



METHODOLOGY

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Rapid bioassay to measure early reactive oxygen species production in *Arabidopsis* leave tissue in response to living *Pseudomonas syringae*

John M Smith^{1,2,3} and Antje Heese^{1,3*}

Abstract

Background: *Arabidopsis thaliana* and *Pseudomonas syringae* pathovar *tomato* (*Pto*) provide an excellent plant-bacteria model system to study innate immunity. During pattern-triggered immunity (PTI), cognate host receptors perceive pathogen-associated molecular patterns (PAMPs) as non-self molecules. *Pto* harbors many PAMPs; thus for experimental ease, many studies utilize single synthesized PAMPs such as flg22, a short protein peptide derived from *Pseudomonas* flagellin. Flg22 recognition by *Arabidopsis* Flagellin Sensing 2 (FLS2) initiates a plethora of signaling responses including rapid production of apoplastic reactive oxygen species (ROS). Assessing flg22-ROS has been instrumental in identifying novel PAMP-signaling components; but comparably little is known whether in *Arabidopsis*, ROS is produced in response to intact live *Pto* and whether this response can be used to dissect genetic requirements of the plant host and live bacterial pathogens *in planta*.

Results: Here, we report of a fast and robust bioassay to quantitatively assess early ROS in *Arabidopsis* leaves, a tissue commonly used for pathogen infection assays, in response to living bacterial *Pto* strains. We establish that live *Pto* elicits a transient and dose-dependent ROS that differed in timing of initiation, amplitude and duration compared to flg22-induced ROS. Our control experiments confirmed that the detected ROS was dependent on the presence of the bacterial cells. Utilizing *Arabidopsis* mutants previously shown to be defective in flg22-induced ROS, we demonstrate that ROS elicited by live *Pto* was fully or in part dependent on *RbohD* and *BAK1*, respectively. Because *fls2* mutants did not produce any ROS, flagellin perception by FLS2 is the predominant recognition event in live *Pto*-elicited ROS in *Arabidopsis* leaves. Furthermore using different *Pto* strains, our *in planta* results indicate that early ROS production appeared to be independent of the Type III Secretion System.

Conclusions: We provide evidence and necessary control experiments demonstrating that *in planta*, this ROS bioassay can be utilized to rapidly screen different *Arabidopsis* mutant lines and ecotypes in combination with different bacterial strains to investigate the genetic requirements of a plant host and its pathogen. For future experiments, this robust bioassay can be easily extended beyond *Arabidopsis-Pto* to diverse plant-pathosystems including crop species and their respective microbial pathogens.

Keywords: ROS, Reactive oxygen, PAMP, FLS2, RbohD, BAK1, flg22, *Pseudomonas syringae*, DC3000, *hrcC*

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Background

Eukaryotes have developed highly effective immune mechanisms for protection against microbial pathogens using pattern-triggered immunity (PTI) as the first line of defense. Pathogen-associated molecular patterns (PAMPs), also referred to as microbe-associated molecular pattern (MAMPs), are highly conserved and essential molecules common to entire classes of microbes but are absent from the host. Host cells utilize pattern recognition receptors (PRRs) to recognize PAMPs as non-self to initiate a large number of signaling responses that contribute to growth restriction of microbial pathogens [1-3]. To evade these host immune responses, pathogenic microbes express and deliver effector molecules into host cells to interfere with PTI [4]. For example, the virulent model bacterium *Pseudomonas syringae* pathovar *tomato* (*Pto*) DC3000 translocates 28 or more effector proteins into plant cells via the type III secretion system (T3SS), some of which are known to suppress PTI [5-7]. Some effector proteins, however, betray the pathogen due to their direct or indirect recognition by cytosolic host resistant proteins resulting in Effector-Triggered Immunity (ETI) [1,5-8]. Non-pathogenic strains lacking functional T3SS such as *Pto* DC3000 *hrcC* (*Pto hrcC*) do not suppress PTI because of their inability to deliver effectors into host cells [6,8].

