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TITLE:

Forward Genetic Screen Using Transgenic Calcium Reporter Aequorin to Identify Novel Targets in Calcium Signaling

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SUMMARY:

A forward genetic screen based on Ca^{2+} elevation as a read-out leads to identification of genetic components involved in calcium dependent signaling pathways in plants.

ABSTRACT:

Forward genetic screens have been important tools in the unbiased identification of genetic components involved in several biological pathways. The basis of the screen is to generate a mutant population that can be screened with a phenotype of interest. EMS (ethyl methane sulfonate) is a commonly used alkylating agent for inducing random mutation in a classical forward genetic screen to identify multiple genes involved in any given process. Cytosolic calcium (Ca^{2+}) elevation is a key early signaling pathway that is activated upon stress perception. However the identity of receptors, channels, pumps and transporters of Ca^{2+} is still elusive in many study systems. Aequorin is a cellular calcium reporter protein isolated from *Aequorea victoria* and stably expressed in Arabidopsis. Exploiting this, we designed a forward genetic screen in which we EMS-mutagenized the aequorin transgenic. The seeds from the mutant plants were collected (M_1) and screening for the phenotype of interest was carried out in the segregating (M_2) population. Using a 96-well high-throughput Ca^{2+} measurement protocol, several novel mutants can be identified that have a varying calcium response and are measured in real time. The mutants with the phenotype of interest are rescued and propagated till a homozygous mutant plant population is obtained. This protocol provides a method for forward genetic screens in Ca^{2+} reporter background and identify novel Ca^{2+} regulated targets.

INTRODUCTION:

A change in cytosolic calcium (Ca^{2+}) concentration upon perception of biotic or abiotic stimulus is

a well-studied early signaling event that activates many signaling pathways[1,2,3,4]. A cell in its basal resting state maintains a lower Ca^{2+} concentration in the cytosol and sequesters excess Ca^{2+} in various intracellular organelles and extracellular apoplast leading to a steep Ca^{2+} gradient[5,6]. Upon signal perception, Ca^{2+} levels rise in the cytosol due to an influx of Ca^{2+} from extracellular and/or intracellular sources and generate a stimuli specific calcium signature[7,8,9]. Ca^{2+} elevations in the cytosol are activated by many stimuli, but specificity is maintained by distinct stores releasing Ca^{2+}, a unique Ca^{2+} signature and appropriate sensor proteins[10,11].

The use of alkylating agent, ethyl-methane sulfonate (EMS) for mutagenesis is a powerful tool in classical forward genetic screens to identify multiple independent genes involved in a process. EMS is a chemical mutagen predominantly inducing C to T and G to A transitions randomly throughout the genome and produces a 1 bp change in every 125 kb of the genome. EMS mutagenesis will induce ≈1000 single base pair changes, either insertion/deletions (InDel) or single nucleotide polymorphism (SNP) per genome[12]. EMS-induced mutations are multiple point mutations with a mutation frequency ranging from 1/300 to 1/30000 per locus. This reduces the number of M_1 plants needed to find a mutation in a given gene. A M_1 seed population range of 2000-3000 is typically used to obtain mutations of interest in *Arabidopsis thaliana*[13,14].

Aequorin transgenics are *Arabidopsis* Columbia-0 (Col-0) ecotype plants expressing p35S-apoaequorin (pMAQ2) in the cytosol[15]. Aequorin is a Ca^{2+} binding protein composed of apoprotein and a prosthetic group consisting of luciferin molecule, coelenterazine. The binding of Ca^{2+} to aequorin, which has three Ca^{2+} binding EF-hands sites, results in coelenterazine being oxidized and cyclized to give the dioxetanone intermediate, followed by a conformational change of the protein accompanied by the release of carbon dioxide and singlet-excited coelenteramide[16]. The coelenteramide so produced emits a blue light (λ_{max}, 470 nm) that can be detected by the luminometer[17]. The extremely fast Ca^{2+} elevations can thus be measured in real time, and exploited for rapid forward genetic screens. This protocol aims to use the specificity of calcium response to identify novel key players that are involved in the Ca^{2+} signature. To achieve this task, we use EMS mutagenesis in transgenic aequorin and identify the SNPs associated with altered Ca^{2+} signaling. The protocol identifies mutants that show no or reduced Ca^{2+} elevations upon stimuli addition. These mutants can then be mapped to identify the genes responsible for the Ca^{2+} response. The method is applicable to any kind of liquid stimuli in plants that results in a Ca^{2+} elevation. Since Ca^{2+} elevation is one of the first responses in the plant defense signaling pathway, the identification of upstream response components can provide candidates for genetic engineering to develop resilient plants.

PROTOCOL:

1. EMS mutagenesis and single pedigree-based seed collection (1-3 months)

1.1. Weigh 150 mg of seeds (~7500) of aequorin for EMS mutagenesis (M_0 seeds). Weigh another 150 mg of seeds to be used as a control.

1.2. Transfer the seeds to a 50 mL tube and add 25 mL of 0.2% EMS (v/v) (CAUTION) (for treatment) or 25 mL of autoclaved water (for control).

NOTE: Ethyl-methane sulfonate is a chemical agent for mutagenizing plant material.

1.3. Seal the tube with parafilm and wrap it in aluminum foil. Rotate the tube end-over-end for 18 h at room temperature.

