

Video Article

# Measuring Spatial and Temporal $\text{Ca}^{2+}$ Signals in Arabidopsis Plants

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## Abstract

Developmental and environmental cues induce  $\text{Ca}^{2+}$  fluctuations in plant cells. Stimulus-specific spatial-temporal  $\text{Ca}^{2+}$  patterns are sensed by cellular  $\text{Ca}^{2+}$  binding proteins that initiate  $\text{Ca}^{2+}$  signaling cascades. However, we still know little about how stimulus specific  $\text{Ca}^{2+}$  signals are generated. The specificity of a  $\text{Ca}^{2+}$  signal may be attributed to the sophisticated regulation of the activities of  $\text{Ca}^{2+}$  channels and/or transporters in response to a given stimulus. To identify these cellular components and understand their functions, it is crucial to use systems that allow a sensitive and robust recording of  $\text{Ca}^{2+}$  signals at both the tissue and cellular levels. Genetically encoded  $\text{Ca}^{2+}$  indicators that are targeted to different cellular compartments have provided a platform for live cell confocal imaging of cellular  $\text{Ca}^{2+}$  signals. Here we describe instructions for the use of two  $\text{Ca}^{2+}$  detection systems: aequorin based FAS (film adhesive seedlings) luminescence  $\text{Ca}^{2+}$  imaging and case12 based live cell confocal fluorescence  $\text{Ca}^{2+}$  imaging. Luminescence imaging using the FAS system provides a simple, robust and sensitive detection of spatial and temporal  $\text{Ca}^{2+}$  signals at the tissue level, while live cell confocal imaging using Case12 provides simultaneous detection of cytosolic and nuclear  $\text{Ca}^{2+}$  signals at a high resolution.

## Video Link

The video component of this article can be found at <https://www.jove.com/video/51945/>

## Introduction

The plant cell responds to the environment via signaling that coordinates cell actions. An early cell signaling event in response to environmental stimuli is a transient  $\text{Ca}^{2+}$  increase. The pattern, or signature of a transient increase in free  $\text{Ca}^{2+}$  concentration is characterized by its amplitude, frequency, and duration. Distinct spatio-temporal  $\text{Ca}^{2+}$  signatures regulate different cellular activities<sup>1</sup>. Specific stimuli, such as heat, cold, salt, drought, light, or plant hormones, may fine-tune the spatio-temporal activity of membrane-localized  $\text{Ca}^{2+}$  channels and/or transporters, resulting in specific  $\text{Ca}^{2+}$  signatures. Although  $\text{Ca}^{2+}$  transporters have been well characterized, little is known about the molecular identities and functions of  $\text{Ca}^{2+}$  channels in plants<sup>1</sup>. Genetic screens for mutants with altered  $\text{Ca}^{2+}$  response to stress stimuli may be an effective approach for identifying the components that compose  $\text{Ca}^{2+}$  signatures. Recently several Aequorin based  $\text{Ca}^{2+}$  detection systems have been developed that facilitate genetic screens for  $\text{Ca}^{2+}$  signaling components in response to pathogen attack and abiotic stress<sup>2-4</sup>.

Aequorin was first used to detect  $\text{Ca}^{2+}$  signals in plants in the early 1990s<sup>5</sup>. Since then, Aequorin has been targeted to different cellular compartments, such as the cytoplasm<sup>5</sup>, nucleus<sup>6</sup>, chloroplasts<sup>7</sup>, tonoplast<sup>8</sup>, mitochondria<sup>9</sup>, and stroma<sup>10</sup>, as well as to different cell types in the root to monitor cell specific  $\text{Ca}^{2+}$  signals<sup>11</sup>. Aequorin based  $\text{Ca}^{2+}$  measurements reveal the spatial and temporal  $\text{Ca}^{2+}$  response of a population of cells to stress stimuli. However, in most cases, the  $\text{Ca}^{2+}$  responses of single cells are unsynchronized in the responding tissue<sup>4</sup>. Therefore, Aequorin  $\text{Ca}^{2+}$  recording does not necessarily report the  $\text{Ca}^{2+}$  signal in individual cells. In recent years, genetically encoded fluorescent protein (FP)-based  $\text{Ca}^{2+}$  indicators, such as yellowameleon (YCs)<sup>12</sup> and CASEs<sup>12,13</sup> have been used to study  $\text{Ca}^{2+}$  signaling with high subcellular resolution. YCs are fluorescence resonance energy transfer (FRET)-based  $\text{Ca}^{2+}$  indicators, containing CFP and YFP variants linked by the  $\text{Ca}^{2+}$ -binding protein calmodulin and calmodulin-binding peptide M13. Calmodulin undergoes a conformational change as it binds to  $\text{Ca}^{2+}$ , thereby brings CFP and YFP closer together, resulting in increased energy transfer (enhanced FRET). The FRET level over time, calculated roughly as the ratio of YFP to CFP signal intensities, reflects intracellular  $\text{Ca}^{2+}$  dynamics. Several YC versions have been used in plants. YC3.6 was targeted to the cytosol<sup>14,15</sup>, nucleus<sup>16</sup>, mitochondria<sup>17</sup>, and plasma membrane<sup>18</sup>, and YC4.6 and D4ER were targeted to the ER<sup>15,19</sup>, and D3cpv was targeted to the peroxisomes<sup>20</sup>. Transgenic plants expressing YCs allow the live-cell imaging of  $\text{Ca}^{2+}$  dynamics within different cellular compartments of different cell types. CASEs (presumably Calcium sensor) are single circularly permuted fluorescent proteins (cpFPs) harboring a calmodulin and calmodulin-binding peptide M13. Upon binding to  $\text{Ca}^{2+}$ , CASEs undergo conformational changes, leading to an increase of fluorescence intensity. The correlation between the CASE's fluorescence response and  $\text{Ca}^{2+}$  concentration allows intracellular  $\text{Ca}^{2+}$

