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Corresponding Author:	Jacqueline Monaghan CANADA
Corresponding Author's Institution:	
Corresponding Author E-Mail:	jm355@queensu.ca
Order of Authors:	Melissa Bredow
	Irina Sementchoukova
	Kristen Siegel
	Jacqueline Monaghan
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TITLE:

Pattern-Triggered Oxidative Burst and Seedling Growth Inhibition Assays in *Arabidopsis thaliana*

AUTHORS AND AFFILIATIONS:

Melissa Bredow[1], Irina Sementchoukova[1], Kristen Siegel[1], Jacqueline Monaghan[1]

[1]Department of Biology, Queen's University, Kingston, ON, Canada

Corresponding author:
Dr. Jacqueline Monaghan (jm355@queensu.ca)

Email Addresses of Co-Authors:
Melissa Bredow (11mb95@queensu.ca)
Irina Sementchoukova (17is12@queensu.ca)
Kristen Siegel (12krs7@queensu.ca)

KEYWORDS:

Plant immunity, microbe-associated molecular pattern, pattern recognition receptor, reactive oxygen species, seedling growth inhibition, *Arabidopsis thaliana*.

SUMMARY:

This paper describes two methods for quantifying defense responses in *Arabidopsis thaliana* following exposure to immune elicitors: the transient oxidative burst, and the inhibition of seedling growth.

ABSTRACT:

Plants have evolved a robust immune system to perceive pathogens and protect against disease. This paper describes two assays that can be used to measure the strength of immune activation in *Arabidopsis thaliana* following treatment with elicitor molecules. Presented first is a method for capturing the rapidly-induced and dynamic oxidative burst, which can be monitored using a luminol-based assay. Presented second is a method describing how to measure immune-induced inhibition of seedling growth. These protocols are fast and reliable, do not require specialized training or equipment, and are widely used to understand the genetic basis of plant immunity.

INTRODUCTION:

To perceive and defend against pathogens, plants have evolved membrane-bound pattern recognition receptors (PRRs) that detect conserved microbial molecules outside the cell known as microbe-associated molecular patterns (MAMPs)[1]. The binding of MAMPs to their cognate PRRs initiates protein kinase-mediated immune signaling resulting in broad-spectrum disease resistance[2]. One of the earliest responses following PRR activation is the phosphorylation and activation of integral plasma membrane Respiratory Burst Oxidase Homolog (RBOH) proteins that catalyze the production of extracellular reactive oxygen species (ROS)[3, 4]. ROS play an important role in establishing disease resistance, acting both as secondary messengers to propagate immune signaling as well as direct antimicrobial agents[5]. The first observation of an immune-

elicited oxidative burst was described using potato tubers of cv. Rishiri following *Phytophthora infestans* inoculation[6]. ROS production has been evaluated in several plant species using leaf discs[7], cell suspension cultures[8], and protoplasts[6]. Described here is a simple method for assaying pattern-triggered ROS production in leaf discs of *Arabidopsis thaliana* (*Arabidopsis*).

As a response to MAMP perception, activated RBOH proteins catalyze the production of superoxide radicals (O_2^-), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) that are converted into hydrogen peroxide (H_2O_2) in the extracellular space[9]. H_2O_2 can be quantified by luminol-based chemiluminescence in the presence of the oxidizing agent horseradish peroxidase (HRP)[10]. HRP oxidizes H_2O_2 generating a hydroxide ion (OH^-) and oxygen gas (O_2) which react with luminol to produce an unstable intermediate that releases a photon of light[10]. Photon emission can be quantified as relative light units (RLUs) using a microplate reader or imager capable of detecting luminescence, which has become standard pieces of equipment in most molecular laboratories. By measuring the light produced over a 40-60-minute interval, a transient oxidative burst can be detected as early as 2-5 minutes after the elicitor treatment, peaking at 10-20 minutes, and returning to basal levels after ~60 minutes[11]. The cumulative light produced over this time course can be used as a measure of immune strength corresponding to the activation of RBOH proteins[12]. Conveniently, this assay does not require specialized equipment or cumbersome sample preparation.

Peaking shortly after MAMP detection, the oxidative burst is considered an early immune response, along with MAPK activation and ethylene production[5]. Later immune responses include transcriptional reprogramming, stomatal closure, and callose deposition[2, 5]. Prolonged exposure to MAMPs continually activates energetically-costly immune signaling resulting in the inhibition of plant growth, indicative of a trade-off between development and immunity[13]. Pattern-triggered seedling growth inhibition (SGI) is widely used to assess immune output in *Arabidopsis* and has been integral to the identification of several key components of immune signaling including PRRs[14–16]. Therefore, this paper additionally presents an assay for the pattern-triggered SGI in *Arabidopsis*, whereby, seedlings are grown in multi-well plates containing standard media or media supplemented with an immune elicitor for 8-12 days and then weighed using an analytical scale.

To demonstrate how ROS and SGI assays can be used to monitor PRR-mediated signaling, three genotypes that represent varying immune outputs were chosen: (1) the wild type *Arabidopsis* accession Columbia (Col-0), (2) the dominant-negative *bak1-5* mutant in which the multi-functional PRR co-receptor Brassinosteroid Insensitive 1-Associated Kinase 1 (BAK1) is non-functional in immune signaling[17,18], and (3) the recessive *cpk28-1* mutant, which lacks the regulatory protein Calcium-Dependent Protein Kinase 28 (CPK28) and displays heightened immune-triggered responses[19,20]. ROS and SGI assays are presented in response to a synthetically-produced elf18 peptide epitope of bacterial Elongation Factor Tu (EF-Tu), recognized in *Arabidopsis* by the PRR EF-Tu Receptor (EFR)[15]. These protocols can be used with other immune elicitors such as the bacterial motility protein flagellin[14] or endogenous Peptide Elicitor Proteins (AtPeps)[16], however, it should be noted that plant responsiveness differs

depending on the elicitor[21]. Together, ROS and SGI assays can be used for the quick and quantitative assessments of early and late PRR-mediated responses.

PROTOCOL:

1. Detection of ROS burst in *Arabidopsis* leaf discs following immune elicitation

1.1. Plant growth and maintenance.

1.1.1. To synchronize germination, stratify *Arabidopsis* seeds by suspending approximately 50 seeds in 1 mL of sterile 0.1% agar [w/v] and store at 4 °C (no light) for 3-4 days.

NOTE: Stratify a wild-type background control (for example, Col-0) and genotypes with high and low immune outputs (for example, *cpk28-1* and *bak1-5,* respectively) to serve as internal controls.

1.1.2. Sow seeds on soil and germinate under standard short-day conditions (22 °C, 10 h light, 150 μE/m^2/s light intensity, and 65-70% relative humidity).

1.1.3. After approximately 7 days, use forceps to transplant individual seedlings to new pots, separated by at least 4 cm to permit full rosette development. Transplant 6-12 seedlings per genotype and return to standard short-day conditions. Regularly water and fertilize plants (1 g/L of 20-20-20 fertilizer every 2 weeks).

1.2. Collect leaf discs in 96-well plates for overnight recovery.

1.2.1. At 4-5 weeks post-germination (**Figure 1A**), use a sharp 4 mm biopsy punch to remove one leaf disc per plant, avoiding the mid-vein and being careful to limit wounding (**Figure 1B**). Place leaf discs in an unused 96-well luminometer plate containing 100 μL of ddH_2O with the adaxial side facing upwards to prevent desiccation[22] (**Figure 1C**). If assessing multiple elicitors, remove 1 leaf disc from the same leaf for each elicitor treatment.

NOTE: Sample leaves that are fully expanded, third to fifth from the top of the rosette, and approximately the same size and age to limit variability. If possible, cut leaf discs in half using a razor blade prior to overnight recovery, as this increases the surface area exposed to elicitor solution[23].

