CHAPTER

Staining and automated image quantification of callose in Arabidopsis cotyledons and leaves

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Chapter outline

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Abstract

Callose is a β-1,3-glucan polysaccharide that is deposited at discrete sites in the plant cell wall in response to microbial pathogens, likely contributing to protection against pathogen infection. Increased callose deposition also occurs in response to the 22-amino acid peptide flg22, a pathogen-associated molecular pattern (PAMP) derived from bacterial flagellin protein. Here, we provide protocols for callose staining using aniline blue in cotyledon and leaf tissue of the model plant *Arabidopsis thaliana*. Aniline blue stain utilizes a fluorochrome that complexes with callose for its visualization by microscopy using an ultraviolet (UV) filter. For robust quantification of callose deposits, we outline an automated image analysis workflow utilizing the freely available Fiji (Fiji Is Just ImageJ; NIH) software and a Trainable Weka Segmentation (TWS) plugin. Our methodology for automated analysis of large batches of images can be easily adapted to quantify callose in other tissues and plant species, as well as to quantify fluorescent structures other than callose.

1 Introduction

Each year, a substantial percentage of crops are lost to pathogenic infection, which can lead to significant economic loss and reduced food security (Savary et al., 2019). Understanding the natural defenses of plants can inspire novel approaches to engineer durable resistance in crop species, contributing to increased agricultural production. A plant's first line of defense includes preformed and induced immune responses, with the latter utilizing the perception of pathogen-associated molecular patterns (PAMPs) by the plant using pattern recognition receptors (PRRs). PRRs initiate an array of immune responses that contribute to pattern-triggered immunity (PTI) to help the host defend itself against non-adpated pathogens (Xin & He, 2013; Yu, Feng, He, & Shan, 2017). One of the best-established microbial PAMP—host PRR system to study immune responses in the model plant, *Arabidopsis thaliana*, is flg22, the 22-amino acid PAMP derived from flagellin, a bacterial protein that serves as the building block for the flagella of pathogenic *Pseudomonas* strains and is necessary for host infection (Xin & He, 2013). flg22 is recognized by the plant PRR, FLAGELLIN SENSING 2 (FLS2), to initiate a myriad of early, intermediate and late defense responses within the host cell (Collins et al., 2020; Yu et al., 2017), including callose deposition (Leslie, Rogers, & Heese, 2016; Smith et al., 2014), which is considered a late PAMP response.

Callose is a β-1,3-glucan polymer of high molecular weight that exists in plant cell walls and is required for normal plant growth and development. Callose is also deposited in wound sites, papillae, and plasmodesmata during plant defense against pathogenic microbes (Ellinger & Voigt, 2014; Nishimura et al., 2003). The exact function of callose in conferring immunity against diverse pathogens is still debated; but stress-induced callose is deposited in cell walls near the neck zone of plasmodesmata to control plasmodesmal permeability and symplastic signaling between

neighboring plant cells (Cheval & Faulkner, 2018; Wu, Kumar, Iswanto, & Kim, 2018). Callose deposition may also help reinforce weak or compromised cell walls and form a cell wall matrix for the deposition of antimicrobial compounds to provide targeted chemical defense at the site of microbial pathogen attack (Luna et al., 2011).

A straightforward method to visualize callose deposition is through aniline blue staining. The fluorochrome present in commercially available aniline blue stain complexes with β-1,3-glucans to fluoresce at a wavelength of 500–506 nm when excited with UV light (Smith & McCully, 1978). While aniline blue can bind other compounds in the plant cell wall, the fluorochrome shows stronger association with β-1,3-glucans due to the greater accessibility of the polymer (Hood & Shew, 1996). Once stained with aniline blue, plant tissues can be imaged with fluorescence microscopy for subsequent quantification of callose deposition.

Here, we present methods for flg22-induced callose in both cotyledons (Fig. 1A) and leaves of mature Arabidopsis plants (Fig. 1B); callose staining using aniline blue; and callose imaging using fluorescence microscopy. We also provide a workflow for the automated quantification of callose staining across large data sets utilizing the free and open-source software Fiji (Fiji Is Just ImageJ) and the Trainable Weka Segmentation (TWS) plugin (https://imagej.net/Trainable_Weka_Segmentation). This Fiji plugin allows the user to train the program to recognize specific features within an image and then uses machine learning algorithms to classify a large set of images. Image analysis can be separated into three main steps: 1. cropping of images to remove background and measure area; 2. segmentation of images to recognize callose deposits; and 3. analysis of particles to quantify results. This plugin is versatile, in that it can be utilized to quantify a multitude of cellular changes that includes quantifying flg22-induced endocytosis of fluorescence protein-tagged FLS2 in endosomal puncta (Leslie & Heese, 2017).

2 Elicitation

In Arabidopsis, the deposition of callose in response to flg22 or other PAMPs is well-established in cotyledons of 7- to 8-day old seedlings and in mature leaves of 5- to 6-week old plants. Callose is a late immune response (Yu et al., 2017), so elicitation can be carried out for 16–24 h in both seedlings and mature leaves to observe a robust callose response. However, because a mutation may lead to constitutive activation of defense responses that are independent of a PAMP, it is critical to include negative control(s) for proper interpretation of callose staining experiments. Negative controls consist of treating tissue with a non-functional flg22 peptide derived from *Agrobacterium tumefaciens*, or with dimethyl sulfoxide (DMSO; solvent for flg22-peptide) (mock) (Korasick et al., 2010). Because flg22-induced callose is dependent on CALLOSE SYNTHASE 12 (CalS12)/GLUCAN SYNTHASE-LIKE 5 (GSL5), also referred to as POWDERY MILDEW RESISTANT 4 (PMR4) (Ellinger & Voigt, 2014; Nishimura et al., 2003), loss of function *pmr4-1* null mutant plants can serve as negative controls (Leslie et al., 2016).