dynamics to be measured quantitatively. The Case12 variant has 12 fold increased fluorescence in the  $\text{Ca}^{2+}$ -saturated forms. *N. benthamiana* plants transiently expressing Case12 or Arabidopsis plants stably expressing Case 12 were used to study  $\text{Ca}^{2+}$  signaling in defense and abiotic stress<sup>4,21</sup>. Asynchronous spatial and temporal  $\text{Ca}^{2+}$  oscillations in cells responding to pathogen attack, or to dehydration stress have been revealed with Case12 based  $\text{Ca}^{2+}$  imaging.

Here, we present detailed instructions for Aequorin based luminescence imaging of tissue- and stimuli specific  $\text{Ca}^{2+}$  dynamics in Arabidopsis seedlings, and for confocal imaging of cytosolic and nuclear  $\text{Ca}^{2+}$  dynamics in Arabidopsis root cells that express Case 12. Luminescence imaging of FAS could be adapted to analyze stress-induced  $\text{Ca}^{2+}$  dynamics in intact plants or tissues not described here, or to screen mutagenized Arabidopsis plant populations for mutants with altered stress induced  $\text{Ca}^{2+}$  signals. The live cell  $\text{Ca}^{2+}$  imaging setup could be adapted to analyze  $\text{Ca}^{2+}$  dynamics within different subcellular compartments or in different cell types using other  $\text{Ca}^{2+}$  indicators.

## Protocol

### 1. Aequorin Based $\text{Ca}^{2+}$ Imaging Using the FAS System

1. Prepare seedlings for luminescence imaging. Sterilize seeds of Arabidopsis plants expressing Aequorin with 10% bleach solution containing 0.01% Triton-100. Sow the sterile seeds on a square plate (10 x 10 cm square Petri dish with grid,) containing full strength MS (Murashige and Skoog Basal Salt Mixture), 1% sucrose, and 1.2% agar. Place plates vertically in a growth chamber after stratification at 4 °C for 2 days (**Figure 1A**).
2. Transfer the seedlings onto a film. Place an adhesive film (**Figure 1B**) on the top of 7-10 day old seedlings growing on the plate. Gently push the film by hand to ensure that seedlings adhere to the film (**Figure 1C**). Peel the film gently so that the seedlings remain adhered to the film (**Figure 1D and 1E**).
3. Incubate the seedlings with cofactor. Place the adhered seedlings onto the square plate (10 x 10 cm) containing 15 ml of 2 µg/ml h-CTZ (coelenterazine) in water. Incubate the seedlings at room temperature for 4 hr to overnight (**Figure 1F**).
4. Prepare for luminescence imaging. Take the film out of the h-CTZ solution and cut it down the middle, forming two pieces. Place each piece of film with seedlings face up in two different plates. Leave the plates in the dark for 5 min.
5. Acquire luminescence images. In the dark, place the two plates next to each other on the stage of the luminescence imaging system (**Figure 1G**). Acquire images immediately upon adding 20 ml of stimuli solution to the plates simultaneously.
6. Analyze luminescence images. Choose the same display range for all luminescence images. Crop the region of interest (ROI) and generate the images as JPEG files (**Figure 2A**). Alternatively, export the images as SPE format files and import them into the ImageJ image analysis software. Set measurements for calculation of the mean gray value of ROI. Select the same size of ROI area and measure the mean gray and present data as bar graphs (**Figure 2B**).