1.4. Allow the seeds to settle. Remove the EMS solution carefully and discard in a waste container containing 1 M NaOH in equal volumes (NaOH helps to neutralize/inactivate EMS making it safe to discard). Discard the used plasticware in a 1 M NaOH solution.

1.4.1. After 24 h, dispose the discards according to hazardous material laboratory practices.

1.5. Wash the mutagenized seeds thoroughly with 40 mL of autoclaved water at least 8 times. Discard EMS containing water in a NaOH waste container as mentioned above.

1.6. For the final wash, add 40 mL of 100 mM sodium thiosulfate and rinse 3 times to remove traces of EMS.

1.7. Soak the seeds in 40 mL of autoclaved water for ~1 h to diffuse EMS out of seeds and then place them on filter paper until completely dry.

1.8. Transfer the seeds to a microcentrifuge tube and stratify the seeds at 4 °C in 40 mL of autoclaved water for 2-4 days. This helps in breaking seed dormancy and ensures homogenous growth.

1.9. Transfer both the mutagenized seeds (M_0) and the control seeds on to soil (soil composition: agropet: soilrite, 1:1) and transfer them to growth rooms with a 16 h light/8 h dark photoperiod, a light intensity of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and ~70% relative humidity.

1.10. To determine if mutagenesis was successful, look for reduced germination speed and seedling growth, and chlorophyll sectoring[18] (**Figure 1A**). Compare the mutagenized plants to the control plants to identify these physiological and developmental differences.

NOTE: Different methods can be used for harvesting seeds from M_1 plants. In this protocol, we have used the single pedigree-based seed collection method. Each M_1 plant is given a unique number, starting from A1 to A3500.

1.11. Maintain individually numbered plants as discrete plant lines (**Figure 1B**).

1.12. Upon maturation, harvest seeds from these individual mutant plants and store as individual M_1 lines (**Figure 2**). From the single pedigree-based seed collection, we obtained around 5000 M_1 lines out of which 3500 M_1 lines were screened.

2. High-throughput screening to select mutants (8 months)

2.1. Identify novel mutants based on the Ca^{2+} response to a selected stimulus. Here, we used H_2O_2 as an example.

2.2. For identifying mutants, screen the M_2 generation. Since recessive mutants segregate at 1/8 frequency in M_2 generation upon EMS mutagenesis[14], screening of 8-12 M_2-segregating plants covers one M_1 line and identifies a homozygous recessive mutant (using 12 seedlings increases the probability of finding a mutant). From each independent M_1 line, test 12 M_2 seedlings for Ca^{2+} response to H_2O_2 (12 M_2 seedlings per M_1 line).

2.3. Use a high throughput seed sterilization and hydroponic plant growth protocol[19]. Place nearly 12-15 M_2 seeds per M_1 line in individual wells of a 24-well tissue culture plate and sterilize using chlorine gas (40 mL of 12% sodium hypochlorite and hydrochloric acid, 3:1, v/v) in a desiccator for 4 h in a fume hood. After the procedure, open the desiccator and leave overnight for chlorine gas to evaporate.

2.4. After sterilization, bring plates outside and add liquid 1/2 MS media (half-strength MS without agar) to individual wells. Seal the plates with parafilm and stratify the seeds for 2-4 days at 4 °C and then move seeds to a growth chamber with 10 h light/ 14 h dark photoperiod at 20-22 °C with 70% relative humidity.

2.5. Once the seedlings are 8-12 days old, place 12 M_2 seedling from each line individually in a 96-well luminometer plate. For measuring Ca^{2+} response to H_2O_2, use a luminometer plate reader.

2.6. After seedling transfer, add 150 µL of 5-10 µM coelentrazine solution (diluted in autoclaved water from a 5 mM stock in methanol) (CAUTION) in individual wells in a dark/low-light area and store in dark at 21 °C for 8 h.

NOTE: Coelentrazine is a prosthetic group that binds to apo-aequorin and reconstitutes it to functional aequorin. Coelentrazine is light sensitive and is hence stored in dark colored bottles, protected from light.

2.7. The next day, perform mutant screen using 10 mM H_2O_2 as a stimulus and measure the subsequent Ca^{2+} response.

2.8. For simultaneous measurement of 24 wells, create an automated kinetic program that measures the background for 1 min, followed by stimuli addition (40 µL) and measurement for 10 min, followed by total aequorin discharge (2 M $CaCl_2$ in 150 µL 20% ethanol) for 1-2 min.

NOTE: An end discharge for the total aequorin is needed to quantify the measured Ca^{2+} and as additional control for functional aequorin. The end discharge is a short run of 1-2 min and does not cause significant plant death. Alternatively, if the plants die after discharge step, then re-screen the specific M_1 line and rescue without discharge. Such mutants can be confirmed in M_3 and M_4 generations using the discharge step.

2.9. Use a 24-well format scanning method that measures each row in 7 s interval with 300 ms integration time per well per measurement point. Use a wild-type seedling as control in each row for comparison and evaluation of the mutant.

NOTE: A single 96 well plate containing M_2 seedlings will cover 8 individual M_1 line (8 M_1*12= 96 M_2) and can be screened in 2.5 h and each day 32 M_1 lines would be screened, 640 M_1 plants per month. The whole screening procedure after the plants are ready would take around 8 months.

2.10. Identify mutants based on loss of or reduced Ca^{2+} response with H_2O_2. Rescue the selected mutants through an antibiotic-based washing process. Wash the seedling with 25 mg/L cefotaxime solution twice and then transfer to rescue medium that contains 25 mg/L cefotaxime in 1/2 MS agar.