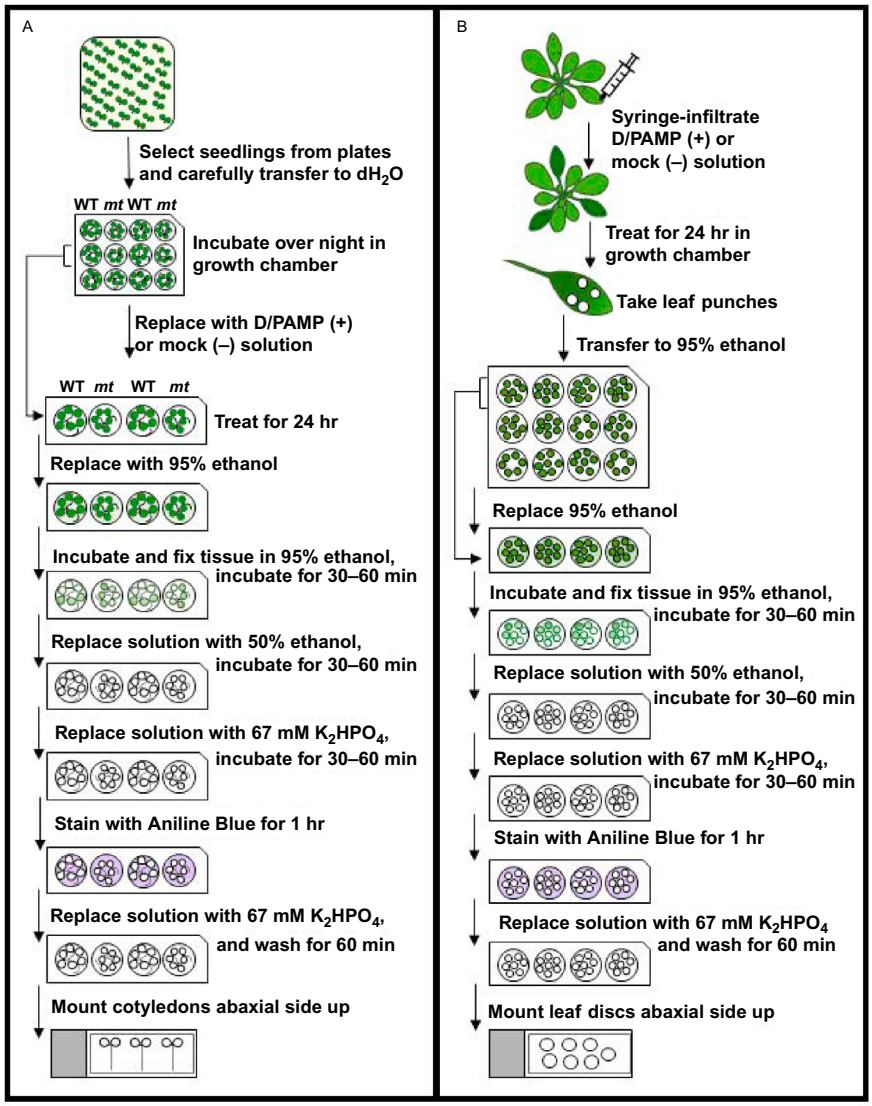

FIG. 1

Flowchart for elicitation, fixation, aniline blue staining and mounting of Arabidopsis tissue. Flow chart for (A) seedlings and (B) leaf tissue.

2.1 Elicitation in cotyledons of seedlings

As shown in Figs. 1A and 2, seedlings are elicited by flotation in liquid solution containing flg22, during which the epidermis of cotyledons and other organs is exposed directly to flg22. In addition, roots take up flg22 from the solution, resulting in flg22-induced responses within tissues other than the epidermis. This flotation method only requires solution changes and is straightforward and easy, but one disadvantage is that the amount of flg22 uptake cannot be regulated directly, resulting in more varied responses between each cotyledon of different seedlings. For seedling assays, we typically select 12 seedlings per genotype and treatment.

2.1.1 Materials

- 7- to 8-day old Arabidopsis wildtype and mutant seedlings ecotype Col-0 grown on ½ MS + 1% sucrose plates (see Section 5.1) at 22 °C and 24-h light photoperiod at 82 μmol m^2 s^{-1}
- Straight tapered flat point forceps (Catalog# 12-000-123, Fisher Scientific)
- 12- or 24-well sterile Falcon™ Polystyrene Microplates (Catalog# 08-772-29 or 08-772-1, respectively; Fisher Scientific)
- Sterile distilled H_2O
- Micropore Surgical Paper Tape (Catalog# 19-061655, Fisher Scientific)
- Elicitation solution (see Section 5.2)
- Dimethyl sulfoxide (DMSO; ≥99.7% purity; Catalog# BP231-100, Fisher Scientific)
- 95% Ethanol
- *Pseudomonas aeruginosa* flg22 peptide (GenScript; see Section 5.2)
- *Agrobacterium tumefaciens* flg22 peptide, if used as a negative control (GenScript; see Section 5.2)

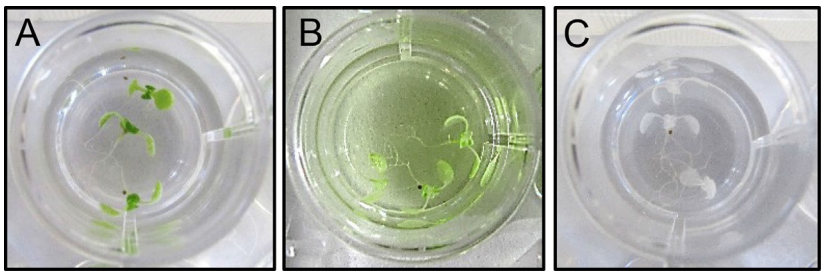

FIG. 2

Representative images of seedlings during elicitation and fixation procedure. (A) After placing 7-day old seedlings in dH_2O overnight to reduce wound responses, seedlings are elicited with 100 nM–1 μM flg22 or mock solution for 24 h. (B) Carefully remove all elicitation solution and incubate seedlings in 95% ethanol to remove chlorophyll and fix tissue. (C) After multiple 95% ethanol changes, make sure that the last ethanol wash results in a clear solution and seedlings appear translucent white before proceeding with aniline blue staining.