### 2. Live Cell Confocal $\text{Ca}^{2+}$ Imaging

1. Prepare seedlings for confocal imaging. Sterilize seeds of Arabidopsis plants expressing case12 with 10% bleach solution containing 0.01% Triton. Sow the sterile seeds on a plate containing full- strength MS salts, 1% sucrose, and 1.2% agar. Place plates vertically in a growth chamber after stratification at 4 °C for 2 days.
2. Setup Imaging Chambers
  1. Assemble an imaging chamber using a slide and coverslip (Chamber A). Place a piece of water soaked cotton wool onto the middle of the slide. Then transfer one or two 5 day old seedlings from the plate onto the top of water soaked cotton wool. Stick small pieces of clay to each corner of a coverslip, and place coverslip on the top of seedlings to create a gap (chamber) between the coverslip and slide (**Figure 3A**, left panel). Connect one end of polyethylene tube (0.58 mm diameter) to a 1 ml syringe and place the other end of tube immediately adjacent to the chamber. Hold the tubing in place with tape (**Figure 3A**, right panel).
  2. Assemble an imaging chamber using a plexiglass chamber (Chamber B).
    1. Spread a thin layer of silicone grease around two polyethylene tubes (0.58 mm diameter) and press the coated tubes into the channels on each side of the plexiglass chamber (**Figure 3B** left panel). Spread a thin layer of silicone grease onto the surface of the chamber that contains the tube grooves and press a coverslip into the grease to seal one side of the chamber opening (cut out).
    2. Transfer a five day old seedling from the plate into the chamber and place a piece of cotton wool soaked with water on the top of the seedling. Spread silicone grease onto the other surface of the chamber, and press another coverslip on top to seal. Connect the end of one of the tubes to a 1 ml syringe. Leave the end of the other tube open (**Figure 3B** right panel).
  3. Prepare an imaging chamber using a chambered cover glass (Chamber C). Sterilize the chambered cover glass with 70% ethanol and leave it on the hood until dry. Add 0.6 ml or 0.2 ml of full strength MS medium containing 1% sucrose and 0.5% phytigel into each well of an 2-well chamber or an 8-well chamber (**Figure 3C** right panel), respectively. Sterilize seeds and sow the seeds directly in a chambered cover glass containing a thin layer of clear gel and let them grow vertically for 5 days (**Figure 3C**).
3. Acquire images using a confocal microscope. To apply stress stimuli, such as salt, cold or peroxide, slowly inject about 200 µl of 150 mM NaCl, ice-cold water or 1 mM  $\text{H}_2\text{O}_2$  solution into the chamber (Chamber A or B) just prior to acquiring images, or gently add 500 µl or 100 µl of stimulus solution to the well of a 2- or 8-well chamber, respectively. Capture images immediately after applying the stimulus solution using an inverted Nikon A1R confocal laser-scanning microscope with a 20X water immersion lens (numerical aperture 0.75). Collect a time series of images at 4 sec intervals with excitation and emission wavelengths of 488 nm and 500 - 550 nm, respectively, and at a pixel resolution of 512 x 512.
4. Image Analysis
 

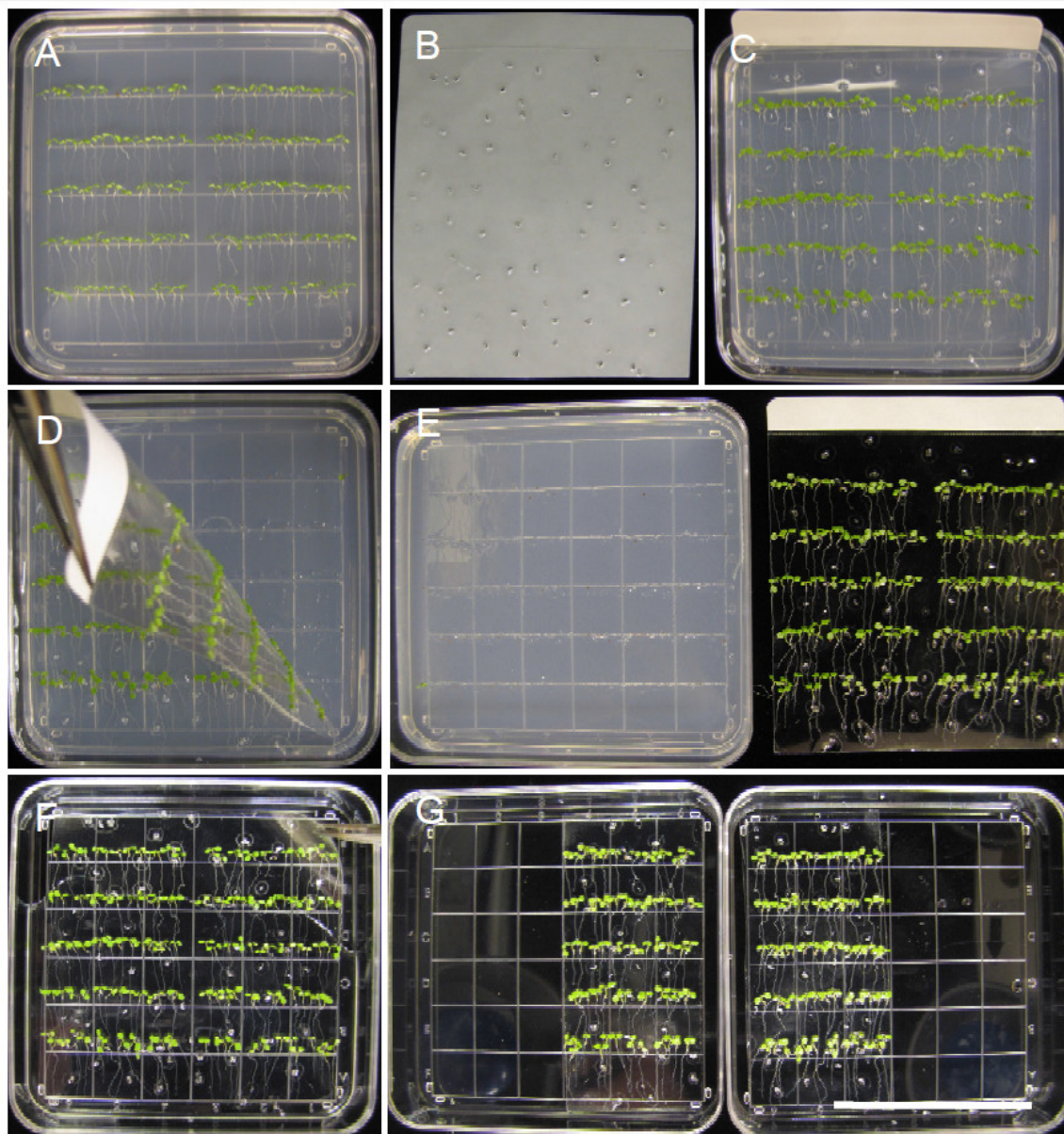
Using Nikon Elements, ROIs were drawn around each cell (or area) of interest. Total intensity within each ROI was measured over time using the Time Measurement dialog box (ImageJ could be used instead). Total intensity measurements were exported and processed in

