Chapter 5

Aequorin Luminescence-Based Functional Calcium Assay for Heterotrimeric G-Proteins in Arabidopsis

Kiwamu Tanaka, Jeongmin Choi, and Gary Stacey

Abstract

Heterotrimeric GTP-binding proteins (G-proteins) and G-protein-coupled receptors are important signaling components in eukaryotes. In plants, the G-proteins are involved in diverse physiological processes, some of which are exerted via changes in the level of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$). Various techniques have been developed to measure the change of $[Ca^{2+}]_{cyt}$, e.g., calcium-sensitive microelectrodes, chemical fluorescent dyes, and biosensors based on luminescent or fluorescent indicators. In this chapter, we describe a protocol for in vivo $[Ca^{2+}]_{cyt}$ measurement in G-protein mutants expressing aequorin, a luminescent-based calcium biosensor, to extend our knowledge about G-protein mediated Ca^{2+} signaling. This method is also applicable to other early signaling events that are mediated by changes in $[Ca^{2+}]_{cyt}$ levels.

Key words Aequorin, Coelenterazine, Cytosolic free calcium ion, Heterotrimeric GTP-binding proteins, Arabidopsis

1 Introduction

Heterotrimeric GTP binding proteins (G-proteins) are well-characterized molecular switches that are activated in response to various extracellular stimuli [1, 2]. Generally, upon signal reception by G-protein-coupled receptors, the Gα subunit dissociates from the Gβγ dimer, and either one or both of these components interact with downstream elements to transmit the signal [3]. Like animals, plants use signal transduction pathways based on G-proteins to regulate many aspects of plant growth and development occasionally via Ca^{2+} signaling. Pharmacological studies targeting the Gα subunit or genetic studies using mutants of the Gα subunit and Gβ subunit have revealed that the G-proteins participate in seed germination, pollen germination, stomatal closure, microbe elicitor responses, etc. by modulation of cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) [4–7]. This suggests that the

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dynamics of $[Ca^{2+}]_{cyt}$ are closely linked to G-protein activity and can be excellent cellular markers to measure the G-protein-mediated signaling in plants.

There are many approaches to measure $[Ca^{2+}]_{cyt}$ such as calcium-sensitive microelectrodes, chemical fluorescent dyes, and biosensors based on luminescent or fluorescent indicators. Luminescent techniques using photoproteins (e.g., aequorin, etc.) have been extensively used to measure $[Ca^{2+}]_{cyt}$ dynamics in plants. Although an extremely sensitive light detector, such as photomultiplier tube or photon-counting camera, is required due to very low light yield, luminescent calcium reporters have several advantages over fluorescent dyes. For example, photo damage associated with excitation illumination is avoided, since the luminescent light emission is not dependent on optical excitation. Luminescence also has an intrinsically high signal-to-background ratio, because there is no autofluorescence and relatively little endogenous, background luminescence. The ability to transgenically express aequorin in plants has made this reporter particularly attractive to plant researchers to measure the cellular Ca^{2+} response to a variety of external stimuli.

Aequorin is a 21.4 kDa photoprotein originally isolated from the coelenterate jellyfish *Aequorea victoria* [8]. In order to be luminescent, the enzyme requires a hydrophobic prosthetic group, coelenterazine, to convert the apo-aequorin to aequorin, which is the active form of the enzyme. Upon Ca^{2+} binding, aequorin oxidizes coelenterazine into coelenteramide with the production of CO_2 and emission of blue light with a wavelength of approximately 470 nm (Fig. 1). The affinity of aequorin for Ca^{2+} is in the low micromolar range, and the enzymatic activity is proportional to cellular Ca^{2+} concentration in the physiological range of 50 nM to 50 μM [9]. Therefore, measurement of light emitted upon oxidation of coelenterazine by aequorin provides reliable quantification of cellular calcium levels. These measurements are comparable to that determined with fluorescent dyes [9].