2.1.2 Protocol

1. Fill each well of a sterile tissue culture plate with 1 mL of sterile dH_2O.

   Note: Volumes of water or other solutions may need to be adjusted depending on well size; but volumes must be consistent between all wells and between different experiments.

   Note: Instead of water, wells may be filled with liquid media similar to growth plate conditions (½ MS + 1% sucrose, no agar) to prevent nutritional deficiencies and/or osmotic shock.
2. Gently lift seedlings in a scooping motion by placing the forceps underneath the cotyledons with one tip of the forceps on either side of the hypocotyl and transfer to well (Fig. 2A). For 12- or 24-well plates, place three or four seedlings per well, respectively.

   Note: Damage caused by forceps will produce wound-induced callose unrelated to flg22-elicitation. The latter will also be stained by aniline blue, and thus may skew results.

   Note: To decrease variability between experiments, keep the time of day when seedlings are picked consistent between experiments.
3. Seal plate with micropore tape. Leave overnight (16 h) in the same growth chamber as they were before (22 °C, 24-h light).

   Note: Leaving seedlings in water or liquid ½ MS overnight will reduce wound responses and is necessary for subsequent flg22-responses.

   Note: In different mutants, effects of light conditions may affect flg22-induced callose differently, and thus may need to be altered.
4. Prepare elicitation solutions using sterile dH_2O (see Section 5.2). Prepare 1 mL of solution per well plus one or two well excess.
   i. Positive treatment: *Pseudomonas aeruginosa* flg22 (active flg22; $flg22^{Paer}$) (or other PAMP); final concentration: 100 nM–1 µM.
   ii. Negative control: *Agrobacterium tumefaciens* flg22 (inactive flg22; $flg22^{Atum}$) at the same concentration as the positive treatment OR mock treatment prepared by adding the same volume of DMSO as used for $flg22^{Paer}$ to sterile dH_2O.

   Note: Other bacterial PAMPs such as bacterial elf26, or the plant-derived damage-associated molecular patterns (DAMP) *At*PEP1, allow for comparing callose deposits in response to diverse D/PAMPs.

   Note: Some mutant seedlings may show constitutive activation of defense responses independent of active D/PAMPs. Therefore, including negative controls are critical for proper interpretation of PAMP-induced callose production.
5. Swiftly but carefully remove dH_2O from individual wells without damaging seedling tissue.

   Note: Make sure to remove all liquid from individual wells including that trapped underneath cotyledons by gently tapping the plate. Insufficient dH_2O removal can result in diluting, thus changing the final flg22-concentration

between wells, which in turn leads to increased variability in responses observed between wells and/or experiments.

Note: Move quickly to avoid drying out of seedling tissue.

6. To elicit callose deposition, add 1 mL elicitation solution to each well and incubate for 24 h in the same growth chamber as they were before (22 °C, 24-h light).

Note: Elicitation time may be shortened to 12–16 h depending on growth conditions, flg22-concentration and genotype.

7. Replace elicitation solution with 95% ethanol to fix seedlings and stop responses (Fig. 2B and C).

2.2 Elicitation in mature leaves

Mature leaves are syringe-infiltrated with elicitation solution (Figs. 1B and 3), which has the advantage that the solution permeates through all tissue for a potentially stronger flg22 response. Infiltrating different leaves of the same plant with both

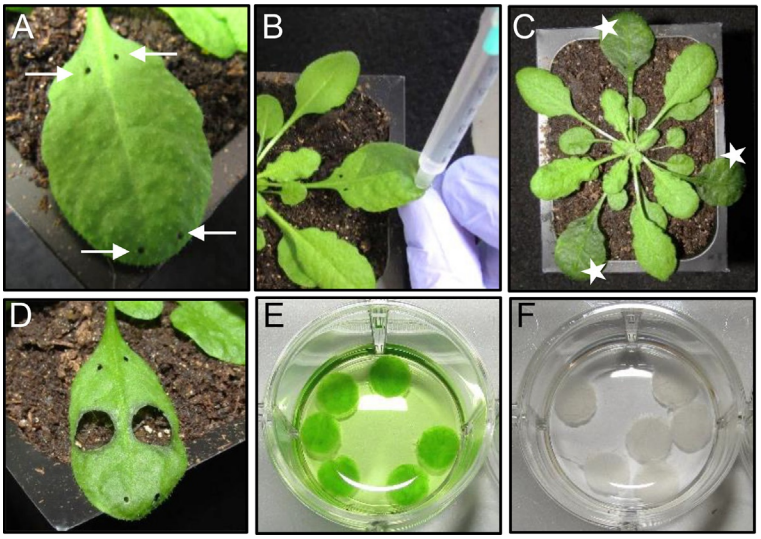

FIG. 3

Representative images for flg22-elicitation, tissue collection and fixation of leaf tissue. (A) In 6-week old plants, immediately prior to leaf infiltration, poke 3–4 holes (arrows) in selected leaves to enable (B) syringe-infiltration with 10 nM–1 μM flg22 (elicitor solution) or mock treatment. (C) Infiltrate three leaves (stars) of similar developmental stage within each rosette. (D) After 24-h elicitation, collect leaf tissue by punching leaf disks on either side of midrib from infiltrated leaves. (E) Transfer leaf disks immediately into 95% ethanol to clear and fix tissue. Change 95% ethanol solution multiple times. (F) For the last wash in 95% ethanol, the solution should remain clear, and all leaf disks should appear translucent white before proceeding to aniline blue staining.

active and inactive elicitation (or mock) solutions should be avoided because it can skew results due to cross-contamination from eliciting solution that may spray or drip onto leaves infiltrated with negative control solution. Furthermore, infiltration of active flg22 in one leaf can induce systemic responses in another leaf over time, potentially contributing to inaccurate results. For mature leaves, we typically infiltrate three fully expanded leaves of at least 3–4 different plants (for a total of 18–24 leaf disks) for each genotype and treatment.