DataGraph.  $\text{Ca}^{2+}$  spike amplitude was defined as peak intensity minus resting intensity, duration as the time between initiation and completion of a spike, and period as the time interval between adjacent spike peaks of two spikes. *t*-test was used to compare the means.

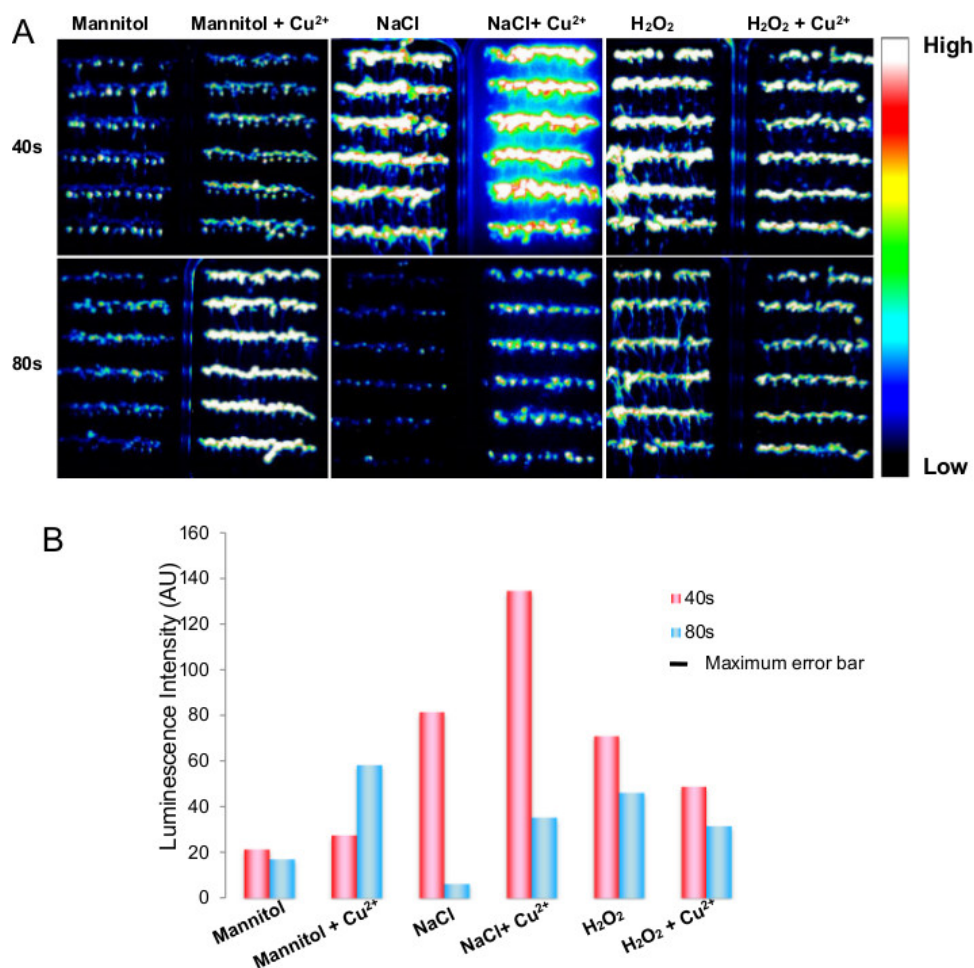
## Representative Results

Mannitol, NaCl and  $\text{H}_2\text{O}_2$  were used as proxies for dehydration, salt and oxidative stress stimuli, respectively. To check if the heavy metal ion  $\text{Cu}^{2+}$  synergizes with any of these three stress stimuli, we compared the  $\text{Ca}^{2+}$  response to each stimuli in the presence or absence of  $\text{Cu}^{2+}$ . As shown in **Figure 2**, FAS luminescence imaging revealed that Arabidopsis seedlings responded differently to dehydration, salt and oxidative stress. For the concentration of stimuli we examined in this study, 75 mM NaCl induced the strongest  $\text{Ca}^{2+}$  response in leaves and roots during the first 40 sec of exposure (**Figure 2A** middle panel and **Figure 2B**), while 1 mM  $\text{H}_2\text{O}_2$  induced a strong  $\text{Ca}^{2+}$  response both in leaves and roots during the first and second 40 sec (**Figure 2A** right panel). The solution of 400 mM mannitol only induced  $\text{Ca}^{2+}$  response in roots during the first 40s and very weak signals in leaves during the second 40 sec.  $\text{Cu}^{2+}$  strongly enhanced the amplitudes and duration of  $\text{Ca}^{2+}$  signals triggered by NaCl and mannitol (**Figure 2A** left panel). It appears that  $\text{Cu}^{2+}$  had inhibitory effects on  $\text{H}_2\text{O}_2$  induced  $\text{Ca}^{2+}$  signals, reducing both the amplitude and duration (**Figure 2A** right panel and **2B**).

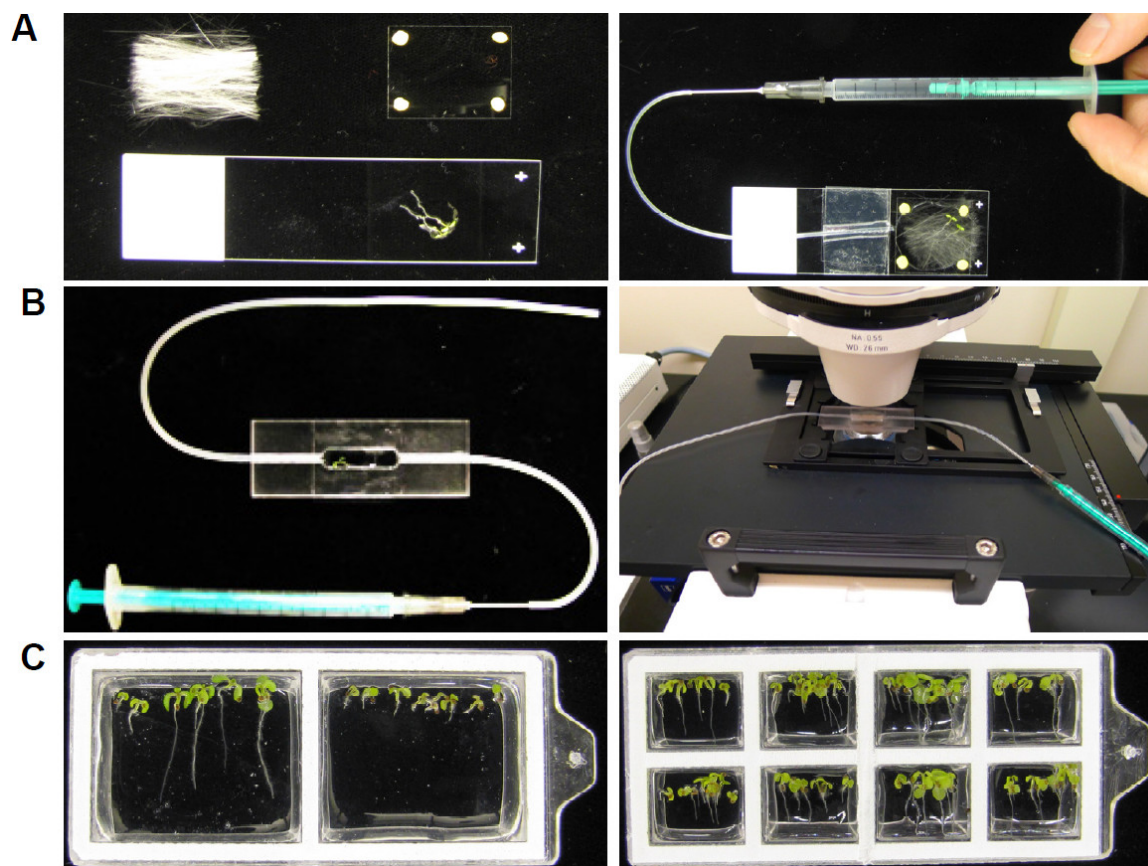