1.2.2. Recover overnight at room temperature to prevent the interference of ROS produced by wounding during leaf disc collection. Cover plates with a lid to prevent evaporation.

1.3. Perform the elicitor treatment and measure ROS production.

1.3.1 Program the plate reader prior to adding the reaction solution, as this will reduce the time between ROS burst initiation and the first measurements recorded. Use a commercial software

that is appropriate for the plate reader. In our case (see **Table of Contents**), click **Settings**, and select the **LUM96** Cartridge and the **Kinetic** Read Type. Click the **Read Area** category and drag to select a subset of wells or the entire plate to be read. Under the **PMT and Optics** tab, set the integration time to 1000 ms. Under the **Timing** tab, set the **Total Run Time** to 40-60 min and the **Interval** to 2 min.

1.3.2. Remove water from each well using a multi-channel pipette.

NOTE: Do not puncture leaf discs, as this may cause wounding stress and result in more variable ROS outputs.

1.3.3. Prepare a reaction solution containing 100 µM luminol, 10 µg/mL HRP, and the desired concentration of elicitor (elf18 at 1 nM, 10 nM, 100 nM, or 1000 nM) in sterile ddH_2O using 10 mL of solution for one 96-well plate.

NOTE: Dissolve lyophilized peptides in sterile H_2O in low-binding tubes to make a 10 mM stock, flash-freeze in liquid N_2, and store at -80 °C. When ready to use, dilute stocks in sterile H_2O to generate a 100 µM working stock and store at -20 °C.

1.3.4. Use a multi-channel pipette to dispense 100 µL of the reaction mixture to each well, adding the solution to all leaf discs of the same treatment at the same time[22]. Include a control reaction (no elicitor) for each genotype to assess basal ROS levels in the absence of elicitation. Immediately measure light emission for all wavelengths in the visible spectrum using a microplate reader.

NOTE: Prepare and apply the reaction solution under low light, as luminol and HRP are light-sensitive reagents. Keep reagents on ice or at -20°C unless in use.

1.3.5. Measure light emission with a 1000 ms integration time in 2 min intervals over a 40-60 min period in a microplate reader in order to capture the dynamic oxidative burst (**Figure 1D**).

NOTE: Use a longer integration time to improve assay sensitivity[11], while ensuring that all samples in a 96-well plate can be measured within one interval.

1.4. Data interpretation.

1.4.1. For each genotype, use a spreadsheet application to calculate the average photon count and standard error at each time point, and display ROS production as a function of time using a scatter plot.

$$Average\ Photon\ Count = \frac{\Sigma\ (Photon\ Count\ of\ 6-12\ leaf\ discs)_t}{number\ of\ leaf\ discs}$$

Where t = each time point

1.4.2. Alternatively, sum the photon counts for each leaf disc and present the average of these values for each genotype using a bar graph with standard error bars or a box and whisker plot.

$$Cumulative\ Photon\ Count = average(\Sigma(Photon\ Count\ of\ each\ leaf\ disc)_{t0-t40})$$

2. Seedling Growth Inhibition Assay

2.1. Sterilize seeds and sow on Murashige and Skoog (MS) plates.

2.1.1. Prepare sterile half-strength (0.5x) MS medium (2.16 g/L) containing 0.8% agar [w/v]. Pour media into plates (90 x 15 mm) in a laminar flow hood.

2.1.2. Sterilize approximately 100 seeds in a microcentrifuge tube by washing with 1 mL of 70% ethanol for 2 min and remove by aspiration. Add 1 mL of 40% bleach and gently rock for 17 mins at room temperature. Remove bleach by aspiration and wash three times in 1 mL of sterile water for 5 min. Resuspend in 1 mL of sterile 0.1% agar.

NOTE: Alternatively, use a chlorine gas seed sterilization protocol[24].

2.1.3. Sow approximately 100 seeds per genotype on MS agar plates using a pipette and seal with micropore tape in a laminar flow hood.

NOTE: Avoid placing seeds in close proximity as this will make transplanting seedlings difficult later.

2.1.4. Stratify seeds by placing plates at 4 °C (no light) for 3-4 days to synchronize germination (**Figure 2A**).

2.2. Transplant seedlings into 48-well plates containing bacterial elicitor peptides.

2.2.1. After stratification, move plates to light under short day conditions (22 °C, 10 h light, 150 µE/m^2/s light intensity, and 65-70% relative humidity) for 3-4 days to allow germination.

2.2.2. In a laminar flow hood, prepare elicitor peptide dilutions (0 nM, 1 nM, 10 nM, 100 nM, and 1000 nM) in sterile 0.5x MS liquid media containing 1% sucrose [w/v], using 25 mL of MS for each 48-well plate. Prepare plates by pipetting 500 µL of MS liquid medium or MS medium containing peptides per well. If available, use a repeat pipettor for plate preparation to expedite the process.

NOTE: For each genotype, grow seedlings in MS without elicitor to account for any inherent differences in seedling growth.

2.2.3. Using sterile forceps carefully transplant one seedling to each well of the same size and age, ensuring that there is no damage to the seedling or breakage to the root and that the root

is submerged in media. For each genotype, transplant a minimum of 6-8 seedlings into each peptide dilution (**Figure 2B**).

NOTE: Transplant seedlings at 4 days post-germination, as short roots are easier to manipulate, and older seedlings may yield less optimal results.

2.2.4. Seal plates with micropore tape and move back to light under standard short-day conditions (22 °C, 10 h light, 150 μE/m^2/s light intensity, and 65-70% relative humidity). Allow seedlings to grow for 8-12 days.

2.3. Determine percent growth inhibition.

2.3.1. Carefully remove seedlings from 48-well plates and dry by dabbing on paper towel. Weigh seedlings on an analytical scale and record values. If available, use an analytical scale equipped with USB output to record fresh weight values on a spreadsheet. Before weighing seedlings, take a photo to visually display growth inhibition (**Figure 2C**).

2.3.2. Determine percent growth inhibition of elicitor-treated seedlings compared to seedlings grown in MS only (**Figure 2D**) as follows:

$$\%\ growth\ inhibition = average\left(\frac{fresh\ weight\ of\ each\ elicitor\ treated\ seedling}{average\ fresh\ weight\ of\ MS\ control\ seedlings}\right)$$

2.3.3. Plot data using a bar graph with standard error bars or using a box and whisker plot to better display inter-experimental variance.

REPRESENTATIVE RESULTS:

Mutant *cpk28-1*[19, 25] and *bak1-5*[17,18] plants were used to demonstrate expected outcomes for genotypes with high and low immune responses, respectively, in oxidative burst and SGI assays relative to a wild-type background control (Col-0). To assess dose-dependent effects, a 10-fold peptide dilution series (1-1000 nM) of elf18 was used. As expected, *cpk28-1* loss-of-function lines had a higher cumulative (**Figure 3A**) and average (**Figure 3B**) ROS burst compared to Col-0, whereas *bak1-5* displayed reduced ROS production at concentrations between 10 nM and 1000 nM (**Figure 3**). Expected differences in SGI could be discerned between all genotypes grown in 100 nM and 1000 nM elf18 (**Figure 4A**), which could also be visually observed in the 1000 nM elf18 treatment (**Figure 4B**). Characteristic of high immune signaling, *cpk28-1* mutants were markedly smaller than Col-0 when grown in 1000 nM elf18, while *bak1-5* mutants displayed weak growth inhibition relative to Col-0 due to disrupted MAMP detection.