2.2.1 Materials

- 5- to 6-week old mature Arabidopsis plants grown on soil at 22 °C, 8-h light/16-h dark cycle photoperiod at 82 μmol m^2 s^{-1}.
- Sterile dH_2O
- Elicitation solution (See Section 5.2)
- DMSO (≥99.7% purity; Catalog# BP231-100, Fisher Scientific)
- Gel loading tips or sterile toothpicks
- 1 mL Syringe without needle (sterile) (Catalog# 14-817-25, Fisher Scientific)
- Kimwipes or equivalent lab wipes
- 12-Well sterile Falcon™ Polystyrene Microplates (Catalog# 08-772-29, Fisher Scientific)
- Straight tapered flat point forceps (Catalog# 12-000-123, Fisher Scientific)
- 95% Ethanol
- #2 or 0.2 cm^2 cork borer for leaf tissue punch (Catalog# 07-865-10B, Fisher Scientific)

2.2.2 Protocol

1. Before preparing elicitation and negative control solutions for infiltration, check that the plant growth chamber is free of pests. Avoid plants that show any symptoms of prior pathogen infection, and any leaves with previous damage, chlorosis or visible cell death that may have caused callose deposition prior to or independent of D/PAMP elicitation, thus skewing results.

   Note: To ease with infiltration, we water plants the day before elicitation. Check, sort and label all plants with information on genotype, concentration and type of elicitation solution prior to preparing the elicitation solution. Exclude plants that have produced inflorescence stems.
2. Prepare 1 mL elicitation solution per plant using sterile dH_2O. If necessary, adjust this volume for future experiments.
   i. Positive treatment: 10 nM–1 μM $flg22^{Paer}$ (or other active D/PAMP)
   ii. Negative control: $flg22^{Atum}$ at the same concentration as positive treatment OR mock treatment with DMSO.

   Note: Determine the optimal flg22 concentration empirically, because different mutants may respond differently to distinct PAMP concentrations. Use serial dilution to prepare different flg22 concentrations to assess whether a mutant has altered callose deposition compared to wild type.
3. Using gel loading tips or sterile toothpicks, carefully punch four small holes in the leaf; two at the apex and two at the base of the leaf blade (Fig. 3A). Avoid ripping or severely damaging leaf tissue.

4. Fill syringe with elicitation solution. Supporting the bottom side of the leaf with a gloved finger, place opening of syringe on one hole in leaf tissue near apex and slowly inject solution (Fig. 3B). Do not press syringe onto tissue too strongly as it will damage surrounding tissue, resulting in callose deposition unrelated to elicitation. Press only enough to create a seal for injecting solution.
5. Repeat infiltration at the other three holes at apex and base. Check that the infiltration solution has spread through the entire blade and entered the petiole. Infiltrated leaf tissue appears darker in color and more translucent than non-infiltrated areas (Fig. 3C). If applicable, mark non-infiltrated areas with a black sharpie and avoid those sections for callose analyses. Alternatively, turn leaf over and very gently place syringe near the uninfiltrated area and inject solution through stomata on the abaxial side.
6. Gently remove excess solution with a Kimwipe without damaging tissue.
7. Repeat infiltration into additional leaves at comparable developmental stages within the same plant (Figs. 1B and 3C).
8. Infiltrate set of plants with active flg22^{Paer} first. Change all tips, syringes, and gloves and wipe down surface before starting from step 3 to infiltrate another set of plants with mock treatment.
9. Cover plant trays with a clear dome lid for 24 h under same growth conditions as before (22 °C, 8-h light).
10. Prior to collecting leaf samples, label 12-well plates with genotype and treatment information using an alcohol-resistant marking pen. Fill each well with 1 mL of 95% ethanol.
11. After 24-h elicitation, collect two leaf disk punches from each infiltrated leaf with one punch from each side of the midrib (Fig. 3D).

    Note: Number of leaf punches may be adjusted depending on leaf size between wild-type and mutant plants. If within an experiment, leaf size across all genotypes are large and of similar size, four disks (two on each side of midrib) may be taken to increase sample number (Fig. 1B). If leaves of mutant plants are too small to punch an entire leaf disk on either side of the midrib, then punch disk(s) across midrib for all plants of all genotypes and treatments to stay consistent within an experiment.

    Note: Make sure to sharpen leaf puncher periodically to prevent extensive tissue damage.
12. Transfer leaf punches using forceps to wells containing 95% ethanol to stop defense/wounding reactions and start fixing leaf tissue.

    Note: Place all leaf punches from same plant into the same well (i.e., six leaf disks per well) (Fig. 3E). This will address reproducibility within an experiment, in that it allows keeping track of whether different plants of the same genotype and treatment behave similarly.

    Note: If leaf disks overlap, use plate with larger well size or add fewer leaf disks per well.

3 Aniline blue staining, tissue mounting and microscopy

Aniline blue is an effective stain for callose in plant tissue and is routinely used to detect flg22-induced callose in both cotyledons and leaf tissue. One disadvantage is that aniline blue is not specific for callose but may complex with other plant cell wall components (Smith & McCully, 1978).

3.1 Materials

- 95% Ethanol
- 50% Ethanol
- Sterile dH_2O
- $10\times$ K_2HPO_4 stock solution (670 mM Potassium phosphate dibasic trihydrate (K_2HPO_4), pH 12; Catalog# P5504, Sigma-Aldrich)
- 67 mM K_2HPO_4 working solution (pH 12; made by $10\times$ K_2HPO_4 stock solution $10\times$ in sterile dH_2O)
- Aniline Blue (Catalog# 415049-50G, Sigma-Aldrich)
- Glycerol (Catalog# BP229-1, Fisher Scientific)
- Parafilm (Catalog# PM992, Bemis™)
- Aluminum Foil
- 1.7 mL microcentrifuge tubes (Catalog# 07-200-534, Fisher Scientific)
- Mounting media (70% glycerol in 67 mM K_2HPO_4, pH 12)
- Premium Superfrost Microscope Slides (Catalog# 12-544-7, Fisher Scientific)
- Cover Slips (50×24 mm; Catalog# 12-545-88, Fisher Scientific)
- Sally Hansen Clear Nail Polish
- Slide box (Catalog# 03-446, Fisher Scientific)
- Leica M205 FA stereoscope with UV fluorescence filter (or equivalent)

3.2 Protocol

1. Clear and fix tissue in 1 mL 95% ethanol on rocker at room temperature (RT) (Figs. 2B and 3E). Change 95% ethanol multiple times and incubate for 30–60 min each time to ensure complete removal of chlorophyll.