Using Case12 expressing Arabidopsis plants, we measured  $\text{Ca}^{2+}$  dynamics in the cytosol and nuclei simultaneously. Molecular weight of Case12 is 46 kDa, therefore transgenic plants expressing Case12 have GFP signals in both the cytosol and nuclei of leaf and root cells (**Figure 4A**). Customized (**Figure 3A** and **3B**) or commercial chambers (**Figure 3C**) were used for live cell imaging with a confocal microscope. Applying 150 mM NaCl to the seedling in the chamber, we observed changes of fluorescence intensity in both the cytosol and nucleus of the individual cells (**Figure 4B** and **C**; Supplementary Movie 1). Using NIS-Elements (Imaging software, Nikon Instruments Inc.), we measured the fluorescence intensity in regions of interest (ROI) over time. The amplitude, duration and frequency of cytosolic and nuclear  $\text{Ca}^{2+}$  oscillation are presented graphically in **Figure 4B**. The amplitude and period of  $\text{Ca}^{2+}$  spikes in the cytoplasm and the nuclei vary greatly between cells of the same root, as well as across cells in different roots. The amplitude and period of cytosolic  $\text{Ca}^{2+}$  spikes were  $60.36 \pm 45.22$  AU (arbitrary unit) and  $58.24 \pm 15.70$  sec, respectively. The amplitude and period of nuclear  $\text{Ca}^{2+}$  spikes were  $316.26 \pm 75.24$  (AU) and  $61.71 \pm 16.31$  sec, respectively. In contrast, the duration of cytosolic and nuclear  $\text{Ca}^{2+}$  spikes were similar between cells, which were  $17.43 \pm 3.67$  sec and  $17.33 \pm 2$  sec, respectively. The amplitude of  $\text{Ca}^{2+}$  spikes decreased over time, which may be due to the change of the focal plane and/ or a loss of response to stress stimuli because of an unfavorable *in vitro* condition. These results show that salt stress triggers  $\text{Ca}^{2+}$  oscillation in both the cytoplasm and nucleus.