FIGURE AND TABLE LEGENDS:

Figure 1. Luminol-based oxidative burst assay following immune induction in *Arabidopsis*. (A) Grow plants on the soil in short day conditions for 4-5 weeks. (B) Use a 4 mm biopsy punch to

collect leaf discs from each plant and recover overnight in ddH_2O. (C) Add reaction solution (100 µM luminol, 10 µg/mL HRP, and the desired concentration of elicitor) and measure light emission over 40-60 min, in 2-min intervals with an integration time of 1000 ms. (D) Determine average photon counts for each genotype relative to Col-0 (shown in green), a high ROS control such as *cpk28-1* (shown in purple), and a low ROS control such as *bak1-5* (shown in orange).

Figure 2. Elicitor-induced seedling growth inhibition assay in *Arabidopsis*. (A) Sow seedlings on MS agar and grow for 3-4 days under standard short-day conditions. (B) Transplant seedlings to 48-well plates containing MS medium or MS containing different concentrations of elf18. (C) After 8-12 days, visually assess the seedling size and then measure fresh weight using an analytical scale to determine percent growth inhibition.

Figure 3. Representative elf18-induced oxidative burst in three *Arabidopsis* genotypes. Four-week-old plants were treated with a dilution series of elf18 (0 nM 'mock', 1 nM, 10 nM, 100 nM, and 1000 nM). Col-0 represents the wild-type background control, with *cpk28-1* and *bak1-5* representing high and low ROS phenotypes, respectively. (A) Total photon count represented as relative light units following elf18 treatment (n=6 leaf discs from independent plants). Statistical differences are represented by lower-case letters and were calculated using a one-way ANOVA with a post-hoc Tukey's Honest Significant Difference test (p<0.05). (B) Average photon count, represented as relative light units, over 40 min following elf18 treatment (n=6 leaf discs from independent plants). Error bars represent standard error of the mean. Similar results were obtained in two of three experiments.

Figure 4. Representative elf18-induced seedling growth inhibition in three *Arabidopsis* genotypes. Wild-type (Col-0), *cpk28-1*, and *bak1-5* seedlings were grown in a 10-fold dilution series (0-1000 nM) of elf18. (A) Percent growth inhibition was calculated by comparing the weight of individual seedlings grown in elf18 (n=6 individual seedlings) to the average weight of seedlings of the same genotype grown in MS only ('mock') (n=6 individual seedlings) over a 10-day period. Statistical differences are represented by lower-case letters and were calculated using a one-way ANOVA with a post-hoc Tukey's Honest Significant Differences test (p<0.05). (B) Visual demonstration of SGI in two representative seedlings of Col-0, *cpk28-1,* and *bak1-5* in response to increasing concentrations of elf18. Similar results were obtained in three of four experiments.

DISCUSSION:

This paper describes two methods for assaying pattern-triggered immune responses in *Arabidopsis*, offering quantitative approaches to evaluating immune output without the use of specialized equipment. In combination, assaying pattern-triggered ROS and SGI can be used to assess short- and long-term responses to microbe perception, respectively.

The major limitation of the oxidative burst assay is variability. For reasons that are not completely understood, absolute RLUs often differ by an order of magnitude between experiments. Because between-experiment variability is high, it is advisable to include internal reference controls with high (*e.g., cpk28-1*) and low (*e.g., bak1-5*) oxidative bursts in addition to a wild-type control (*e.g.,* Col-0). However, measures can be taken to increase reproducibility between experiments.

Environmental conditions such as temperature, humidity, photoperiod, and light intensity should be identical between replicates. The age and health of plants should also be considered when sampling. Leaf discs can be collected from plants that are between 4 and 7 weeks of age grown under short day conditions (6-10 hours of light). Anecdotally, the most consistent results were found with plants older than 6 weeks post germination but not yet flowering or senescing. It is important to grow plants in clean environmental chambers so that they are not exposed to common glasshouse pests such as powdery mildew or chewing insects. Since seedlings for SGI are grown in sterile MS media, and for a shorter time, environmental fluctuations are largely not a concern. However, variation can occur if seedlings become damaged while transplanting, if seedlings selected for elicitor treatments are different sizes, or if the growth media becomes contaminated. It is recommended to include the internal reference control genotypes described above in SGI experiments as well.

Elicitor concentration is another important consideration when conducting immune assays. Elicitors are perceived by PRRs resulting in a rapid and robust ROS burst peaking 10-20 minutes after elicitor treatment (**Figure 2B**). However, the magnitude of the burst is dependent on both the elicitor as well as the plant genotype. Therefore, an elicitor dose series, as presented in **Figures 3** and **4**, is recommended in order to identify a suitable elicitor concentration. Pattern-triggered ROS can also be assayed by inoculating leaf discs with live microbes[23, 26] or microbial extracts[26–28]. For example, transient and dose-dependent ROS bursts can be detected in *Arabidopsis* in response to virulent pathovars of *Pseudomonas syringae*, peaking at 35-40 minutes and reaching basal levels approximately 70 mins after elicitation[23, 29]. In response to avirulent bacteria[29] or the fungal pathogen *P. infestans*[30, 31], a second more pronounced accumulation of ROS is produced that can be monitored 6-10 hours after inoculation. For weaker elicitors, such as fungal chitin[32] or chitosan[33], more sensitive luminescent indicators can be used, such as the luminol derivative L-012, however, the background signal produced is often higher[32, 34]. Importantly, the plant ecotype will also dictate its responsiveness to specific elicitors[35, 36]. For example, while bacterial flagellin is capable of eliciting immune responses in several *Arabidopsis* ecotypes including Col-0, the Wassilewskija (Ws-0) ecotype expresses a non-functional variant of the PRR Flagellin-Sensing 2 (FLS2) and is therefore insensitive to flagellin[14, 37].

Immune-induced ROS can also be observed in leaf discs of other dicotyledonous plants including *Brassica napus*[38], tomato[39], *Nicotiana benthamiana*[22, 40, 41], and several other Solanaceous species[41]. Additional luminol-based ROS detection assays have been developed for dicotyledonous plants using tissue extracts[10], cell suspension cultures[8, 42], and protoplasts[43], and are particularly useful in systems where leaf disc protocols are not effective[42]. For example, elicitor-induced ROS bursts have been described using cell suspension cultures in rice[42, 44] and wheat[45, 46], as well as the gymnosperm *Araucaria angustifolia*[47] and the moss *Physcomitrella patens*[48]. SGI has not been used as broadly to measure immune signaling. However, growth inhibition in response to elicitors has been demonstrated in *N. benthamiana*[41] and *B. napus*[38, 49]. Rapid growth inhibition has also been demonstrated in *P. patens* in response to fungal chitin occurring within 2 mins of exposure, which can be observed using time-lapse photography[48].

With modification, both SGI and oxidative burst assays can be used for high-throughput screens

to identify immune regulators. For example, mutagenized populations *of Arabidopsis* can be grown on MS medium and flooded with elicitors to identify insensitive mutants[15, 50–53]. Alternatively, mutagenized populations can be assessed for elicitor-triggered ROS, which has been successfully executed with both leaf-discs[54] and whole seedlings grown on MS plates[19]. Another useful screening method is the transient expression of proteins in *N. benthamiana* for ROS analysis prior to the development of transgenic overexpression lines[22, 40, 55]. However, intra-experimental variation is higher than in stable *Arabidopsis* lines due to differential protein expression in *N. benthamiana* leaves[22], although this can be partially mitigated by infiltrating reference controls on the same leaf as experimental samples.

In summary, immune-induced oxidative burst and SGI assays are quick and reliable methods for assessing PRR-mediated signaling in *Arabidopsis.* These methods can be extended to other systems and used for large-scale screens to uncover novel immune regulators.

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None.