In this chapter, we describe an in vivo $[Ca^{2+}]_{cyt}$ measurement technique using transgenic Arabidopsis expressing aequorin [10]. By way of example, we compare the calcium response of Arabidopsis G-protein mutants to those of wild-type plants [11]. Since the Gα subunit (GPA1) and Gβ subunit (AGB1) are encoded by single-copy genes in the Arabidopsis genome, analysis of single mutants in these genes provides a rigorous means to study their involvement in various signaling pathways. In order to trigger $[Ca^{2+}]_{cyt}$ changes, plants were treated with either extracellular ATP [12] or other microbial elicitors (Figs. 2 and 3). The method described here is generic and applicable for other studies using different mutant backgrounds that exhibit a change in the $[Ca^{2+}]_{cyt}$ levels [13–18].

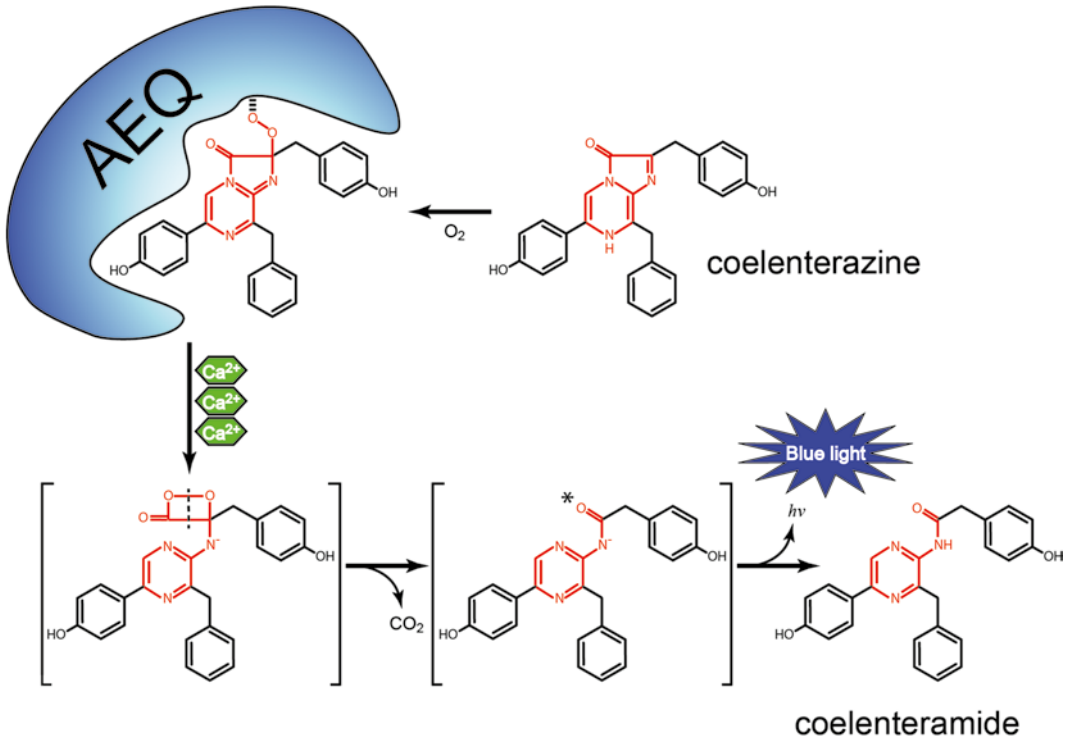

Fig. 1 Diagram showing Ca^{2+}-dependent light generation by aequorin. Apo-aequorin is converted to the active form, aequorin (AEQ), when reconstituted by a luminophore coelenterazine in the presence of oxygen (O_2). Upon binding of three molecules of Ca^{2+} to the respective EF-hands on the aequorin protein, coelenterazine is oxidized and cyclized to give the dioxetanone intermediate, followed by a conformational change of the protein accompanied by the release of carbon dioxide (CO_2) while producing the singlet-excited coelenteramide (*asterisk*) that emits *blue* light ($hv_{\sim 470nm}$). The part of the coelenterazine molecule where the changes occur is indicated with *red* color