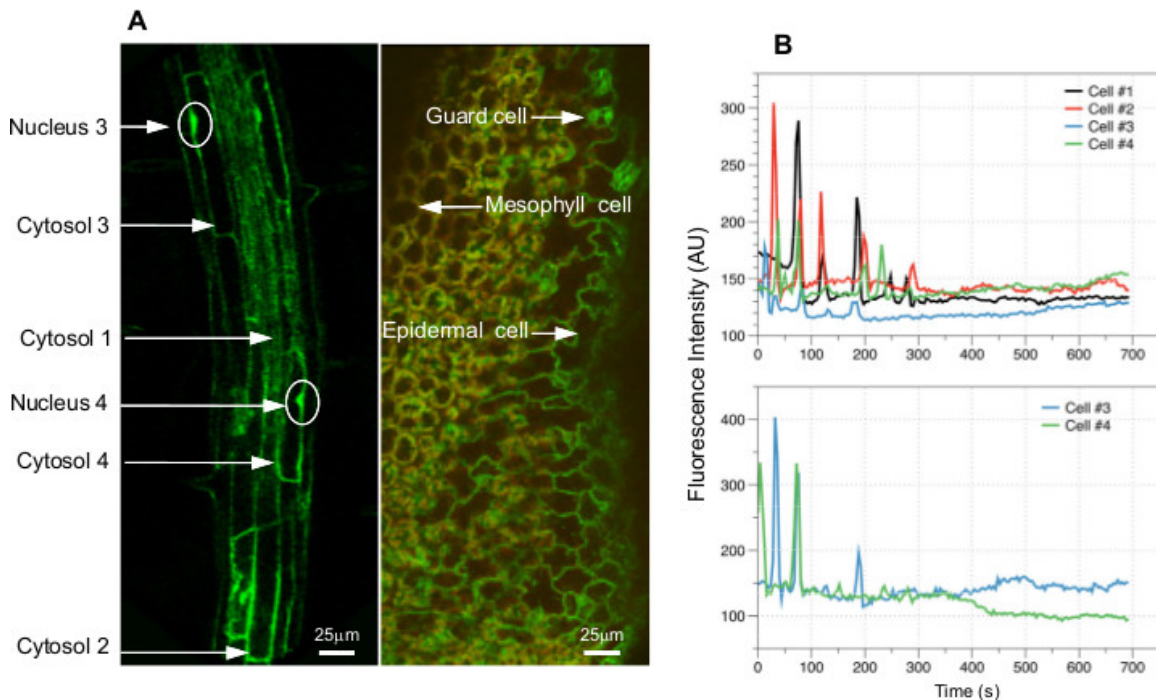
**Figure 1. Aequorin based FAS system for measuring spatial-temporal  $\text{Ca}^{2+}$  dynamics in response to stress stimuli.** **A)** Grow Arabidopsis seedlings vertically on a plate containing MS media. **B, C)** Place an adhesive film (B) on the top of seedlings (C). **D, E)** Transfer 10 day old seedlings onto the adhesive film. **F)** Incubate seedlings adhered to the film with 2  $\mu\text{g}/\text{ml}$  of h-CTZ in water for 4 hr. **G)** Cut the film down the middle and place each piece into a different empty plate. Leave the plates in the dark for 5 min. Apply simultaneously control and stimuli solutions (be sure the seedlings are entirely covered), and acquire images immediately. The scale bar is 5 cm.