2.11. Grow the plates at a 10 h light/ 14 h dark photoperiod to obtain a mutant plant. The cefotaxime wash helps to remove any harmful micro-organisms on the seedling. Since the seedlings after reconstitution are kept un-sealed, minimize contamination when transferring back to sterile condition.

2.12. Transfer the mutant plant to soil for obtaining the homozygous M_3 population.

3. Data analysis and mutant identification (1-3 months)

NOTE: The readings provided from the above method are in relative light units (RLU).

3.1. For calculating $[Ca^{2+}]_{cyt}$ concentration, use the following concentration equation[20].

$pCa = 0.332588(-\log (L/L_{max})) + 5.5593$

Luminescence counts (L) and total remaining counts (L_{max}).

3.2. Convert the obtained pCa values to $[Ca^{2+}]_{cyt}$ values in µM by multiplying by 10^6. Then graphically plot against an aequorin (transgenic Col-0 aequorin) control for identifying putative H_2O_2 mutants to Ca^{2+} response.

3.3. Rescue seedlings showing a loss of or reduced response to H_2O_2 application rescued.

3.4. Upon maturation, harvest seeds from the rescued mutant and re-screen for response to H_2O_2 in M_3 seedlings. If all 12 M_3 seedlings show a Ca^{2+} response similar to the mother plant, consider the population to be a homozygous M_3 mutant population. If all 12 seedlings do not show

such a response, then take them to M_4 generation and re-screen to obtain homozygous population.

3.5. Once a homozygous plant line has been obtained, identify the gene leading to altered phenotype using next generation sequencing.

REPRESENTATIVE RESULTS:

The EMS population was screened for H_2O_2 induced Ca^{2+} elevation. As discussed earlier, 12 individual M_2 seedlings were screened from each M_1 line. In **Figure 3**, one such M_1 line is plotted with each panel showing 12 individual M_2 seedlings. A wild-type aequorin is used as control for comparing and evaluating the mutant response. A recessive mutant segregates in the ratio of 1:7 (mutant: non mutant). When screening 12 individual seedlings per M_1 line, we can identify 1 or 2 mutants per line. We have identified 2 putative mutants from 12 M_2 seedlings (**Figure 3**). These mutants are further taken to M_3 and M_4 and a homozygous population is generated. The homozygous mutant is further mapped to identify the causal gene.

FIGURE AND TABLE LEGENDS:

Figure 1: EMS mutagenized Arabidopsis. (**A**) To determine a successful EMS mutagenesis, we looked for chlorophyll sectoring in the mutagenized plant population (indicated by arrow). Statistically, 0.1 to 1% of M_1 plants must show chlorotic sectors. (**B**) Individual potting of M_1 plants was done to perform a single pedigree-based seed collection.

Figure 2: **A schematic representation of the forward genetic screen methodology.** 7500 transgenic Arabidopsis plants expressing cytosolic Aequorin (Aeq) in Col-0 are mutagenized with ethyl methanesulphonate (EMS) in the M_0 generation. Around 5000 seedlings from M_1 generation are propagated individually by single pedigree method and propagated to M_2 generation. 12 plants from each M_2 line are screened for the phenotype of interest (around 3000-3500 M_1 lines). Mutant for the phenotype of interest is rescued and propagated to the M_3 generation and screened for homozygosity.

Figure 3: Identification of mutants with altered response to H_2O_2 treatment. A representative figure to depict mutant selection from a single segregating M_1 line is shown. The panels show the screening result (12 segregating M_2 seedlings per individual M_1 line) upon stimulation with 10 mM H_2O_2. WT (Aequorin) control (green line), average of all 12 M_2 seedlings is the mean response (red line) and A1-X (black line) are individual seedlings numbered from 1-12. For each graph, the y-axis is the $[Ca^{2+}]_{cyt}$ (µM) and the x-axis is time (min). $[Ca^{2+}]_{cyt}$ levels were calculated from relative light units (RLUs).

DISCUSSION:

EMS mutagenesis is a powerful tool to generate mutations in population. The classical forward genetic screens using EMS has been an effective tool to identify novel genes for two major reasons: firstly, they do not require any prior assumptions on gene identity and secondly, they do not introduce any bias. There are several methods to generate a screening populations like

EMS, T-DNA insertions, radiations etc. Out of all the methods, EMS-based mutagenesis has few advantages over the other methods. First, it is easier to generate a mutant population by exposure to EMS as described in the current protocol[21,22]. Second, a sufficiently large number of mutant seed population can be generated, which can be used for multiple screens for one or more stimuli. Third, a weak allele of an essential gene generated due to a missense mutation can be identified. Fourth, an array of gene function effects can be identified using the EMS screen including complete loss-of-function, partial function loss, altered function and a constitutive gene function. It can help in identification of double mutants that is not feasible by other mutagenesis methods[23,24,25.] The single pedigree based M_1 seed collection used in this protocol is also advantageous as it allows one to go back to the mother population to identify the same mutant again, if the progeny is lost in the subsequent future generations. It offers the possibility for recovering mutations that are infertile when homozygous and can be recovered via the heterozygous siblings of the mutant plants. Secondly, this strategy guarantees the independence of all mutants isolated when compared to bulking of seeds in M_1 generation. It ensures that mutations isolated from the M_2 collection are different alleles at the same locus rather than the same mutational event[26].