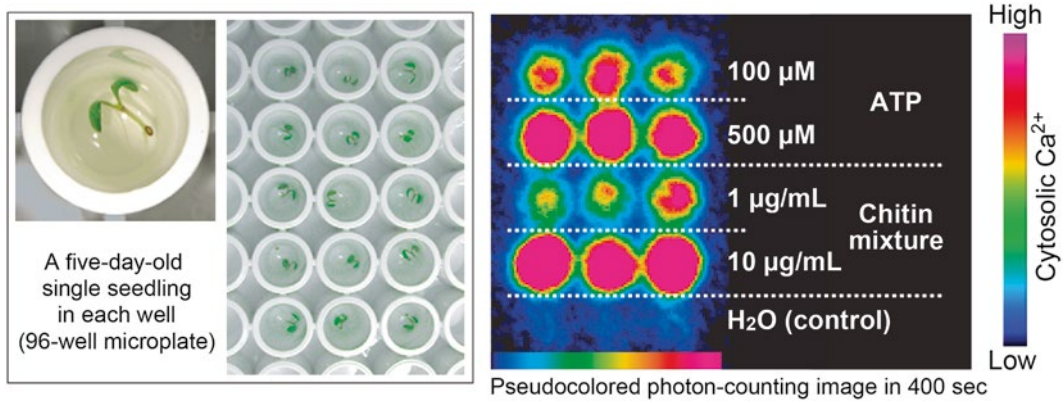

Fig. 2 An example of aequorin-based Ca^{2+} measurement in a 96-well white plate. *Left* picture shows the individual aequorin Arabidopsis seedlings in each well of the microplate. The *right* picture shows a pseudocolored photon-counting image (integrated over 400 s) after addition of ATP or chitin mixture. $[Ca^{2+}]_{cyt}$ has been coded according to the scale