**Figure 2. Comparison of spatial-temporal Ca<sup>2+</sup> response of 10 day seedlings to different stress stimuli. A)** A time series of luminescence images of seedlings subjected to 400 mM mannitol and 400 mM mannitol plus 1 mM CuCl<sub>2</sub> (A, left panel), 75 mM NaCl and 75 mM NaCl plus 1 mM CuCl<sub>2</sub> (A, middle panel), and 1 mM H<sub>2</sub>O<sub>2</sub> or 1 mM H<sub>2</sub>O<sub>2</sub> plus 1 mM CuCl<sub>2</sub> (A, right panel). Upper and lower panels of A are luminescence images acquired during first 40 sec and second 40 sec, respectively. An intensity scale bar indicates an increase of luminescence signals from low (black) to high (white). **B)** Bar chart of average luminescence intensity of seedlings in response to the indicated stress stimuli.



**Figure 3. Setup of a live cell confocal imaging experiment.** **A)** A customized chamber is built with a slide, coverslip with four pieces of clay and cotton wool. Two 5 day old seedlings are placed on the slide. One end of a polyethylene tube is placed next to the assembled chamber and fixed with a pieces of tape while the other end is connected to a 1 ml syringe. **B)** A customized slide made with plexiglass has a open chamber in the middle and two channels that are connected to the two sides of channel. One coverslip is placed on the top of the slide and two polyethylene tubes are placed in the channel. Coverslip and tubes are fixed in the position with silicone grease. The end of one of the tubes is connected to a 1 ml syringe and the end of another tube remains open. A 5 day old seedling is placed in the chamber and a coveslip is put on the top of the seedling and sealed with silicone grease. The plexigalss slide with the assembled chamber is placed on the stage of the microscope. **C)** Arabidopsis seedlings are grown in two wells (left) or 8 wells (right) of the chambered cover glass containing a thin layer of 0.6% of phytigel in MS media.



**Figure 4. Cytosolic and nuclear  $\text{Ca}^{2+}$  oscillation in response to salt stress.** **A)** Confocal images show that Case12 is expressed in the cytoplasm and nuclei of root cells (left panel) and leaf cells (right panel) of transgenic plants expressing Case12. The image on the right panel is a merged image of red autofluorescence of the chloroplasts and green fluorescence of Case12. **B)** Salt stress (150 mM NaCl) induced  $\text{Ca}^{2+}$  oscillation in the cytosol (upper panel) and nuclei (lower panel) of root cells. Locations of the measured cells are indicated by arrows with numbers on panel.

## Discussion

We have demonstrated a FAS system for recording the spatial-temporal  $\text{Ca}^{2+}$  response of Arabidopsis seedlings. This FAS  $\text{Ca}^{2+}$  recording system provides a simple, sensitive and robust approach that could be adapted for measuring  $\text{Ca}^{2+}$  dynamics triggered by various stimuli in addition to the abiotic stress stimuli that are presented here. Using this system, we can easily compare tissue- or stimuli-specific spatial-temporal  $\text{Ca}^{2+}$  dynamics at the whole plant level. The high sensitivity of FAS allows the use of low intensity stimuli for examining  $\text{Ca}^{2+}$  responses, which is important for avoiding artifacts caused by physical damages to cells when high intensity stress is used. Seven to 10 day old Arabidopsis seedlings showed tissue or stimuli-specific  $\text{Ca}^{2+}$  response, though seedlings at different physiological stages may have different sensitivity and specificity in response to a given stimulus. Therefore it is preferable to use 7-10 day old seedlings to increase the throughput of FAS measurements, in which around 100 seedlings could be adhered onto one piece of film. Together with easy and simple performance, high sensitivity and reproducibility, the FAS system could also be adapted for screening EMS mutagenized mutants for altered  $\text{Ca}^{2+}$  responses. For screening purposes, it is crucial to have uniform seedlings that are well grown on the plate and adhered onto the film. Four to five hours incubation of film adhered seedlings with h-CTZ is usually sufficient for reconstitution of aequorin. Using the adhesive film with holes is preferable because reaction of apoaequorin and cofactor requires oxygen. For the comparison of tissue or stimuli-specific  $\text{Ca}^{2+}$  responses, using groups of seedlings originally from the same film is critical because the state of seedlings and/or the reconstitution condition may affect the total amount of functional aequorin. When these requirements are met, the difference of luminescence response is proportional to the tissue- or stimulus-specific  $\text{Ca}^{2+}$  response.