The genes identified through EMS mutagenesis screens are dependent on the phenotype used for screening the population. The faster the phenotype of interest can be screened, the easier is to identify novel pathways. Ca^{2+} is a ubiquitous secondary messenger that is among the first signaling cascades to be activated. It acts as a mediator for plant response against a wide array of biotic and abiotic stimuli. Additionally, the calcium reporter aequorin can be localized to various sub-cellular compartments and organelles[27,28,29]. This opens avenues for identifying roles of protein localized in these compartments in calcium response dynamics[30,31,32].

Forward genetic screens based on EMS-mutagenesis in aequorin and using Ca^{2+} as readouts have remained contemporary since their discovery. The advantages of this method have outweighed the pitfalls. However, few limitations of the technique still need to be carefully evaluated. The screen is labor and time-intensive and requires identification of mutant plants from a vast mutagenized population. Hence, detailed planning based on resource availability, work personnel requirement and space constrains must be done before embarking on the experimental plan. The second major challenge with the aequorin-based Ca^{2+} screens is possibility of false positives without a discharge step. Hence a very short discharge step is included in the protocol. Random mutations can also lead to generation of sterile plants that cannot rescued due to multiple mutations. Thirdly, Ca^{2+} signature is highly tissue specific and consistency in screening must be ensured[4].

Not many forward genetic screens have identified receptors, channels, pumps and transporters of Ca^{2+} as use of Ca^{2+} as a screening phenotype in forward genetics was rare. Stimuli (e.g., H_2O_2) induced Ca^{2+} elevation is used as marker for a forward genetic screen in our methodology, to identify new genetic components involved in the process. A similar strategy using EMS mutagenized aequorin population has led to the discovery of many receptors like DORN1 which is eATP receptor[33], calcium channel OSCA[34], LORE receptor involved in lipopolysaccharide sensing[35] and the ribonuclease PARN1[36]. A recent study published by Wu et al. has used a very

similar methodology of screening EMS-mutagenized aequorin plants upon H_2O_2 elicitation to identify the novel hydrogen peroxide sensor HPCA1[37]. Hence the protocol using EMS mutagenesis in Ca^{2+} reporter background is a promising method for novel gene discovery involved in stimuli sensing.

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DISCLOSURES:

None of the authors have any conflicts of interest to declare.