REFERENCES:

1. Couto, D.E., Zipfel, C. Regulation of pattern recognition receptor signalling in plants. *Nature Reviews Immunology.* **16**, 537-552 (2016).
2. Boller, T., Felix, G. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annual Review of Plant Biology*. **60**, 379-406 (2009).
3. Marino, D., Dunand, C., Puppo, A., Pauly, N. A burst of plant NADPH oxidases. *Trends in Plant Science*. **56** (8), 1472-1480 (2012).
4. Kadota, Y., Shirasu, K., Zipfel, C. Regulation of the NADPH Oxidase RBOHD during Plant Immunity. *Plant and Cell Physiology*. **56** (8), 1472-1480 (2015).
5. Yu, X., Feng, B., He, P., Shan, L. From chaos to harmony: responses and signaling upon microbial pattern recognition. *Annual Review of Phytopathology*. **55**, 109-137 (2017).
6. Doke, N. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. *Physiological Plant Pathology*. **23** (3), 345-357 (1983).
7. Bindschedler, L. V. *et al.* Peroxidase-dependent apoplastic oxidative burst in Arabidopsis required for pathogen resistance. *The Plant Journal*. **47** (6), 851-863 (2006).
8. Keppler, L.D. Active Oxygen Production During a Bacteria-Induced Hypersensitive Reaction in Tobacco Suspension Cells. *Phytopathology*. **110** (3), 759-763 (1989).
9. Wrzaczek, M., Brosché, M., Kangasjärvi, J. ROS signaling loops - production, perception,

394 regulation. *Current Opinion in Plant Biology*. **16** (5), 575-582, (2013).
395 10. Warm, E., Laties, G.G. Quantification of hydrogen peroxide in plant extracts by the
396 chemiluminescence reaction with luminol. *Phytochemistry*. **21** (4), 827-831, (1982).
397 11. Trujillo, M. Analysis of the lmmunity-Related Oxidative Bursts by a Luminol-Based Assay.
398 *Methods in Molecular Biology*. **1398**, 323–329 (2016).
399 12. Nühse, T. S., Bottrill, A, R., Jones, A. M. E., Peck, S. C. Quantitative phosphoproteomic
400 analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune
401 responses. *The Plant Journal.* **51** (5), 931-940.
402 13. Belkhadir, Y., Yang, L., Hetzel, J., Dangl, J.L., Chory, J. The growth-defense pivot: Crisis
403 management in plants mediated by LRR-RK surface receptors. *Trends in Biochemical Sciences*. **39**
404 (10), 447-456 (2014).
405 14. Gómez-Gómez, L., Felix, G., Boller, T. A single locus determines sensitivity to bacterial
406 flagellin in Arabidopsis thaliana. *The Plant Journal*. **18** (3), 277-284 (1999).
407 15. Zipfel, C. *et al.* Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts
408 Agrobacterium-Mediated Transformation. *Cell*. **125** (4), 749-760 (2006).
409 16. Krol, E. *et al.* Perception of the Arabidopsis danger signal peptide 1 involves the pattern
410 recognition receptor AtPEPR1 and its close homologue AtPEPR2. *Journal of Biological Chemistry*.
411 **285** (18), 13471-13479 (2010).
412 17. Schwessinger, B. *et al.* Phosphorylation-dependent differential regulation of plant
413 growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. *PLoS*
414 *Genetics*. **7** (4), e1002046 (2011).
415 18. Roux, M. *et al.* The *Arabidopsis* Leucine-Rich Repeat Receptor–Like Kinases BAK1/SERK3
416 and BKK1/SERK4 Are Required for Innate Immunity to Hemibiotrophic and Biotrophic Pathogens.
417 *The Plant Cell*. **23** (6), 2440-2455 (2011).
418 19. Monaghan, J. *et al.* The calcium-dependent protein kinase CPK28 buffers plant immunity
419 and regulates BIK1 turnover. *Cell Host and Microbe*. **16** (5), 605-615 (2014).
420 20. Wang, J. *et al.* A Regulatory Module Controlling Homeostasis of a Plant Immune Kinase.
421 *Molecular Cell*. **69** (3), 493–504 (2018).
422 21. Mott, G.A. *et al.* Genomic screens identify a new phytobacterial microbe-associated
423 molecular pattern and the cognate Arabidopsis receptor-like kinase that mediates its immune
424 elicitation. *Genome Biology*. **17**, 98 (2016).
425 22. Sang, Y., Macho, A.P. Analysis of PAMP-Triggered ROS Burst in Plant Immunity. *Methods*
426 *in Molecular Biology*. **1578**, 143–153 (2017).
427 23. Smith, J.M., Heese, A. Rapid bioassay to measure early reactive oxygen species production
428 in Arabidopsis leave tissue in response to living Pseudomonas syringae. *Plant Methods*. **10** (1), 6
429 (2014).
430 24. Lindsey, B.E., Rivero, L., Calhoun, C.S., Grotewold, E., Brkljacic, J. Standardized Method for
431 High-throughput Sterilization of Arabidopsis Seeds. *Journal of Visualized Experiments*. **128** (2017).
432 25. Matschi, S., Werner, S., Schulze, W.X., Legen, J., Hilger, H.H., Romeis, T. Function of
433 calcium-dependent protein kinase CPK28 of Arabidopsis thaliana in plant stem elongation and
434 vascular development. *The Plant Journal*. **73** (6), 883–896 (2013).
435 26. Felix, G., Duran, J.D., Volko, S., Boller, T. Plants have a sensitive perception system for the
436 most conserved domain of bacterial flagellin. *The Plant Journal*. **18** (3), 265-276 (2002).
437 27. Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., Felix, G. The N Terminus of

Bacterial Elongation Factor Tu Elicits Innate Immunity in Arabidopsis Plants. *The Plant Cell.* **16** (12), 3496-3507 (2004).
28. Zipfel, C. *et al.* Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature*. **428** (6984), 764-767 (2004).
29. Mur, L.A.J., Kenton, P., Draper, J. In planta measurements of oxidative bursts elicited by avirulent and virulent bacterial pathogens suggests that H2O2 is insufficient to elicit cell death in tobacco. *Plant, Cell and Environment*. **28** (4), 548-561 (2005).
30. Kobayashi, M. *et al.* Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. *The Plant Cell*. **19** (3), 1065–1080 (2007).
31. Yoshioka, H. *et al.* Induction of Plant gp91 *phox* Homolog by Fungal Cell Wall, Arachidonic Acid, and Salicylic Acid in Potato. *Molecular Plant-Microbe Interactions*. **14** (6), 725-736 (2001).
32. Klauser, D., Flury, P., Boller, T., Bartels, S. Several MAMPs, including chitin fragments, enhance AtPep-triggered oxidative burst independently of wounding. *Plant Signaling and Behavior*. **8** (9), e25346 (2013).
33. El Gueddari, N.E., Rauchhaus, U., Moerschbacher, B.M., Deising, H.B. Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. *New Phytologist*. **156** (1), 103-112 (2002).
34. Daiber, A. *et al.* Detection of superoxide and peroxynitrite in model systems and mitochondria by the luminol analogue L-012. *Free Radical Research*. **38** (3), 259-269 (2004).
35. Bauer, Z., Gómez-Gómez, L., Boller, T., Felix, G. Sensitivity of Different Ecotypes and Mutants of Arabidopsis thaliana toward the Bacterial Elicitor Flagellin Correlates with the Presence of Receptor-binding Sites. *Journal of Biological Chemistry*. **276** (49), 45669-45676 (2001).
36. Vetter, M.M. *et al.* Flagellin perception varies quantitatively in arabidopsis thaliana and its relatives. *Molecular Biology and Evolution*. **29** (6), 1655-1667 (2012).
37. Chinchilla, D. The Arabidopsis Receptor Kinase FLS2 Binds flg22 and Determines the Specificity of Flagellin Perception. *The Plant Cell*. **18** (2), 465-476 (2006).
38. Lloyd, S.R., Schoonbeek, H., Trick, M., Zipfel, C., Ridout, C.J. Methods to Study PAMP-Triggered Immunity in *Brassica* Species. *Molecular Plant-Microbe Interactions*. **27** (3), 286-295 (2014).
39. Clarke, C., Vinatzer, B. Characterizing the Immune-Eliciting Activity of Putative Microbe-Associated Molecular Patterns in Tomato. *Methods in Molecular Biology*. **1578**, 249–261 (2017).
40. Gimenez-Ibanez, S., Hann, D.R., Chang, J.H., Segonzac, C., Boller, T., Rathjen, J.P. Differential Suppression of Nicotiana benthamiana Innate Immune Responses by Transiently Expressed Pseudomonas syringae Type III Effectors. *Frontiers in Plant Science*. **9**, 688 (2018).
41. Wei, Y. *et al.* The Ralstonia solanacearum csp22 peptide, but not flagellin-derived peptides, is perceived by plants from the Solanaceae family. *Plant Biotechnology Journal*. **16** (7), 1349-1362 (2018).
42. Melcher, R.L.J., Moerschbacher, B.M. An improved microtiter plate assay to monitor the oxidative burst in monocot and dicot plant cell suspension cultures. *Plant Methods*. **12**, 5 (2016).
43. Perraki, A. *et al.* Phosphocode-dependent functional dichotomy of a common co-receptor in plant signalling. *Nature*. **561** (7722), 248–252 (2018).
44. Yamaguchi, K., Kawasaki, T. Chitin-Triggered MAPK Activation and ROS Generation in Rice Suspension-Cultured Cells. *Methods in Molecular Biology*. **1578**, 309–316 (2017).