   Note: Residual chlorophyll appears as a milky substance during imaging using a UV filter and interferes with callose detection and quantification.
2. Once tissue is translucent white (Figs. 2C and 3F), wrap plate with parafilm and leave on rocker at 4 °C until ready for staining. Samples can be kept in 95% ethanol for up to a week at 4 °C.

   Note: Make sure to periodically change or replenish 95% ethanol to avoid tissue drying.

3. Rinse tissue with 1 mL 50% ethanol. Discard solution and incubate tissue in 1 mL 50% ethanol for 30–60 min on rocker at RT.

   Note: For this and subsequent steps, change liquid in one well at a time to prevent drying out of tissue.

4. Rinse tissue with 1 mL 67 mM K_2HPO_4 (pH 12). Discard solution and rehydrate tissue in 1 mL 67 mM K_2HPO_4 (pH 12) for 30–60 min on rocker at RT.

   Note: Prepare 10 × K_2HPO_4 (pH 12) stock solution using KOH pellets to adjust pH. Filter-sterilize 10 × and 1 × K_2HPO_4 solutions through a 0.2 µm filter to remove any dust particles as they may interfere with imaging.

   Note: For efficacy of aniline blue to stain callose deposits and to retain its buffering capacity, K_2HPO_4 must be at a basic pH of 12 and at a final concentration of 67 mM, respectively.

5. Prepare 0.01% (w/v) aniline blue in 67 mM K_2HPO_4 (pH 12) (aniline blue staining solution) as described in Section 5.3. To stain tissue, exchange 67 mM K_2HPO_4 (pH 12) solution with 1 mL aniline blue staining solution and incubate for 60 min on rocker at RT. Because aniline blue may be light sensitive, wrap plate in aluminum foil.

   Note: Prepare 1–2 mL excess of aniline blue staining solution. For consistency, all samples within an experiment must be stained with the same preparation of 0.01% aniline blue solution.

6. Rinse samples in 1 mL 67 mM K_2HPO_4 (pH 12). Discard solution and wash stained tissue in 1 mL 67 mM K_2HPO_4 (pH 12) for 60 min on rocker at RT.

   Note: Plant tissue is easiest to mount immediately after staining. However, plates can be sealed with parafilm and left overnight on a rocker at 4 °C. Tissue becomes less rigid over time in 67 mM K_2HPO_4 (pH 12), making it more difficult to mount.

7. Prior to mounting tissue onto microscope slides, prepare mounting solution.

   Note: For 10 mL mounting solution, use 7 mL 100% glycerol, 1 mL 10 × K_2HPO_4 (pH 12) stock solution and 2 mL dH_2O and store at 4 °C for up to 1 month.

8. Mount seedlings on Premium Superfrost Microscope Slides by spreading out 100–150 µL of mounting media onto labeled slide. Gently place tissue into mounting media on slide with abaxial side facing up, spread out and flat. Use one slide for tissues from one well (Fig. 1). Place coverslip in one smooth motion beginning at one edge of slide to prevent trapping of air bubbles. Seal edges of coverslip with nail polish. Store slides in slide box at 4 °C.

   Note: Placing a black background under slides makes it easier to visualize cleared translucent tissue to arrange on slide.

   Note: Use a fine pipette tip, potentially under a dissecting microscope, to ensure that cotyledons or leaf disks are not folded over or overlap with other tissues such as roots.

   Note: For consistent data collection, it is critical that all cotyledon or leaf samples are placed on slides with the abaxial (lower surface) facing up. This is because adaxial (upper) and abaxial (lower) surfaces are developmentally distinct, and thus respond differently to flg22-elicitation. In addition,

developmentally deposited callose accumulates at the bases of trichomes, which are more abundant on adaxial (upper) epidermis of cotyledons or leaves, interfering with quantification of stress-induced callose.

Note: Slide boxes may be wrapped in plastic wrap to reduce condensation on slides.

9. For callose detection, examine mounted tissue using a microscope equipped with a mercury lamp and a "DAPI" filter set or equivalent filter set for detecting aniline blue complexed with β-1,3-glucan (excitation and emission maxima 390 nm and 500–506 nm, respectively) (Smith & McCully, 1978). We use a Leica M205 FA stereoscope with a mercury lamp and UV filter (excitation filter 390 nm; longpass emission filter 420 nm). Set microscope magnification to fit one entire cotyledon of the largest genotype (Fig. 4A) or one leaf disk in microscope field of view. Use grayscale during image acquisition and exposure that creates the best contrast between callose deposits (bright white) and the leaf tissue (gray) (Fig. 4A and B).

