disrupted plant genes. For T-DNA mutants, the pSKI015 activation tagging vector was used [11] and ten T2 individuals were pooled to make one pool.

43.3.3 Plant Handling

Seeds were dispensed in eppendorf tubes (about 50,000 seeds/g) and were surface-sterilized with commercial bleach supplemented with 0.01% Triton X-100 for 5 to 10 min, and then washed with sterile water four to five times. One droplet of 0.3% low-melting agarose can be added into the

sterilized seeds for easier handling during plating. Seeds were plated onto 0.6% agar medium with 3% sucrose (pH 5.7) in 150 × 15 mm petri dishes with a transfer pipette. As many as 500 seeds can be plated per 150 × 15 mm round plate. After being kept at 4°C for 2 to 4 days to break the seed dormancy, the plates were placed at 22°C under continuous light for germination and growth. Approximately 1-week-old seedlings were used for luminescence imaging. When needed, seedlings on agar medium were transferred to soil, and then allowed to grow in a growth chamber with cycles of 16-h light at 22°C and 8-h dark at 18°C.

43.3.4 Stress Treatments

Based on characterization of bioluminescence from *RD29A::LUC* plants (wild-type), 1-day cold treatment for cold stress (0°C), 3-h incubation under light after 100 μM ABA spray for ABA treatment, and 5-h incubation under light after 300 mM NaCl application for salt stress were chosen. Because of the short half-life of the luciferase enzyme (about 3 h) and nontoxicity of the marker, the same seedlings can be used repeatedly for several different stress applications.

43.3.4.1 No Stress

Constitutive expression of *RD29A::LUC* should be detected without stress treatment. One-week-old seedlings were first subjected to luminescence imaging without stress. The perturbations in the environment during manipulation may cause some luminescence induction. Therefore, care should be taken when screening for constitutive luciferase-expressing mutants.

43.3.4.2 Cold Stress

After imaging of seedlings without stress, the plates were placed at 0°C for 1 day. In our conditions, sometimes the 0°C 1-day incubation was not sufficient to induce strong luminescence. In such cases, more prolonged incubation such as 2 days at 0°C resulted in better luminescence images. Since the enzyme activity is reduced in cold conditions, the plates were occasionally warmed at room temperature for as long as 30 min. This usually gives higher luminescence signals.

After luminescence imaging, the plates were placed at 22°C under continuous light for at least 24 h to allow the luminescence signal to disappear.

43.3.4.3 ABA Treatment

After incubation of the plates under continuous light, 100 μM ABA was sprayed onto the seedlings to sufficiently wet each seedling. Before ABA treatment, the luminescence images of plates can be taken to ensure that no luminescence signal remains from the cold treatment. 100 μM ABA was prepared by diluting with sterile water from the stock solution of 10 mM (±)-*cis,trans*-abscisic acid (Sigma Chemical Co., St. Louis, MO) in ethanol. The stock solution should be stored at −20°C and the working solution can be stored at 4°C.

After ABA-sprayed plates were incubated at 22°C under continuous light for 3 h, the luminescence images were taken. After imaging, the plates were, again, placed at 22°C under continuous light.

43.3.4.4 NaCl Treatment

Seedlings on plates were carefully transferred onto filter paper saturated with nutrient solution supplemented with 300 mM NaCl. After 5 h, the images were taken. It should be noted that recovering the putative mutant after NaCl treatment might be difficult due to the severity of NaCl stress. In addition, the putative mutants previously marked after cold or ABA treatment may be lost because seedlings may be floating and moving in the 300 mM NaCl solution. Therefore, the NaCl treatment was usually applied only during the secondary screening process with progeny from the putative mutants.

43.3.5 Luminescence Imaging

After each treatment, the plates were sprayed evenly with 1 m*M* luciferin. The 1 m*M* luciferin solution was freshly prepared from 100 m*M* luciferin (Promega Co., Madison, WI) stock solution. The stock solution prepared in sterile water was stored at −80°C in 100 μl aliquots and diluted with 0.01% Triton X-100 to make 10 ml of 1 m*M* luciferin. The working solution of luciferin was kept at 4°C in the dark during use. Luciferin-sprayed plates were kept in dark for 5 min for luciferase enzyme reaction and decay of autofluorescence from chlorophyll. During the 5-min incubation, a background image was taken with an empty plate. A background image is generally needed because CCD cameras have some internal noise. Therefore, subtraction of background signal from the raw luminescence image enhances the image qualities. After a 5-min incubation of the plates, the plates were placed under the CCD camera in the dark. To prevent chlorophyll autofluorescence from interfering with the luminescence image, the plates should not be exposed to light after luciferin is applied. Luminescence images were acquired with 5-min exposure. Some representative mutants are shown in Figure 43.3.