Aequorin based measurement of  $\text{Ca}^{2+}$  signals reflect the response of a population of the cells. However, considering the heterogeneity of cell types and/or the different accessibility of cells in the tissues to the stimuli, the results of Aequorin based  $\text{Ca}^{2+}$  measurements do not equate to the behavior of any one cell. Understanding  $\text{Ca}^{2+}$  dynamics at the cellular level requires a  $\text{Ca}^{2+}$  indicator that can be detected with subcellular resolution. Live cell confocal imaging of fluorescence protein (FP)-based  $\text{Ca}^{2+}$  indicators provides an advanced  $\text{Ca}^{2+}$  detection system for recording cellular  $\text{Ca}^{2+}$  dynamics with cellular resolution in a desired tissue. Here, using live cell confocal imaging and FP-based  $\text{Ca}^{2+}$  indicators, we recorded subcellular  $\text{Ca}^{2+}$  transients in both the cytoplasm and nuclei simultaneously. Two types of FP-based  $\text{Ca}^{2+}$  indicators have been implemented in plants with different characteristics. Compared to YCs, single GFP-based  $\text{Ca}^{2+}$  indicator has some advantages: 1. Case12 does not require YFP and CFP filter sets or the many assumptions and complex calculations needed to measure FRET<sup>22</sup>. Case12 has single excitation/emission maxima and can be easily detected using standard GFP filter set. 2. Case12 is stable under physiological pH, and is a relatively a small protein (46 kDa). When expressed in plants, non-targeted Case12 appears in the cytoplasm as well as the nuclei, so Case12 transgenic plants can be used to track cytosolic and nuclear  $\text{Ca}^{2+}$  dynamics simultaneously. A drawback of this reporter is that it is a non-ratiometric indicator, so fluorescence levels are also affected by factors that are unrelated to  $\text{Ca}^{2+}$  concentration, such as the expression level of Case12 and local pH. Nevertheless, Case12 is adequate to determine cellular  $\text{Ca}^{2+}$  concentration for the comparison of spatial-temporal  $\text{Ca}^{2+}$  dynamics in the cytoplasm and organelles between materials with different genetic backgrounds under the same experimental conditions. In the future, new GFP based  $\text{Ca}^{2+}$  indicators with higher signal-to-noise ratio, fast kinetics and greater response, like GCaMP3<sup>24</sup> and GCaMP5<sup>25</sup> demonstrated in animals, could be better indicators for recording cellular or subcellular  $\text{Ca}^{2+}$  dynamics in plants.

The Aequorin and Case12 detection systems described here provide nondestructive *in vivo* approaches for the measurement of spatial-temporal  $\text{Ca}^{2+}$  dynamics at the whole seedling and subcellular levels, respectively. In both systems, the physiological state or growth conditions could affect the  $\text{Ca}^{2+}$  responses of the seedlings or cells. If physically damaged or not grown in a good condition, cells could have weak or even no  $\text{Ca}^{2+}$  responses. In addition, the duration of spatial and temporal  $\text{Ca}^{2+}$  signals are also affected by the conditions of the seedling during the measurements. The luminescence or fluorescence signals may decrease over time due to stimuli-induced damage, and/or the laser-induced photo-damage in the case of confocal imaging. Although stimulus-induced damage cannot be avoided, phototoxicity can be reduced by limiting the intensity and/or frequency of laser exposure. In addition, Aequorin based  $\text{Ca}^{2+}$  recording system requires a reconstitution step. Factors that interrupt the diffusion of CTZ to the cell and/or reaction of apoaequorin with CTZ would affect luminescence signals that are related directly to  $\text{Ca}^{2+}$  concentration. Therefore the concentration of CTZ, as well as the duration and conditions of CTZ incubation need to be optimized for best sensitivity and reproducibility. Genetically encoded  $\text{Ca}^{2+}$  indicators may inaccurately report cellular  $\text{Ca}^{2+}$  concentrations for other reasons, including unintended targeting to specific subcellular compartments, temperature sensitivity, or interference of the reporter with the cellular  $\text{Ca}^{2+}$  signaling machinery<sup>23</sup>. We found that Aequorin or Case12 expressing plants have no obvious morphological or conditional phenotypes such as altered stress tolerance. Therefore it is unlikely that these genetically encoded  $\text{Ca}^{2+}$  indicators have major interferences with  $\text{Ca}^{2+}$  signaling in plants.

In summary, we present here detailed instructions for the use of two  $\text{Ca}^{2+}$  detection systems for measuring spatial and temporal  $\text{Ca}^{2+}$  signals at both the whole plant seedling and subcellular levels. These two systems can be used to determine tissue (or cell type) or stimuli-specific  $\text{Ca}^{2+}$  dynamic patterns with high sensitivity and reproducibility. Therefore they are powerful tools for elucidating the gene networks that underlie  $\text{Ca}^{2+}$  signaling in response to various environmental cues.

## Disclosures

The authors have nothing to disclose.

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