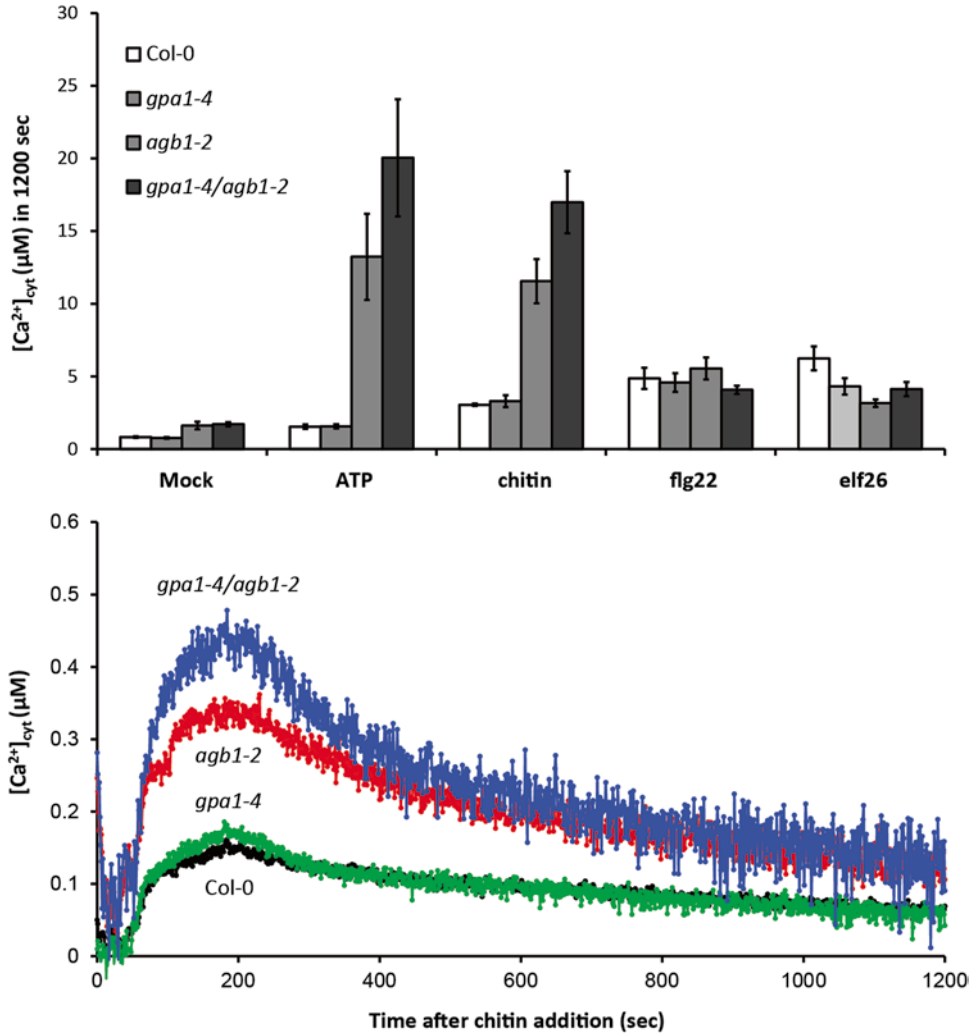

Fig. 3 Measurement of stimuli-induced $[Ca^{2+}]_{cyt}$ changes using Arabidopsis in G-protein mutants expressing aequorin. Histogram represents integrated $[Ca^{2+}]_{cyt}$ values over 1,200 s after various stimuli treatments: mock (2 mM MES buffer), ATP 100 μM, chitin mixture 10 μg/mL, flg22 100 nM, and elf26 100 nM. Each value represents the mean of six seedlings ±SE. *Line graph* shows kinetic differences in $[Ca^{2+}]_{cyt}$ responses to chitin treatment. Note that the *agb1* mutant seedlings exhibited hyper-responsiveness to extracellular ATP or chitin, suggesting that the Gβ-subunit is negatively involved in the cellular response to these stimuli

2 Materials

2.1 Plant Materials and Plant Growth

1. Transgenic Arabidopsis expressing the photoprotein apoaequorin (*see* **Note 1**).
2. Heterotrimeric G-protein mutants confirmed by PCR-based genotyping using gene specific primers (*see* **Note 2**).

3. Half-strength Murashige and Skoog (MS) medium containing 1 % (w/v) sucrose, 1 % (w/v) agar, 0.05 % (w/v) MES (pH 5.7), and Gamborg's B5 vitamins.
4. Growth chamber set at 22 °C under long-day conditions (16 h light at ~80 μE/m^2/s and 8 h dark).

2.2 Aequorin Luminescence-Based Calcium Assay

1. Coelenterazine stock solution: 1 mM native coelenterazine (Nanolight Technology) dissolved in ethanol. This is 100× stock solution and diluted to the specific concentration needed. This solution should be stored in the dark at −20 °C and, under these conditions, is stable up to two weeks (*see* **Note 3**).
2. Reconstitution buffer: 2 mM MES (pH 5.7 with 10 N KOH), 10 mM $CaCl_2$. Immediately prior to use, add coelenterazine from the stock solution (**item 1** of Subheading 2.2) to a final concentration of 10 μM (*see* **Note 4**).
3. 96-well white polystyrene microplates with opaque flat bottom (*see* **Note 5**).
4. A CCD camera with a fully light-tight dark box (Photek 216; Photek, Ltd.) (*see* **Note 6**).
5. Multichannel pipettes: to apply the chemical solutions to multiple wells at the same time.
6. Discharging solution: 2 M $CaCl_2$ dissolved in 20 % (v/v) ethanol.

3 Methods

3.1 Preparation of the Aequorin Transgenic Lines in the G-Protein Mutant Background Through a Genetic Cross

To transfer an aequorin gene into the G-protein mutants, F_2 generation pools from a cross between each mutant and the aequorin transgenic Arabidopsis line are subjected to screening as follows. T-DNA insertion of the mutants is evaluated by PCR-based genotyping. Introgression of the aequorin gene is confirmed by bioluminescence of aequorin itself (*see* **Note 7**).

1. Grow the F_2 seedlings for ~14 days in the growth chamber.
2. Harvest a small piece of leaf tissue from each seedling and incubate with 50 μL of the reconstitution buffer in each well of a 96-well plate. After >6 h incubation, apply the discharging solution to each well and monitor the bioluminescence signal. If the seedling is not expressing aequorin, no bioluminescence will be visible from the tissue. Select only those seedlings that show strong bioluminescence consistent with aequorin expression.
3. Harvest leaf tissue from the selected seedlings from **step 2** above and extract genome DNA. Use this DNA to genotype the plants by PCR using gene-specific primers and a T-DNA

left-border-specific primer (*see* **Note 2**). Select those plants that possess a homozygous T-DNA insertion and then grow to obtain additional seeds in the next generation.