45. Ortmann, I., Conrath, U., Moerschbacher, B.M. Exopolysaccharides of Pantoea agglomerans have different priming and eliciting activities in suspension-cultured cells of monocots and dicots. *FEBS Letters*. **580** (18), 4491-4494 (2006).
46. Ortmann, I., Sumowski, G., Bauknecht, H., Moerschbacher, B.M. Establishment of a reliable protocol for the quantification of an oxidative burst in suspension-cultured wheat cells upon elicitation. *Physiological and Molecular Plant Pathology*. **64** (5) 227-232 (2004).
47. Dos Santos, A.L.W., El Gueddari, N.E., Trombotto, S., Moerschbacher, B.M. Partially acetylated chitosan oligo- and polymers induce an oxidative burst in suspension cultured cells of the gymnosperm Araucaria angustifolia. *Biomacromolecules*. **9** (12), 3411-3415 (2008).
48. Bressendorff, S., Rasmussen, M., Petersen, M., Mundy, J. Chitin-Induced Responses in the Moss Physcomitrella patens. *Methods in Molecular Biology*. **1578**, 317–324 (2017).
49. Lloyd, S.R., Ridout, C.J., Schoonbeek, H. Methods to Quantify PAMP-Triggered Oxidative Burst, MAP Kinase Phosphorylation, Gene Expression, and Lignification in Brassicas. *Methods in Molecular Biology.* **1578**, 325–335 (2017).
50. Gómez-Gómez, L., Boller, T. FLS2: An LRR Receptor-like Kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Molecular Cell*. **5** (6), 1003-1011 (2000).
51. Li, J. *et al.* Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR. *Proceedings of the National Academy of Sciences of the United States of America*. **106** (37), 15973-15978 (2009).
52. Lu, X. *et al.* Uncoupling of sustained MAMP receptor signaling from early outputs in an Arabidopsis endoplasmic reticulum glucosidase II allele. *Proceedings of the National Academy of Sciences of the United States of America*. **106** (52), 22522–22527 (2009).
53. Nekrasov, V. *et al.* Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO Journal*. **28** (21), 3428-3438 (2009).
54. Boutrot, F. *et al.* Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. *Proceedings of the National Academy of Sciences of the United States of America*. **107** (32), 14502-14507 (2010).
55. Kadota, Y. *et al.* Direct Regulation of the NADPH Oxidase RBOHD by the PRR-Associated Kinase BIK1 during Plant Immunity. *Molecular Cell*. **54** (1), 43–55 (2014).

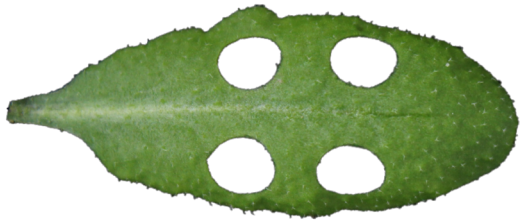
Figure 1

A

4-5 week old plants
(with 6-12 individuals per genotype)