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Figure 1

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Figure 2

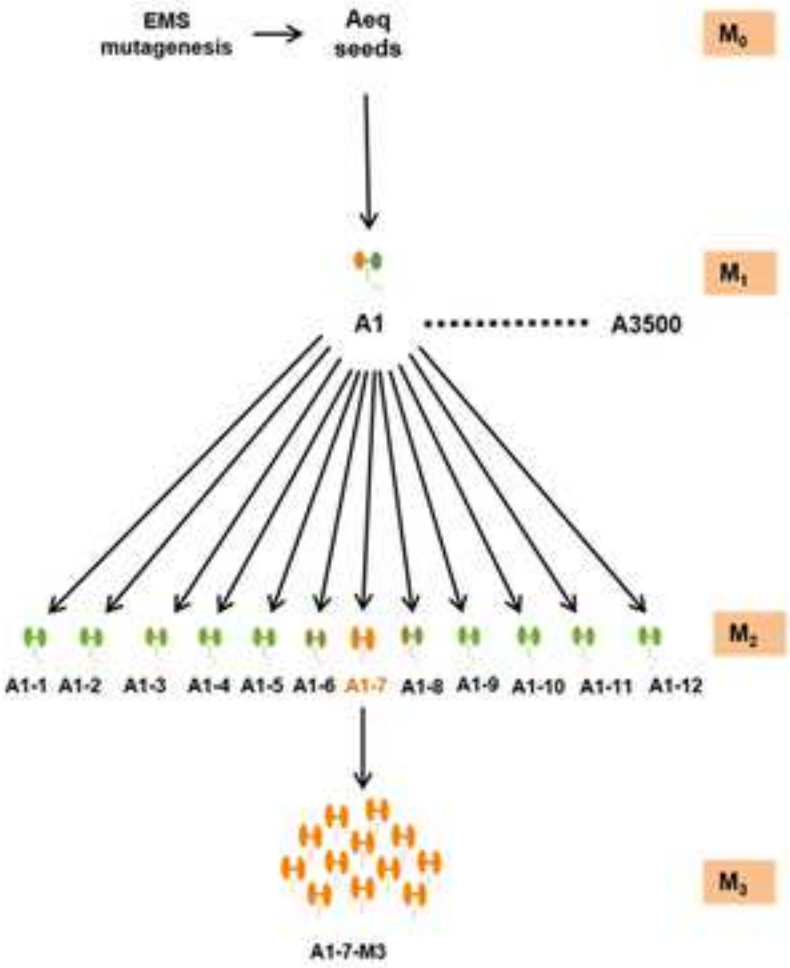

Figure 3

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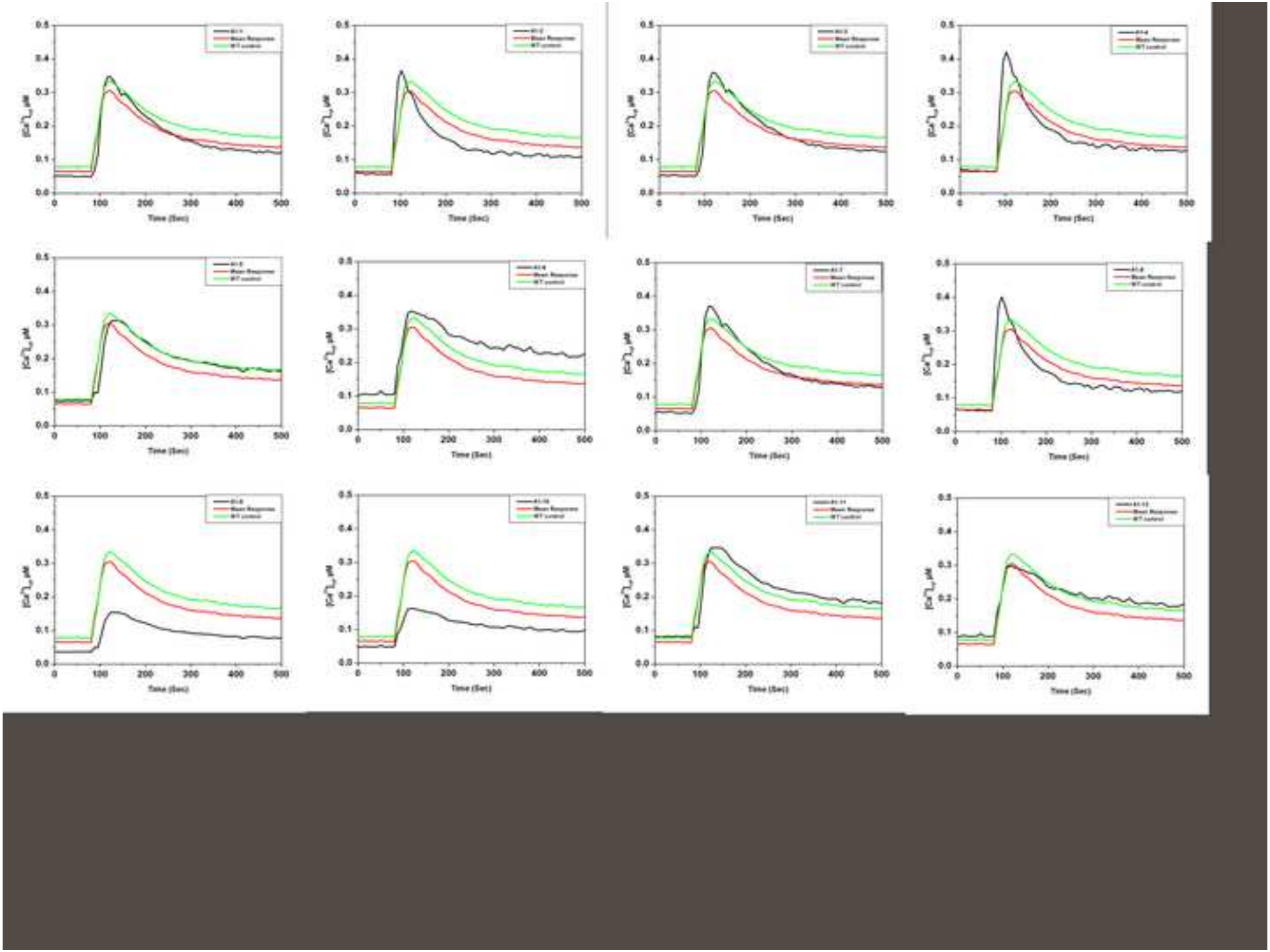

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials-dmupdate.xlsx

Name of Material/ Equipment	Company	Catalog Number
24 well tissue culture plate	Jetbiofil	11024
96 well white cliniplate	Thermo Scientific	9502887
Aequorin		
Agropet	Lab Chem India	
Calcium chloride	Fisher Scientific	12135
Coelenterazine	PJK	55779-48-1
Ehtylmethane sulfonate	Sigma Aldrich	M0880-5G
Ethanol	Analytical reagent	1170
Hydrochloric acid	Merck Life Sciences	1.93001.0521
Hydrogen peroxide	Fisher Scientific	15465
Luminoskan ascent	Thermo Scientific	5300172
MES buffer	Himedia	RM1128-100G
Murashige and skoog media	Himedia	PT021-25L
Sodium hydroxide	Fisher Scientific	27805
Sodium hypochlorite	Merck Life Sciences	1.93607.5021
Sodium thiosulfate	Fisher Scientific	28005
soilrite	Lab Chem India	
Square pots	Lab Chem India	
Sucrose	Sigma Aldrich	S0389
Taxim	Alkem	7180720

Comments/Description
for growing seedlings
for luminometer measurements
for plant growth
for discharge solution
prosthetic group for aequorin
for seed mutagenesis
for discharge solution
sterlization solution
as stimulus for Calcium elevation
aequorin luminescence measurement
plant growth
plant growth
for neutralizing EMS
sterlization solution
for seed washing in step 1.6
for plant growth
for plant growth
plant growth
for seedling rescue

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Your revision is due by **Apr 21, 2020**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Furthermore, please submit a high resolution version of your video (up to 2 GB) here: https://www.dropbox.com/request/45OXmm22KBvpW96vBXCx?oref=e

Sincerely,

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Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Unfortunately, there are a few sections of the manuscript that show overlap with

previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please revise the following lines: first 7 lines of the introduction, third sentence of the last paragraph of the Discussion. The lines are highlighted in the attached iThenticate report.