4. In the F3 generation, homozygosity is confirmed by the same method described above (*see* **steps 2** and **3** of Subheading 3.1).

3.2 In Vivo Aequorin Reconstitution and Monitoring of $[Ca^{2+}]_{cyt}$ Responses

Once homozygous aequorin lines are successfully obtained in the various mutant backgrounds, they can be used for measurement of cytoplasmic calcium levels. First, reconstitute the aequorin–coelenterazine complex in vivo by incubating the seedlings in the reconstitution buffer. In a side by side manner, measure the calcium levels of the mutant plants in direct comparison to control experiments using non-transgenic wild-type plants. This is important to verify that aequorin bioluminescence is triggered by increased $[Ca^{2+}]_{cyt}$, and not due to reactive oxygen species (ROS)-triggered coelenterazine chemiluminescence (*see* **Note 8**).

1. Sow the seeds along a straight line on an MS solid medium in a petri dish.
2. After incubation at 4 °C for 3 days to synchronize seed germination, place the petri dish vertically in a plant growth chamber at 22 °C under lights.
3. Transfer a 5-day-grown seedling from the petri dish to an individual well of a 96-well microplate containing 50 μL of reconstitution buffer with coelenterazine and incubated overnight in the dark at room temperature.
4. After overnight incubation (*see* **Note 9**), place the 96-well plate under the camera and monitor the basal level to be stable before chemical applications.
5. Apply chemicals of your interest at the desired final concentrations (e.g., add 50 μL of double strength of the final concentrations), and then continue photon counting for a reasonable term (Fig. 2). We recommend the use of multichannel pipettes to simultaneously apply the chemical solutions to all wells.
6. At the end of each experiment, add an equal volume of the discharging solution (e.g., 100 μL) to discharge out remaining aequorin (*see* **Note 10**). Continue photon counting after discharge until the luminescence signal is well below the baseline level.

3.3 Calculation and Data Analysis: Converting Photon Counting Data into Ca^{2+} Concentration

Several procedures have been published to convert photon counts into $[Ca^{2+}]$ [19–22]. In our case, we use an equation adapted from Allen et al. [19] to determine in vivo $[Ca^{2+}]$ as follows:

1. Obtain the counting data as photons per second (L). In the case of measuring integrated $[Ca^{2+}]$ (Fig. 3), acquire the sum of the all photon counting data during the experiments.

2. Obtain total counting data for the entire sample over the course of the experiment until all the aequorin was discharged (L_{max}).
3. Input the data into the following equation: $[Ca^{2+}](nM) = \{[X^{1/3} + (X^{1/3} \times 55) - 1]/(1 - X^{1/3})\}/0.02$ (*see* **Note 11**), where X is the amount of light per second divided by the total light emitted after that time point until all the aequorin was discharged, i.e., $X = L/L_{max}$.
4. For all treatments, at least six replicates should be performed per group, and the individual $[Ca^{2+}]$ from repeat experiments averaged (Fig. 3).

4 Notes

1. In the examples shown here, we used transgenic aequorin lines in the wild-type Arabidopsis Col-0 background [10], in which 95–98 % of the aequorin is expressed in the cytoplasm [23]. There are other transgenic Arabidopsis lines expressing aequorin targeted to specific organelles, such as mitochondria [24], vacuolar microdomain [23], chloroplast [18, 25], and nucleus [26]. Aequorin can be fused with other reporter proteins such as green fluorescent protein (GFP) [27, 28], which can be used to localize the protein and analyze Ca^{2+} mobility in tissue or at a cellular level by bioluminescence resonance energy transfer.
2. Mutant lines are available at Arabidopsis Biological Resource center (https://abrc.osu.edu). In the examples shown here, we used *gpa1-4* (CS6534) and *agb1-2* (CS6536). Homozygous T-DNA insertions are selected by PCR using gene-specific primers [29, 30] and a T-DNA left border-specific primer (5′-GCGTGGACCGCTTGCTGCAACT-3′). Genomic DNA is isolated according to the protocol described by Edwards et al. [31].
3. Coelenterazine can also be dissolved in methanol, but never in organic solvents such as dimethylformamide and dimethylsulfoxide because coelenterazine spontaneously oxidizes. All the procedures using coelenterazine should be conducted under dim light. Prices of the chemical differ widely in companies although in our limited survey we found no differences in quality or purity. In addition to the native coelenterazine substrate, synthetic derivatives are available. For instance, *h-coelenterazine* and *cp*-coelenterazine have been used to demonstrate small changes in $[Ca^{2+}]_{cyt}$ in plants due to the increased Ca^{2+} sensitivity of aequorin.