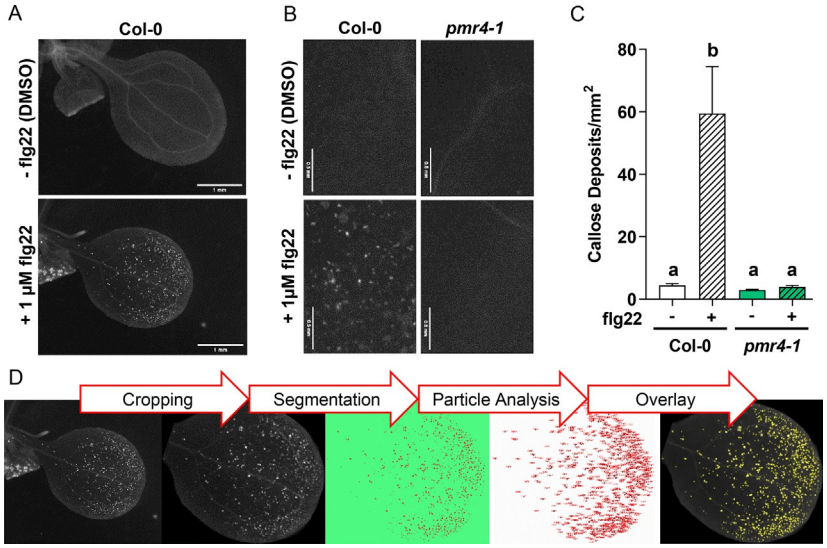

FIG. 4

Representative images of callose deposition and subsequent quantification process using Fiji. (A) Wild-type Col-0 cotyledon after 24 h treatment without (− flg22, DMSO) or with flg22 (+ 1 µM flg22). Scale bar, 1 mm. (B) Close-up images of Col-0 and *pmr4-1* cotyledons after 24 h treatment without (− flg22, DMSO) or with flg22 (+ 1 µM flg22). Scale bar, 0.5 mm. (C) Automated large-scale image quantification of callose deposits/area (callose deposits/mm^2) identified differences in callose deposition between different genotypes (Col-0, *pmr4-1*) and different treatments (−/+ 1 µM flg22 for 24 h). Values are mean ± SEM with $n = 22–24$ cotyledons per treatment. Different letters indicate statistically significant differences as determined by one-way Anova with Tukey multiple comparisons test ($P < 0.05$). (D) Representative step-by-step images generated during in Fiji quantification process for cropping, segmentation and particle analysis with a final image showing the overlay of detected callose deposits onto cropped image.

Note: Acquire all images within an experiment at the same magnification and exposure time. Try to take all images during the same microscope session to reduce image variability, which can affect subsequent quantification.

4 Quantification of callose deposits

Quantification of callose deposits across large sets of image data allows for comparisons of responses to flg22 and mock treatments between wild-type and mutant genotypes such as Col-0 and *pmr4-1*, respectively (Fig. 4B and C), with the ability to discern subtle but significant differences between different genotypes (Leslie et al., 2016). Our preferred program for callose quantification is Fiji, a powerful tool for scientists to conduct advanced, large-scale image analysis (Schindelin et al., 2012). Here, we employ Fiji to create classifiers with the help of the existing Trainable Weka Segmentation (TWS) plugin. In the initial step, a single callose image is utilized to establish a Fiji-derived workflow that allows the user to process large batches of callose datasets. This processing pipeline is easily adaptable for each experiment due to the creation of a segmentation classifier that can discern callose deposits from the other features in an image. Each image is cropped and measured individually, but this process can be made more efficient by designing a Fiji macro that condenses multiple steps into one and analyzes particles for a batch set of images. As part of Fiji, the TWS plugin saves time and produces less biased analysis of data due to the development of a classifier that can be applied to a whole set of images. In Fig. 4D, we provide a quantification workflow that utilizes Fiji for image cropping/measuring, segmentation, and particle analysis. Fig. 5 highlights specific tools in Fiji and the TWS plugin relevant to callose quantification across large batches of images.

4.1 Materials

- Computer (preferably with touch screen capabilities)
- Fiji (https://imagej.net/Fiji) including the TWS plugin (https://imagej.net/Trainable_Segmentation)
- Microsoft Office Excel (any version after MSOffice Excel 95)
- GraphPad Prism 8 or other graphing program
- MiniTab or other statistical analysis program

4.2 Protocol

1. Launch Fiji program and open a single image.
   Note: If Fiji shows a dialog box requesting to update Fiji upon opening this program, click "never"; otherwise the subsequent protocol may not work properly and needs to be adjusted.
2. Setting Measurements: In Fiji, go to *Analyze* ➔ *Set Measurements* (Fig. 5A, blue box). Check the boxes for "Display Label" and "Area."