Authors: The revisions suggested by the editor has been made.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

Authors: Thermo scientific was removed from line 2.5

4. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
5. 1.6: How much is used to wash?

Authors: The description has been added to line 1.6.

6. 1.8: What does this mean and how is it done?

Authors: The description has been added to line 1.8.

7. 2.3: How much chlorine gas is added?

Authors: The description has been added to line 2.3.

8. Please discuss limitations of the protocol in the discussion.

Authors: Limitations have been added in the discussion, paragraph 3.

9. Please do not abbreviate journal titles in the references.

Authors: The corrections have been done.

10. Please remove the legends from the figure files. The text in the manuscript is

sufficient.

Authors: The corrections have been done and the legends have been removed from figure files.

Changes to be made by the Author(s) regarding the video:
1. Main & Chapter Title Cards
• Start & End of Video Main Title Cards: Please freeze or eliminate text movement on these cards. These cards need to be the most readible and be free of distractions. The same effect was applied to the chapter title cards, but you needn't remove it from those cards.
• @00:52 End of introduction and into the first chapter title card (and for all chapter title cards): Consider fading into and out from chapter title cards. At the beginning and end of segments, consider fading to and from white. It would look smoother and little more polished.
• @08:35 Editing error: The outro title card fades into a few frames of the "I. An Introduction" chapter title card.
2. Editing Style
• 06:57 Jump cuts like this are not allowed. Consider rescaling the second clip that starts at "collection of seeds", so that it looks like a different clip, if this can be done without losing too much quality.
3. Narration Coverage & Performance
• @00:57 There seems to be some clothing or papers shuffling around here. You should probably re-record this line.
• @01:15 Add just a little silent space between the lines "...be careful, because it is carcinogenic." and "Protective gears should be worn...", because they are too smashed up next to each other right now.
• @01:28 There is a paper heard shuffling in the narration audio here.
• @01:46 There's a stray sound heard at the beginning of the narration here "Wash the mutagenized seeds thoroughly..."
• @03:10 "Each emb-" Stray audio clip right before the Chapter III title card comes in.
• @06:20 Some possible furniture moving is heard in the background here.
4. Graphics Usage & Placement
• @05:13 The grid graphic here abruptly switches. An on-screen text label would be helpful, even though the narration is explaining. These graph grids may serve us better without the movement as well, as the movement may be distracting us from the meaning.
• @05:44 The first spreadsheet here doesn't seem show much, as it quickly leaves the screen. Consider holding on it longer and providing a verbal explanation, or; if it doesn't need to be here, consider eliminating it.
• @05:56 Consider fading in and out the graphics here. Hard cuts are very jarring when it comes to graphics.

Authors: All the above mentioned corrections have been made in the video file.

Please upload a revised high-resolution video
here: https://www.dropbox.com/request/45OXmm22KBvpW96vBXCx?oref=e

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The methods article by Mittal et al. explains a very key and always in use methodology for the identification of novel targets in calcium signaling by a classical EMS- based forward genetic screen. It explains how to mutagenized, collect and test Arabidopsis plants to create a population suitable for a forward genetic screen. Plus the screen process based on large scale calcium measurement in aequorin-containing seedlings is explained, as well as the possible results and applications of it.
The methodology is well explained and I find it undoubtedly useful for the community. The key references for the field are cited and details are appropriate and correctly explained. I am really glad that this video will be made part of the collection.

Some points could be though a bit clarified and small inaccuracies occur in the manuscript.

Major Concerns:
Comment 1
In point 2.6 authors say that coelenterazine is diluted in water, when it is not soluble unless you add methanol (preferred) or ethanol. Could they comment on this? Also, they say that this compound is photosensitive, so it should said that it is better to add it in darkness or at least with little amount of light to avoid degradation in the process.

Authors: The stock solution of coelenterazine is made in 100% methanol. Only the working solution is diluted with water for overnight incubation according to previous protocols (Ranf et al. 2012). The suggestion on low-light area has been added to the protocol.

Comment 2
This is quite important: in point 2.7 the volume (nor concentration) of H2O2 used is not specified, but this makes a difference for point 2.8. The idea behind the discharge of the aequorin is indeed as mentioned in the video to calculate the total amount of aequorin in the plant; this and only this will allow the correct precise measurement of calcium levels, hence making comparison between plants possible. Discharge is made by adding a double volume of 20% EtOH to burst cells, let aequorin out and 2M CaCl2 is added to saturate aequorin calcium binding sites. The final concentrations are hence 10% EtOH and 1M CaCl2. However seedligns are in 150 µl solution before adding H2O2, then 150 µl discharge solution is added, and wonder what are the final discharge concentrations.

Authors: 40 µL of H_2O_2 solution was added. Our discharge solution is 2M $CaCl_2$ in 20 % ethanol.The final volume after discharge is 340 µL and the final ethanol

concentration in this solution is 0.88 M in 8.6% of ethanol. This is also one of the reasons why we are able to rescue plants.

Comment 3
This comment relate to point 2.11 in the protocol. Could the seedling rescue part be explained better? How is the antibiotic helping the seedling recovery? Plus, linked to comment 2, if discharge is correctly done, cells were burst throughout the process, so how do seedlings get rescued? This part is not clear and need more explanation.