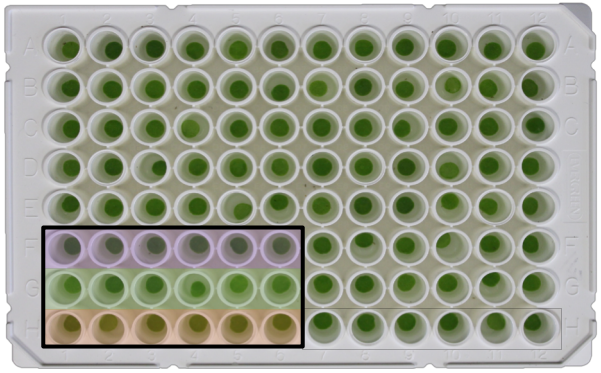
Collect
leaf discs

B



Recover
overnight in
ddH_2O

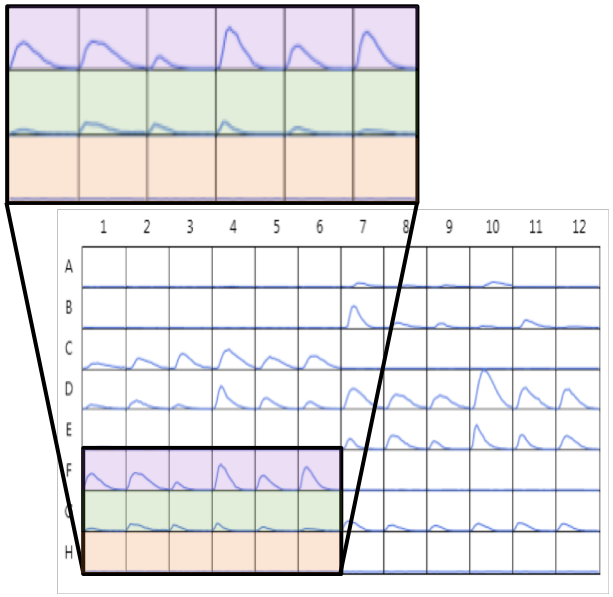
C

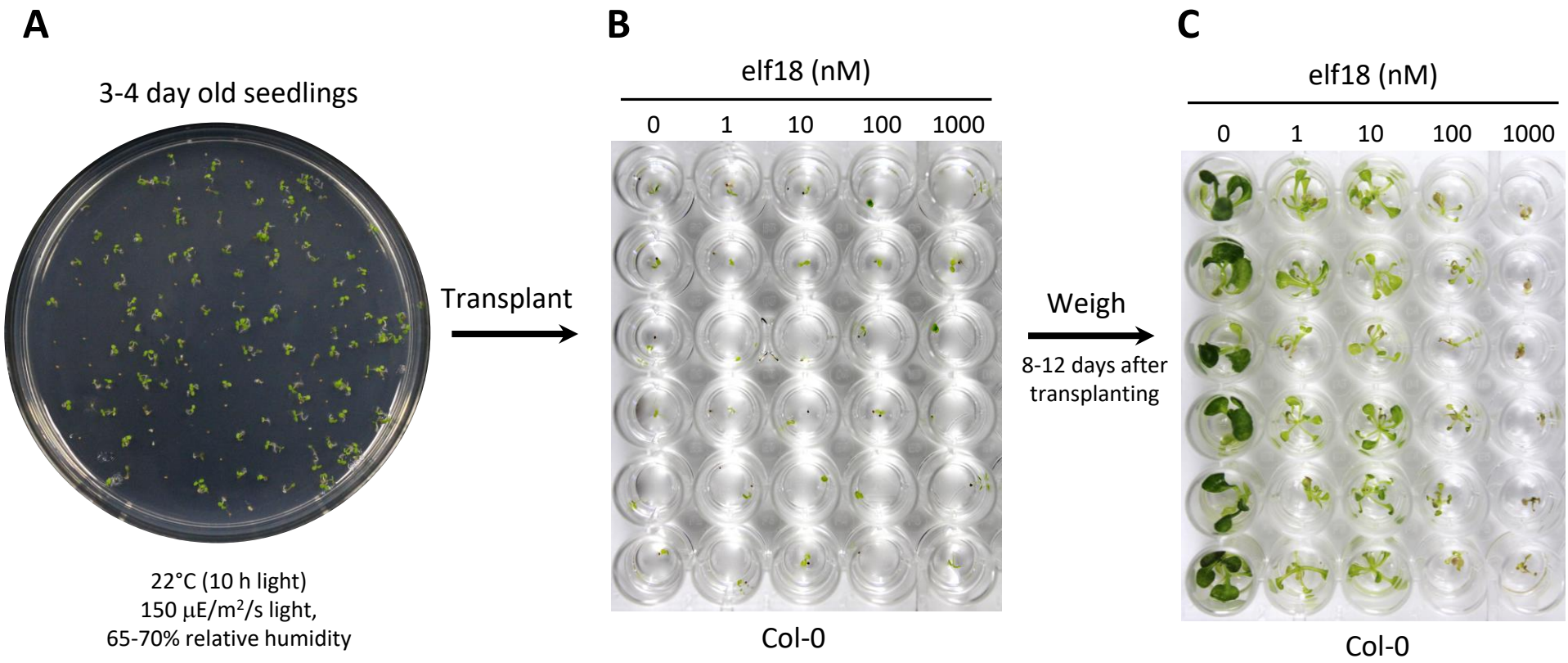


Col-0 *cpk28-1* *bak1-5*

Add reaction
solution and
measure light
emission

D





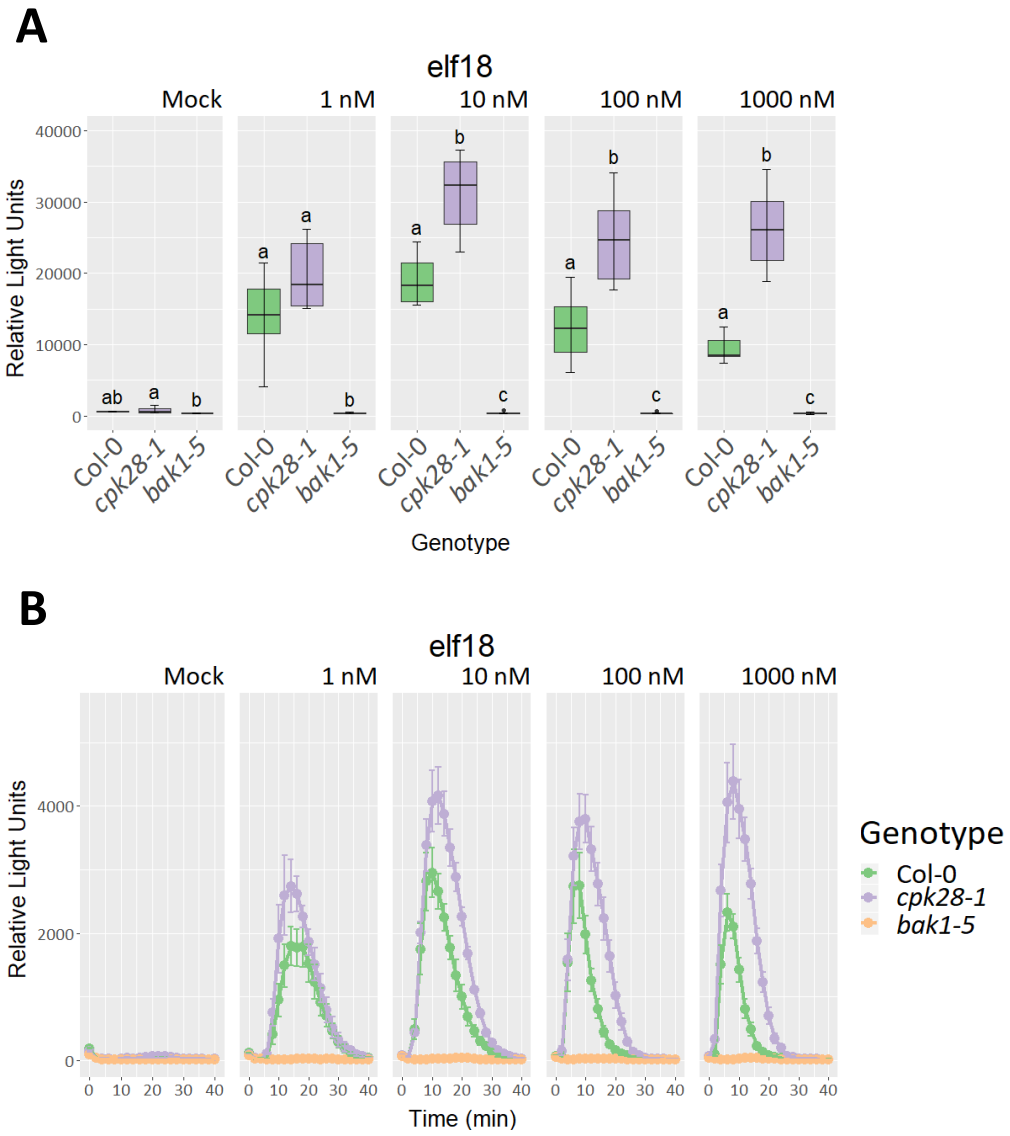


Figure 4

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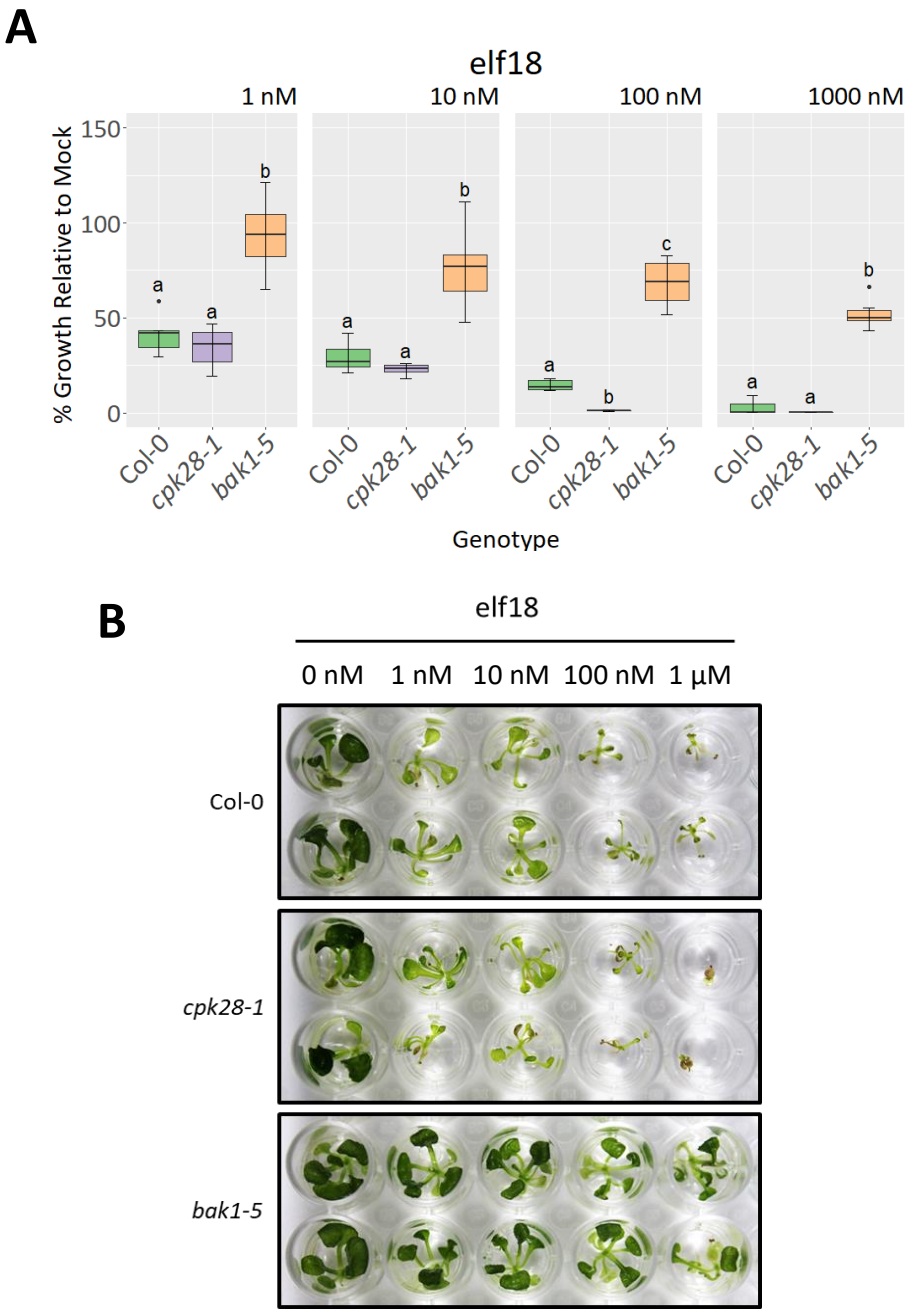

Table of Materials

Click here to access/download;Table of Materials;Materials Sheet Revised.xlsx

Name of Material/ Equipment	Company	Catalog Number
20-20-20 Fertilizer	Plant Prod	10529
4 mm Biopsy Punch	Medical Mart	232-33-34-P
48-Well Sterile Plates with Lid	Sigma-Aldrich	CLS3548
Analytical Scale with Draft Sheid	VWR	VWR-225AC
BioHit mLine Mechanical 12 Multichannel Pipette (30-300 uL)	Sartorius	725240
elf18 (Ac-SKEKFERTKPHVNVGTIG)	EZ Biolab	cp7211
Forceps	Fisher Scientific	22-327379
Horseradish Peroxidase	Sigma-Aldrich	P6782
Luminol	Sigma-Aldrich	A8511
Murisage and Skoog Basal Salts	Cedarlane Labs	MSP09-100LT
Soil	SunGrow Horticulture	Sunshine Mix #1
SpectraMax Paradigm Multi Mode Microplate Reader with LUM Module	Molecular Devices	Must request a quote
Sucrose	Sigma-Aldrich	S0389-1KG
White Polystyrene 96-Well Plates	Fisher Scientific	07-200-589

Comments/Description

Mix 1g/L in water and apply to plants every 2 weeks for optimal growth.
A cork borer set with a 0.125 cm^2 surface area can also be used.

Any standard analytical scale can be used for growth inibition assays, however, a direct computer output is optimal.
Any multichannel pipette can be used, as can a single pipetter if necessary.
Store 10 mM stock peptide at -80C in low protein binding tubes. When thawed, store 100 uM working stock at -20C.

Dissolve in pure water. Store at -20C and away from light.

Dissolve in DMSO. Store at -20C and away from light.
Store at 4C.

Other soil types can also be used to grow Arabidopsis. Mix with water when filling pots.

Any plate reader capable of detecting luminescence can be used for these assays.
Store at room temperature.

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Author(s): Melissa Bredow, Irina Sementchoukova, Kristen Siegel, Jacqueline Monaghan

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Name:	Jacqueline Monaghan
Department:	Biology Department
Institution:	Queen's University
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Dear Editor,

We thank-you for your helpful editorial comments, and appreciate critiques made by two external reviewers. Please see our point-by-point responses to each comment below (in blue). All changes have been tracked in our revised manuscript.

On behalf of all authors,
Jacqueline Monaghan

Editorial comments:

Changes to be made by the Author(s):

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- All authors have proofread the manuscript.

2. Please provide an email address for each author.

- We have provided e-mail addresses for all authors.

3. Please provide at least 6 keywords or phrases.

- We have now included the following keywords in addition to those already provided: *Arabidopsis thaliana* and pattern recognition receptor.

4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

- We removed all personal pronouns from the text.