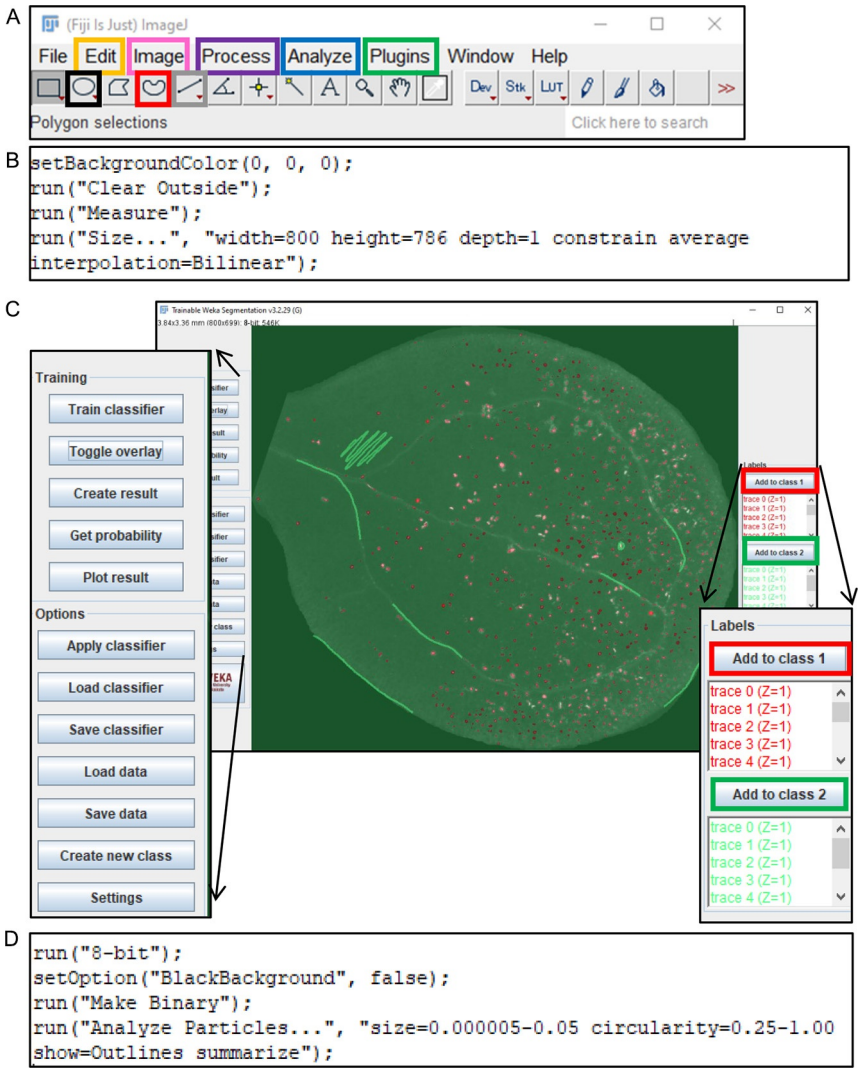

FIG. 5

Images of Fiji program relevant for quantification process. (A) Ribbon of Fiji when using Windows operating system to highlight specific control tabs: Edit (yellow), Image (pink), Process (purple), Analyze (blue), Plugins (green); and tools for area selection: Oval (black), Free Hand (red) and Line Selection (gray) (B). Example of dialog box with a defined "clear measure resize" macro created using the macro recorder. (C) Representative image of the TWS Fiji plugin to select callose puncta with Oval selection tool to add to class 1 (red box; included features). Excluded features or background are selected using Line selection tool and added to class 2 (green box; excluded features). (D) Example of a dialog box with the "particle analysis" macro that was created using the macro recorder.

3. Setting scale: Open the corresponding data file that contains all parameters used during image acquisition. If applicable, convert meters per pixel to millimeters (mm) per pixel. In Fiji, go to *Analyze* ➔ *Set Scale*. Enter mm per pixel value as "Known Distance" and set "Distance in pixels" and "Pixel Aspect Ratio" to 1. Set "Unit of Length" to mm. Record "Scale" value at bottom to refer to later. Check the box for Global, which will apply this scale to subsequent images. Open a new image, and when dialog box pops up, uncheck "Disable Global Calibration" and check "Do Not Show This Message Again." Check scale of new image to make sure correct scale was applied.

   Note: If you close the Fiji app at any time during quantification, you will need to set the scale again.
4. Creating "Clear Measure Resize" Macro (Fig. 5B): Use *Freehand Selections Tool* on Ribbon (Fig. 5A, red box) to trace outline of plant tissue, excluding regions where tissue is folded, overlapping, or torn. Open *Plugins* ➔ *Macros* ➔ *Record* (Fig. 5A, green box). This will record further actions made in Fiji. Go to *Edit* ➔ *Clear Outside* (Fig. 5A, yellow box). This will exclude everything outside of selection. Go to *Analyze* ➔ *Measure* to measure area of selection displayed in results window. Go to *Image* ➔ *Adjust* ➔ *Size* (Fig. 5A, pink box). If raw image is 1600×1200 pixels wide, we change "Width" to 800 and check boxes for "Constrain Aspect Ratio" and "Average when Downsizing" to decrease image processing time. Change Interpolation to "Bilinear." Click OK. Click Create in upper right corner of Recorder window. In Macro Script Window that pops up, go to *File* ➔ *Save As* and name macro "Clear Measure Resize." This macro can be used for future experiments and does not need to be adjusted every time.
5. Cropping: Create a copy of the original folder with all the images from experiment and rename it "cropped version." Open the first image file from "cropped version" folder. In Fiji, go to *Plugins* ➔ *Macros* ➔ *About Startup Macros*. Within Macros Script window, go to *File* ➔ *Open* and choose "Clear Measure Resize" macro. Choose *Freehand Selections Tool* on Ribbon. Trace outline of plant tissue, excluding regions where tissue is folded, overlapping, or torn. Press "Run" on Macro Script window. Save and overwrite image file. Repeat cropping protocol for each image in "cropped version" folder. Copy all labels and area measurements from Results window into an Excel spreadsheet titled Callose Quantification.

   Note: Crop images in alphabetical order by name because later steps will batch process the folder in that same order. Alternatively, arrange them in alphabetical order after pasting in Excel.
6. Segmentation: Create a new folder titled "Segmentation Results." Open a single cropped version .tif file from previous step that is a good representative of callose deposition. Reset the scale by going to *Analyze* ➔ *Set Scale*. If width of original raw image has been changed from 1600 to 800 pixels (see step 4 Section 4.2), divide "Distance in Pixels" by 2 and replace value. Check box for Global to apply new scale to all images. To start creating a classifier, go to

Plugins ➔ *Segmentation* ➔ *Trainable Weka Segmentation*. For callose deposits, use *Oval tool* (Fig. 5A, black box) to highlight one punctum and then click "add to class 1" on right side of window (Fig. 5C, red box). Repeat for a few differently sized callose deposits. For plant tissue, use *Line tool* (Fig. 5A, gray box) to highlight a feature without callose and click "add to class 2" (Fig. 5C, green box). It is best if selected *tool* matches relative shape of feature, in that the *Oval tool* is best for circular puncta such as callose deposits, and the *Line tool* is best for vasculature. Repeat for a few different features such as vasculature or edges of tissue. After sorting a small number of features into classes, click "Train Classifier." Refine classifier by repeating the sorting of features and retraining the classifier until almost all callose deposits are recognized with minimal other features being recognized. Click "Save Classifier" if you want to be able to use this classifier again. Click "Apply Classifier." Select all cropped images from previous step and click Open. When asked "Do you want the results to be stored on the disk instead of opening them in Fiji?" select yes and select "Segmentation Results" folder as the save location. When asked "Create Probability maps instead of segmentation?" select no. Wait for segmentation analysis to finish processing all selected images.