Authors: The end discharge is a short run of 1-2 min and hence, although it does cause some damage to the plant tissue, there is no instantaneous plant death and hence the plant can be rescued by quickly removing it from the solution and washing it several times with distilled water. Cefotaxime wash helps remove any harmful micro-organisms which might have accumulated in the solution and as result on the seedling surface. Since the seedlings after reconstitution were kept un-sealed, it was important to remove these micro-organisms to minimize contamination when transferring back to sterile condition

Minor Concerns:
Comment 4
In the introduction, at the end of the first paragraph, with ref 9 also McAinsh and Pitmann 2009, New Phytologist should be cited. In the same sentence, the comma after cytosol should not be there (typo).

Authors: Reference has been added and the typo corrected in the end of the first paragraph of introduction.

Comment 5
The seed sterilization described in 2.3 should take place under the hood (and this needs to be also correct in the video) as the vapors are toxic.

Authors: Our plates are sealed and kept in a dessicator in a fume hood. We have included this is the text. Additionally, the seed sterilization protocol was adapted from Ranf et al. 2012 wherein they have clearly outlined that once sterilized due to the presence of the breathable seal the plates can be stored on the shelf without any contamination.

Comment 6
Point 1.2 in the protocol: the role of the control plants should be specified, either here or in point 1.10.

Authors: Description has been added to point 1.10.

Comment 7
In point 1.4, specify that NaOH neutralizes the EMS (I think it is important), and in point 1.5 you need to specify where you discard the water full of EMS (I am guessing in NaOH as well).

Authors: Description has been added to point 1.4.

Comment 8
A new paper on calcium and H2O2 perception (Wu et al., 2020, Nature) came out and it should be added to the last paragraph of the discussion, this will further show how the topic and the technique are still of high interest.

Authors: Description has been added at the end of the discussion.

Comment 9
This is just a suggestion, maybe in the discussions the authors could mention that aequorin has been targeted to different tissues and organelles, and this would be an additional application of this method to answer an even wider range of scientific questions.

Authors: Description is added to the third paragraph of discussion.

Comment 10
Throughout introduction, many of the references only refer to biotic stimuli inducing calcium signals, some more abiotic stimuli shall be mentioned as well.

Authors: The references have been updated at reference number 4, 6 and 9.

Comment 11
To be extremely accurate, in the material section pots and soil should be included.

Authors: These have been added in the attached material file.

Comment 12
Detail: keywords in the first table and in the second page are not corresponding, the ones in the second page being more appropriate.
Authors: We did not understand the comment. There is only 1 set of key words in the MS first page

Reviewer #2:
The manuscript and video show useful method and I just found one significant flaw. The authors should address this critical problem and minor comments shown below.

Protocol 2.8 - After discharging with 2M CaCl2 and 20% ethanol, the plants will died and cannot be rescued because all cellular constituents are discharged out during this process. This is a significant flaw in the method. The authors should revise this point. The most of cases, the discharging process is included after the 2nd screening (M3 generation), but never in the 1st screening using M2. This point MUST BE corrected in the manuscript to be accepted.

Authors: The end discharge as mentioned in the manuscript is a short run of 1-2 min and hence, although it does cause some damage to the plant tissue, there is no instantaneous plant death and hence the plant can be rescued by quickly removing it from the solution and washing it several times with distilled water. The quick rescue step provided us with the opportunity to rescue plants which were true mutants to calcium response and hence we decided to discharge them during the screen instead of confirming the mutants in the 2nd screening.

Below are less major comments and advice.

There are many grammatical and typographical errors. While the meaning of the sentences is understandable, the English is "clunky" throughout the manuscript, for example, "we EMS-mutagenized…" and "a 96-well high-throughput Ca2+ measurement protocol" and much more. The authors should ask a native English speaker or a professional company to improve the manuscript writing. In addition, multiple gaps throughout the text and figure legends should be fixed.

Authors: We have carefully proof read all language errors

The reference #9 is not appropriate to cite since it is just a conference abstract. Instead, the authors should cite Plieth (Plant Physiol, 2016) and Marcec et al. (Plant Sci, 2019), in which Ca signature and its sensors were well summarized.

Authors: The review from Plieth, 2016, seems to be more about the mechanism involved in calcium signaling rather than focusing on the various components involved in the signaling pathway and hence has been omitted. The Marcec etal. 2019 review is more informative and topical and hence has been included in the manuscript reference after removal of reference #9.

Second paragraph in the Introduction section, the description sounds all about Arabidopsis. Please specify.

Authors: The paragraph details as to how EMS mutation works and the numbers presented are mathematical probabilities and not specific to any plant species except for the last sentence wherein it pertains to Arabidopsis. This correction has been made and updated in the manuscript.

Third paragraph in the Introduction section, the authors should add more detail to information about the reaction of aequorin system, especially explaining "intramolecular reaction" by citing Tanaka et al. (Methods in Molecular Biology, 2013). https://www.researchgate.net/publication/255178058_Aequorin_Luminescence-Based_Functional_Calcium_Assay_for_Heterotrimeric_G-Proteins_in_Arabidopsis/figures

Authors: The reference suggested by reviewer has been added and we have explained the intra molecular reaction as described by the above manuscript in the third paragraph of introduction.

EMS is toxic. So, the authors should include a procedure how to waste and detoxicate in NaOH in the video.

Authors: The procedure for EMS disposal has been elaborated more in the revised version of the protocol (Points 1.4 and 1.5). Inclusion of EMS waste disposal in the video would be a detour to the main flow of steps and will hinder the understanding of the protocol. EMS disposal can be a short protocol in good laboratory practices and hence does not fall in the scope of this protocol. Hence, the authors think that to maintain the continuity of the protocol it can be omitted.