4. The solution should be prepared immediately before use since coelenterazine is very sensitive to light and easily oxidizes. Once dissolved in aqueous solution, the chemical will start to be oxidized due to interaction with ambient oxygen. The best results are obtained with 1–10 μM of coelenterazine at final concentration.
5. White plates are commonly used for luminescent assays because of their reflective property; white plates reflect light within each well and will maximize the light output signal.
6. Recombinant aequorin emits ~5×10^{15} photons/mg protein [32]. In plant tissues, the actual levels of aequorin are relatively low: a few pg protein/mg fresh weight of tissue [33]. Therefore, very sensitive light counting equipment is required to detect the blue light. We used an image-intensified CCD camera. This is a very sensitive device since the signal-to-background ratio is improved by increasing the strength of the signal from the light sensors to detectors using microchannel plates. This device has an advantage of a quick scan speed to detect rapid changes in light emission in contrast to the cooled CCD camera.
7. It will be easier to screen the aequorin transgenic lines if the location of the aequorin transgene on the genome is determined in advance. We identified the insertion position of the aequorin used in our experiments by using TAIL-PCR. The gene is inserted at 4,341,822 bp on chromosome 1. This knowledge allows us to design primers to detect the transgene by PCR-base genotyping. Therefore, it would be difficult to establish useful mutant lines if the mutation were closely linked to the site of aequorin insertion on chromosome 1.
8. Coelenterazine is an effective antioxidant used as a chemiluminescent indicator for ROS production [34]. Indeed, in nature, coelenterazine have originally been used in ROS detoxification system by ancient marine organisms. Although light release from aequorin-bound coelenterazine is triggered by Ca^{2+}, any free coelenterazine can produce light when exposed to ROS. To avoid this problem, plants should not be stressed during the reconstitution step. Additionally, a control experiment is required using non-transgenic wild type plants in parallel and under identical condition.
9. Coelenterazine is a membrane-permeable molecule. Therefore, it is not necessary to use surfactants and vacuum infiltration to facilitate loading the molecule into tissue. However, different from the case of in vitro reconstitution (saturated after 6 h), aequorin activity continues to increase over a 24 h time period during in vivo reconstitution of 7-day-old Arabidopsis

seedlings [23]. The optimal incubation time for reconstitution is best obtained empirically in each lab, although for the example shown here we incubated the seedlings for 18–24 h.

10. To estimate the remaining aequorin after the experimental treatment, the tissues are immersed in a solution containing a massive amount of ethanol and Ca^{2+}. The ethanol destroys the cell membrane allowing Ca^{2+} penetration and discharge of the remaining aequorin. The discharging data is used for normalization of the results (*see* **steps 2** and **3** of Subheading 3.3).
11. The equation was adopted from a method based on the calibration curve of Allen et al. [19]: $L / L_{max} = \left\{\left(1 + K_R \times [Ca^{2+}]\right) / \left(1 + K_{TR} + K_R \times [Ca^{2+}]\right)\right\}^3$, where K_R is the dissociation constant for the first Ca^{2+} to bind to aequorin, and K_{TR} is the binding constant of the second Ca^{2+} to bind to aequorin; $K_R = 2 \times 10^6$ M^{-1} and $K_{TR} = 55$ M^{-1}. The values are specific for the affinity between aequorin and coelenterazine derivatives, e.g., *cp*-coelenterazine: $K_R = 26 \times 10^6$ M^{-1} and $K_{TR} = 57$ M^{-1} [35].

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