7. Creating "Callose Analyze Particles" Macro (Fig. 5D): Open a single segmentation result .tif file from previous step. Open *Plugins* ➔ *Macros* ➔ *Record*. This will record further selections made in Fiji. Go to *Image* ➔ *Type* and select "8-bit." If image is not already binary, go to *Process* ➔ *Binary* ➔ *Make Binary* (Fig. 5A purple box). Go to *Analyze* ➔ *Analyze Particles*. For "Size (mm^2)" box, input range of Area of callose deposits. Change "Circularity" to 0.25–1. Change "Show" to "Outlines." Check the box for "Summarize" only. Click Okay. Create and Save macro as stated in step 4 and title this macro as "Callose Analyze Particles."

   Note: You can find the Area of callose deposits by using *Freehand Selection Tool* and outlining callose deposit and going to *Analyze* ➔ *Measure*. Measure area of largest and smallest callose deposit to create range. The size range of 0.000005–0.005 mm^2 generally works for the experiments conducted in our lab.

8. Analyze Particles: Create a new folder titled "Analyze Particles Results." Open a single segmentation result .tif file from step 6. Check scale so that it is the same value as segmentation step and apply Global. Go to *Process* ➔ *Batch* ➔ *Macro*. Set the input folder as "Segmentation Results" and set the output folder as "Analyze Particles Results." Click "Open" at the bottom left and select the macro "Callose Analyze Particles." Click "Process." Copy the values in Results window and paste into Excel file next to previous Area data.
9. Calculations: To calculate callose deposits/area (callose deposits/mm^2) in the Excel file, take the particle count (found in step 8) values divided by the total area of the leaf tissue (found in step 5).
10. Graphing: Use Column Graph in GraphPad Prism 8 or any other graphing program to graph callose deposits/area (callose deposits/mm^2) on y-axis and

genotypes and treatments on x-axis (Fig. 4C). Use the online tool GraphPad Outlier Calculator (https://www.graphpad.com/quickcalcs/Grubbs1.cfm) to identify and if necessary, exclude outliers within datasets.

11. Statistical Analysis: For experiments with multiple genotypes and treatments, as seen here, use One-way Analysis of Variance (ANOVA) statistical analysis with Tukey multiple comparisons test (Fig. 4C). We use MiniTab for our data analysis, but other statistical tools (e.g., R, PAST4) can be used.

5 Precursor techniques

5.1 Preparing ½ MS + 1% sucrose plates

For a final volume of 700 mL, add 1.51 g Murashige and Skoog (MS) Basal Salt Mixture (Catalog# M5524-50L, Sigma-Aldrich) to 530 mL dH_2O. When completely dissolved, adjust pH to 5.7 using KOH. Bring volume to 630 mL and transfer to 1 L bottle with stir bar. Add 4.2 g BD Difco™ Agar Technical Solidifying Agent (Catalog# DF0812-17-9, Fischer Scientific) and mix gently on stir plate. After sterilizing in an autoclave for 30 min at 103 kPa, 121 °C, let medium cool down to about 60 °C on stir plate. From now on, work under sterile conditions in laminar flow hood. To the solution, add 70 mL of filter-sterilized 10% sucrose (w/v) for a final concentration of 1% sucrose (w/v). Close bottle and stir gently on stir plate. Pour ~32 mL of solution into each of about 20 100 × 100 mm square polystyrene petri dishes. Let plates dry in laminar hood for 30 min or until solidified and cooled down. Store plates wrapped in plastic bags at 4 °C for up to 1 month.

5.2 Preparing elicitation solution

D/PAMPs peptides including active $flg22^{Paer}$ (QRLSTGSRINSAKDDAAGLQIA) and inactive $flg22^{Atum}$ (ARVSSGLRVGDASDNAAYWSIA) are made by Genscript (Piscataway, NJ, USA) at a guaranteed purity of ≥90%. To increase consistency between experiments utilizing different peptide batches, we prepare stock solutions, for which lyophilized flg22 peptide is dissolved in DMSO to a concentration of 10 mM, adjusted to 100% purity. Because of a potential risk of contamination, it is critical to test peptides for purity and cellular responses when preparing peptide stocks (Mueller et al., 2012). All peptide stocks are prepared using filter tips and stored in low-binding tubes as 10–30 µL aliquots at −20 °C. We make single-use small aliquots to avoid multiple freeze-thaw cycle that may cause peptide degradation. For a 100 nM flg22 elicitation solution, we add 10 µL of 100 µM flg22 (1000 × stock solution) to 10 mL of dH_2O (RT) and mix the solution gently. We use serial dilution to prepare different flg22-concentrations to test for dose-dependent callose deposition.

5.3 Preparing aniline blue staining solution

Prepare a 1% aniline blue (w/v) stock solution fresh for each experiment. Dissolve dye at 10 mg/1 mL in 67 mM K_2HPO_4 (pH 12) in a 1.7 mL microcentrifuge tube by vortexing. Before subsequent dilutions, centrifuge the resuspended dye for 2 min at 17,000 $\times g$ to remove any undissolved dye that will appear as bright dots in microscopy and interfere with image quantification. Dilute 1% stock solution to a final concentration of 0.01% aniline blue (w/v). For example, for a 24-well plate, we make 25 mL solution (for 24 + 1 extra well) consisting of 250 μL of 1% aniline blue (w/v) + 24.75 mL of 67 mM K_2HPO_4 (pH 12). Prepare aniline blue solution in darkness OR wrap tubes in aluminum foil because aniline blue may be light sensitive.

6 Conclusion

In this chapter, we provide protocols for aniline blue staining of the cell wall polysaccharide, callose, and subsequent large-scale automated image quantification using Fiji. This quantification platform has the advantage of being an open-source and freely available program. Here, we focus on quantifying flg22-induced callose deposits in Arabidopsis cotyledons and mature leaves, but this automated image quantification workflow can be easily adapted to quantify other plant cellular features such as altered subcellular localization of fluorescently-tagged proteins in plant organelles or assessing cell death in diverse tissues and organisms.

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