Video at 3'11" - Voice was cut in the middle. Need to be fixed or removed

Authors: This has been corrected.

Protocol 1.10 - Additionally, abortion rate should be evaluate to know success rate of mutagenesis.

I do not like the authors' labeling way, M1 to M5000, because these are confusing with the numbering of generation M1 and M2… The authors should come up with another idea, for example M1-1 to M1-5000 or something similar.

Authors: We did account for seed lethality during the process. And as mentioned in the protocol 1.14, we could only screen 3500 out the total 5000 lines. The remaining 30% lines either did not germinate or did not survive for us to screen.

For the labelling we have taken into account the kind suggestion of the reviewers and modified our Figure 2 and named individual plants as A1- A500

Protocol 2.2 - "8" makes sense but where is the number "12" come from? Explain.

Authors: Explanation has been added at protocol 2.2

Protocol 2.6 - Use the technical term "reconstitution" for the process from apo-aequorin to the active form of aequorin.

Authors: Corrected at protocol 2.6.

Protocol 3.2 - It would be great if the authors show how to convert pCa to [Ca2+]cyt for the beginners.

Authors: The video at 5'46" shows as to how the obtained luminescence units have been converted to Ca^{2+} concentration values. With the formula, it is just the placement of values and multiplying with 10^6 to obtain a calcium concentration in µM.

Protocol 3.4 - Any threshold to distinguish positive or negative for mutant screening?

Authors: We use a threshold discharge value of 30 for seedlings to distinguish positive from negative. A mutant identified as positive in M_2 screen after a short discharge step is again confirmed in subsequent M_3 and M_4 generation.

Reviewer #3:
Manuscript Summary:
This kind of protocol has been provided by many labs.

Major Concerns:
1. The title and abstract needs to be more specific and should mention peroxide (H2O2) because their screening method is not generally applicable (e.g., this method could not be used for cold treatment).

Authors: This protocol is applicable for every Aequorin based screen and using a liquid stimuli. Even cold water of a specific temperature can be used for such a screen. So it is not specific to H_2O_2 and we cannot include that in our title. Our screening method is based on a luminometer. The only other screening system one can use is based on an Aequorin imaging platform for single photon counting using a CCD camera and that should be an entirely different protocol.

Aequorin based screening methods have been used to identify Ca^{2+} signaling response upon simulation with various stresses e.g. H_2O_2- based identification of

HPCA1 (Wu et al., 2020), LPS-based identification of LORE (Ranf et al. 2015), sorbitol-based identification of OSCA1 (Yuan et al. 2014).

2. The title is not accurate. The method does not identify novel targets as the title says, rather the method identifies upstream regulators of calcium signaling. That is, the method should identify mutants that are upstream of [Ca+2] change and not targets that are downstream of [Ca+2] change.

Authors: Calcium signature can be modulated by both upstream and downstream components that regulate a "channelosome" or even calcium sensors. The method described in this protocol aims at identifying novel components involved in Ca^{2+} signaling pathways. These can either be receptor kinase (e.g. HPCA1; Wu et al. 2020), receptor-like kinase (e.g. LORE; Ranf et al. 2015), nuclease (eg. PARN1) or a transferase protein (e.g. MOCA1; Jiang et al. 2019) to name a few. Since the screen uses Ca^{2+} response as a read-out, all early signaling genes which lead to the activation of the response cascade are majorly identified.

3. At 6:31 in the video, the authors should add advice, "reconfirm the putative mutants in the next generation in a secondary screen" before going on to identify the causative mutation in those mutants. Many putative mutants could be false positives.

Authors: The audio has been edited to include this information.

Reviewer #4:
Manuscript Summary:
This manuscript gives a brief introduction about using EMS-mutagenized Arabidopsis aequrion seedlings to screen genetic components involved in generation of cytosolic Ca2+ transient rise. It gives an clear working flow and can be used as an preliminary guidance for interested people. The whole manuscript is well written.

Major Concerns:
(1) Based on my own experiences in this techniques, the screening is not as easy as it is presented in this manuscript. Therefore, I would like to suggest the authors to list major problems or technical challenges they were confronted with, if they did.

Authors: Description added in the text in paragraph four of discussion.

(2) 2.2 Please give a brief explanation about" the 1/8 frequency."

Authors: Description added in the text at protocol 2.2.

(3) 2.4 How can the solution contamination be avoided during the 8-12 days hydroponic growth.

Authors: Description added in the text at protocol 2.4.

Minor Concerns:
1.2 "ml" should be "mL".

Authors: This has been corrected

2.6, 5mM should be "5 mM"

Authors: This has been corrected.

3.6. It seems it is not a complete sentence. "either" ?

Authors: “Either” has been deleted From protocol 3.6.

Author Bio

1) Deepika Mittal - PhD student (5th year) working on identification of novel calcium regulated jasmonate perception modules in Arabidopsis at NIPGR, New Delhi.

2) Shruti Mishra – PhD student (1st year) at NIPGR working on mobile calcium signals upon herbivory and its generation.

3) Ramgopal Prajapati- PhD student (5th year) working on role of calcium signaling in plant defense against herbivory.

4) Dr. Jyothilakshmi Vadassery – PI of the lab working on role of calcium in regulating plant defense against herbivory