5. 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. For example: plantprod 20-20-20, sunshine mix, Microsoft excel, etc.

- We removed “Sunshine Mix #1” from Step 1.1.2, rephrased Step 1.1.3 to exclude reference to PlantProd (“***with 1 g/L of 20-20-20 fertilizer every 2 weeks***”), and rephrased Step 2.3.1 to exclude reference to Microsoft Excel (“...***on a spreadsheet***”).

- Sunshine Mix #1 and PlantProd fertilizers have been added to the Table of Materials and Reagents.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

- All text is now written in imperative tense and phrases “should be”, “could be”, and “can be” have been removed.

7. The Protocol should contain only action items that direct the reader to do something.

- Protocol text now only states action items.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

9. 1.1.3: how do you transplant? Please quantify sufficient spacings

- We have expanded on how to transplant seedlings:

  “After approximately 7 days, ***use forceps to transplant individual seedlings, separated by at least 4 cm*** *to permit full rosette development.”*

10. 1.2: How do you identify leaf disc?

- Step 1.2 is a section heading and therefore does not contain any specific details however this information can be found in step 1.2.1:

  “...use a sharp 4 mm biopsy punch to remove one leaf disc per plant, avoiding the mid-vein and being careful to limit wounding.”

11. 1.2.2: When was an elicitor added? What concentration?

- Since this section of the protocol details leaf disc collection and recovery we have not yet described adding elicitor treatments to leaf discs. However, we now include the specific elicitor concentrations used, in step 1.3.2:

  “Prepare a reaction solution containing 100 µM luminol, 10 µg/mL HRP, and the desired concentration of elicitor ***(For example, elf18 at 1 nM, 10 nM, 100 nM, or 1000 nM)*** *in sterile ddH_2O.”*

12. 1.2.3: Is this done before adding the elicitor?

- Section 1.2 describes leaf disc collection and recovery prior to measuring elicitor-induced ROS production. To make this clearer, we have now changed

the title of section 1.3 from “Measure ROS response following elicitor treatment” to “Add elicitor treatment and measure ROS production”.

13. 1.3.2: What is the volume of the solution prepared? Please mention the elicitor and concentration used in your study. What is the control in this case? Do you use multichannel pipette for this step? What absorbance is used to measure the light emission?

- This section has been rephrased to address reviewer comments as follows:
    - *“Prepare a reaction solution containing 100 µM luminol, 10 µg/mL HRP, and the desired concentration of elicitor* ***(elf18 at 1 nM, 10 nM, 100 nM, or 1000 nM)*** *in sterile ddH_2O* ***using 10 mL of solution for one 96-well plate.****”*
    - We have now included information on controls in step 1.1.1:

      “Note: Stratify a wild-type background control (for example, Col-0) and genotypes with high and low immune outputs (for example, cpk28-1 and bak1-5, respectively) to serve as internal controls.”

    - We have also now included information on controls in step 1.3.2:

      *“*Note: Include a control reaction (no elicitor) for each genotype to assess basal ROS levels in the absence of elicitation.