In our system, 5-min exposure was sufficient to detect luminescence emitted from seedlings. However, the luminescence intensity is dependent on the nature of promoter and/or the position of transgene in the plant genome. Indeed, another *RD29A::LUC* line in the Columbia ecotype shows lower luminescence intensity than that in C24 ecotype, thus requiring longer exposures. Lower luminescence intensities were also observed in the *DREB1A::LUC*, *DREB1B::LUC*, and *DREB1C::LUC* lines. After imaging, the plates were aligned with the images and the putative stress mutants showing

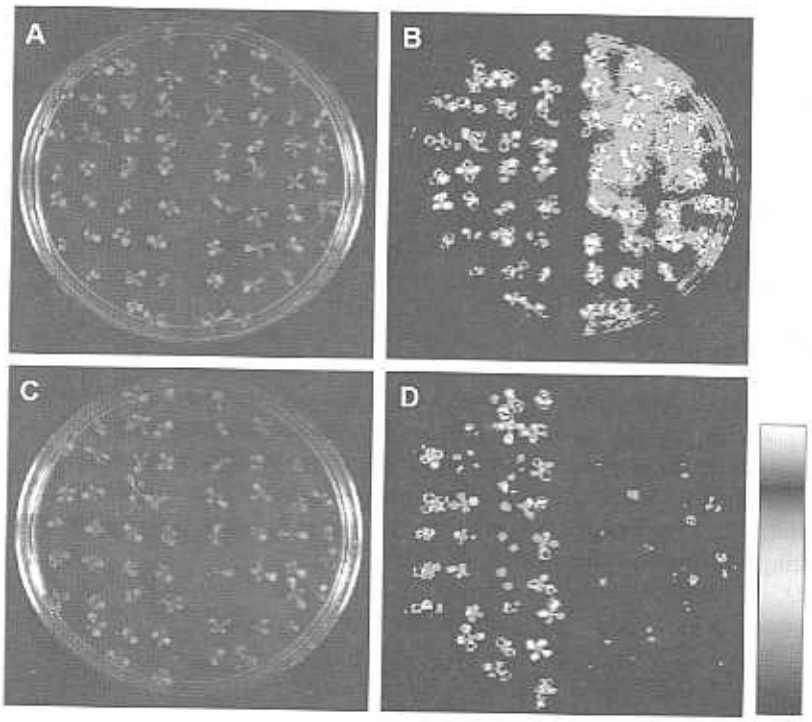

FIGURE 43.3 (Color figure follows p. 266.) Stress mutants showing altered RD29A::LUC expression. (A) The plate corresponding to luminescence image in B; (B) Mutants (right half) showing higher RD29A::LUC expression than wild-type (left half) after ABA treatment; (C) the plate corresponding to luminescence image in D; (D) Mutants (right half) showing lower RD29A::LUC expression than wild-type (left half) after ABA treatment. Right spectrum bar shows color changes depending on luminescence intensity; as intensity goes higher, color changes from black to white.

altered luminescence intensity—either higher or lower than wild-type (*RD29A::LUC* line)—were identified. For convenience in aligning with images, plates can be marked on the side with tape before luciferin application. After all stress imaging, all putative mutants were transferred to soil to produce seeds.

43.3.6 Secondary Screening

The progenies from the putative mutants were retested to confirm the mutant phenotypes. The plates were divided into eight to ten sections and each mutant progeny (about 30 seeds) was plated in each section. One section of wild-type plants should be included on each plate to compare with the mutants. If a mutant is real- and true-breeding, its progeny section will show all higher or lower intensity relative to the wild-type control. However, it should be noted that our T-DNA lines were generated with an activation tagging vector. Therefore, one may see a 3:1 segregation, if the mutation is dominant.

43.4 CONCLUSION

How cells perceive environmental signals and how the signals are transduced to activate adaptive responses have long been of interest to scientists. Molecular genetic approaches coupled with a chimeric transgene of the signal-inducible promoter fused with luciferase are valuable tools to study signal transduction. Here we have provided an example of luciferase imaging application to generate large numbers of stress-signaling mutants. This method can be applied to studies of any signaling pathway. However, because of the technical limitations of the CCD camera sensitivity, luminescence emitted from the plants should be strong enough to be detected. It is also helpful to have a basic knowledge of which treatment conditions are the most effective and how long after each treatment the luminescence signal is the highest before a large-scale screening is carried out.

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