    - A multichannel pipette is indeed used to distribute the reaction solution to the 96-well plate, and is referenced in this step: “*Use a multichannel pipette to dispense 100 µL of reaction mixture to each well…*”
    - We have now included the spectrum at which light emission is measured as “***all wavelengths in the visible spectrum***”.

14. 1.3.3. How is this set up?

- Microplate reader set-up will vary based on the manufacturer and model. Since JoVE manuscripts cannot contain commercial language we have not included this information.

15. 1.4.1 How do you calculate the average photon count? Is this displayed on the software?

- We have now included formulas for calculating both average and cumulative photon count in Step 1.4.1.

16. 2.2. Please include volume and concentration for the same.

- It is now specified in step 2.2.2 to prepare 25 mL of peptide solution for each 48-well plate. Recommended peptide dilutions are also provided (1 nM, 10 nM, 100 nM, and 1000 nM).

17. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next.

- We have highlighted all critical steps for the video protocol.

18. Please expand the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.

- We have expanded the results section to better describe how the results are interpreted and how they demonstrate the technique as follows:

  "cpk28-1[19, 26] and bak1-5[17] were used to ***demonstrate expected outcomes for loss-of-function lines with high and low immune responses, respectively, in oxidative burst and SGI assays relative to a wild-type background control (Col-0). To identify an appropriate elicitor concentration to distinguish between immune outputs, a*** *10-fold peptide dilution series (1-1000 nM) of elf18 was used. Concentrations between 10 nM and 1000 nM could differentiate between all three genotypes in oxidative burst assays using elf18 (Figure 3)* ***and would therefore act as suitable elicitor treatments. As expected, cpk28-1 loss-of-function lines had a higher cumulative and average ROS burst than Col-0, indicative of a high immune output, whereas bak1-5 displayed reduced oxidative burst production due to low immune signaling (Figure 3). Differences in SGI could be discerned between all genotypes*** *with 100 nM and 1000 nM elf18 (Figure 4),* ***which could also be visually observed in the 1000 nM elf18 treatment*** *(Figure 4B).* ***Characteristic of high immune signaling, cpk28-1 loss-of-function lines were markedly smaller than Col-0 with continuous MAMP detection, while bak1-5 mutants displayed weak growth inhibition relative to Col-0 due to a low immune output****."*

19. Please format the units as mg/mL and not mg ml-1. Please leave a single space between number and units.

- We have ensured that there are no superscripts notations for units in the text or the material sheet. Note that we also changed light intensity units from the format μE m^{-2} s^{-1} to the format μE/m^2/s.

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21. Please alphabetically sort the materials table.

- The materials table has been sorted alphabetically.

Reviewers' comments:

Reviewer #1:
This manuscript describes two very useful methods for monitoring the production of ROS and measuring SGI. The authors provide very detail and complete protocols which others can follow the methods easily without doubt. However, there are some places need to improve to make the manuscript more complete.

Minor Concerns:

1.Line 122,"Collect leaf discs in 96-well plates for overnight recovery. "Why not place the leaf discs in reaction solution after removing from the plant but recovering in ddH2O overnight?

- We include this information in step 1.2.2: *“Recover overnight at room temperature to prevent the interference of ROS produced by wounding during leaf disc collection.”*

2.Line 127,"adaxial side facing upwards", please explain why.

- We have modified this statement to clarify that leaf discs do not need to be submerged but rather need to be in contact with the water surface to prevent desiccation during recovery: *“Place leaf discs in an unused 96-well luminometer plate containing 100 µL of ddH_2O adaxial side facing upwards* ***to prevent desiccation*** *(Figure 1C)”.*

- We reference the following article: Sang and Macho (2016) Methods in Molecular Biology (https://www.ncbi.nlm.nih.gov/pubmed/26867635)

3.Line 137,"Cover plates with a lid" explain whether this lid needs to protect from light, because light could affect the production of ROS

- Normal light conditions as well as low light conditions are conducive to recovery prior to oxidative burst assays. We have now included the following statement to clarify that lids are used to prevent evaporation of water during overnight recovery: *“Recover overnight at room temperature to prevent the interference of ROS produced by wounding during leaf disc collection.* ***Cover plates with a lid to prevent evaporation.****”*

4.Line 164 to 165,"Measure light emission with a 1000 ms integration time in 2 min intervals over a 40-60 min period in a microplate reader". please explain why to choose 1000 ms integration time in 2 min intervals over a 40-60 min.

- We have modified this sentence as follows: *"Measure light emission with a 1000 ms integration time in 2 min intervals over a 40-60 min period in a microplate reader* ***in order to capture the dynamic oxidative burst*** *(Figure 1D)."*

- We have also included a note which provides further detail on the choice of integration time:

  "Note: Use a longer integration time to improve assay sensitivity[11], while ensuring that all samples in a 96-well plate can be measured within one interval."

Reviewer #2:
The manuscript entitled "Pattern-triggered oxidative burst and seedling growth inhibition assays in Arabidopsis thaliana" describes two method for evaluating early and late immune responses; reactive oxygen species (ROS) production and seedlings growth inhibition (SGI), respectively, triggered by pattern-recognition receptor activation in Arabidopsis.

The manuscript is written very well and concisely with various useful information. All the experimental steps are also explained very well. With many valuable information written in the manuscript, not only the protocol part but also introduction and discussion, the manuscript have value for researchers exploring plant immunity. I support publishing this article after addressing the following minor points;

Point 1
The arrangement of Figures appears to be odd since the numbering does not match to the order in which they appear in the text. (Fig 1 > Fig. 3 > Fig. 2 > Fig. 4 in the text) This point should be considered according to the JoVE guideline.

- Thank you for catching this- we have corrected the figure numbering in the text.

Point 2, page 2, PROTOCOL 1.2.2
The leaf disks are usually hydrophobic. Is there any method to "ensure that leaf discs are submerged" without physical wounding damages?

- We have modified this statement to clarify that leaf discs do not need to be submerged but rather simply need to be in contact with the water surface to prevent desiccation: *"Place leaf discs in an unused 96-well luminometer plate containing 100 µL of ddH_2O adaxial side facing upwards* ***to prevent desiccation*** *(Figure 1C)".*

- We reference the following article: Sang and Macho (2016) Methods in Molecular Biology (https://www.ncbi.nlm.nih.gov/pubmed/26867635)

Point 3, page 3, PROTOCOL 1.2.3
Is the plate stored for recovery in a dark condition or in a normal short day growth chamber? Since immunity is affected very much by the presence of light, it would be useful to describe precisely about the "recovering" condition.

- Normal light conditions as well as low light conditions are conducive to recovery prior to oxidative burst assays. We have now included the following statement to clarify that lids are used to prevent evaporation of water during overnight recovery: *"Recover overnight at room temperature to prevent the interference of ROS produced by wounding during leaf disc collection.* ***Cover plates with a lid to prevent evaporation.****"*

Point 4, page 3, PROTOCOL 1.3.2
Does the 10 mM peptide stock solution require sterilization, for example, by sterile filtration? Is the use of sterile ddH20 for dissolving the lyophilized peptide sufficient to avoid contamination? It might be useful to explain the requirement of sterilization here clearly because this would be a big issue in the SGI assay that require 8-12 days incubation.

- We have not found it necessary to sterilize peptide solutions by filtration, however, we use sterile water for resuspending the lyophilized solution and we have clarified this in the text.

Point 5, page 5, PROTOCOL 2.2.4
Hydroponic culturing of Arabidopsis seedlings is usually performed with gentle agitation. Does the incubation for 8-12 days here require gentle agitation/shaking of the plate?

- Over the 8-12 days that seedlings are growing in MS liquid media agitation/shaking is not required.