In plants, only few PAMP/PRR pairs involved in PTI have been identified and characterized [1,2,9,10]. In the model plant *Arabidopsis thaliana*, the best studied PTI-system is perception of bacterial flagellin by Flagellin Sensing 2 (FLS2), a plasma membrane localized PRR [2,11]. Flagellin is the main proteinaceous component of extracellular flagellum filaments essential for the mobility and ability of bacteria such as *Pto* to infect host plants [12,13]. Binding of flagellin or flg22, a 22-amino acid peptide derived from the consensus sequence for the most highly conserved region in the N-terminus of eubacterial flagellin [14], to the extracellular domain of FLS2 occurs within the plant apoplast and leads to a variety of early and late signaling responses [11,15,16], all of which are dependent on BRI1-Associated Receptor Kinase 1 (BAK1) [17,18]. One of the best characterized and robust early PAMP-signaling events is the rapid and transient accumulation of apoplastic reactive oxygen species (ROS). Assessing PAMP-elicited ROS has proven to be a valuable tool in identifying and characterizing novel PAMP-signaling components and specific amino acids necessary for their function [18-23]. Production of rapid apoplastic ROS in response to PAMP peptides is solely dependent on the plasma membrane localized NADPH respiratory burst oxidase homolog D (RbohD) [22,24,25]. Although the exact role of ROS production in innate immunity remains unclear, ROS initiates a plethora of downstream signaling events, some of which are essential in establishing defense mechanisms to prevent the spread of bacterial pathogens [26,27].

Over the past decade, studies utilizing commercially synthesized flg22 peptide have greatly aided in increasing our insight into early and late signaling events and in identifying required signaling components and their contribution to PTI [2,28]. Other efforts exploited boiled bacterial extract to investigate various PAMP-induced responses in leaves or cultured plant cells [14,29-31]. Boiling bacterial cells results in the release of both extra- and intracellular PAMPs, thus making it difficult to determine the biologically relevant order of PAMP recognition by specific host PRRs. The disadvantage of utilizing cultured cells as opposed to leaf tissue is that because of the unavailability of *Arabidopsis* mutant cell culture lines, cell culture limits the ability to assess the genetic requirement of the plant host for the response of interest. In contrast, the genetic plant host-pathogen interplay between *Arabidopsis* plants and *Pseudomonas* can be interrogated *in planta* due to the availability of large number of *Arabidopsis* ecotypes and mutant lines. When investigating responses induced by living bacteria in plant tissue (*in planta*), most efforts have focused on later responses such as accumulation of the defense hormone salicylic acid (SA) [12-24 hours post infection (hpi)], transcriptional changes of the late gene marker *PRI* (24 hpi) or changes in resistance to bacterial infection measured 3 days post-infection (dpi). Only more recently, attention has been given to identifying early signaling events and their genetic requirements induced by living bacterial pathogens on plant host leaves [32-35], the tissue that serves as the primary source for bacterial pathogen infection.

Here, we describe advancement of a fast and convenient *in planta* bioassay that allows for quantitative assessment of early ROS production in *Arabidopsis* leaf tissue, the primary site of *Pto* infection, induced by living *Pto* strains. Importantly, we provide necessary control experiments showing that *in planta*, early ROS production was dependent on the presence of *Pto* cells. By utilizing *Arabidopsis* mutants previously shown to be affected in flg22-induced ROS production, we demonstrate that early ROS produced in response to live *Pto* strains was fully dependent on *RbohD* and *FLS2* and partially dependent on *BAK1*. No statistical differences were observed between ROS induced by *Pto* DC3000 and *hrcC* cells, thus the virulence-promoting T3SS does not appear to have an influence on early ROS production. Because of the ease in setting up ROS assays in a 96-well plate assay, this quantitative analysis is highly suitable to screen within a relatively short period of time large populations of *Arabidopsis* accession lines in combination with diverse *Pto* mutant strains to define the genetic requirements of host and bacterial pathogen.

Results and discussion

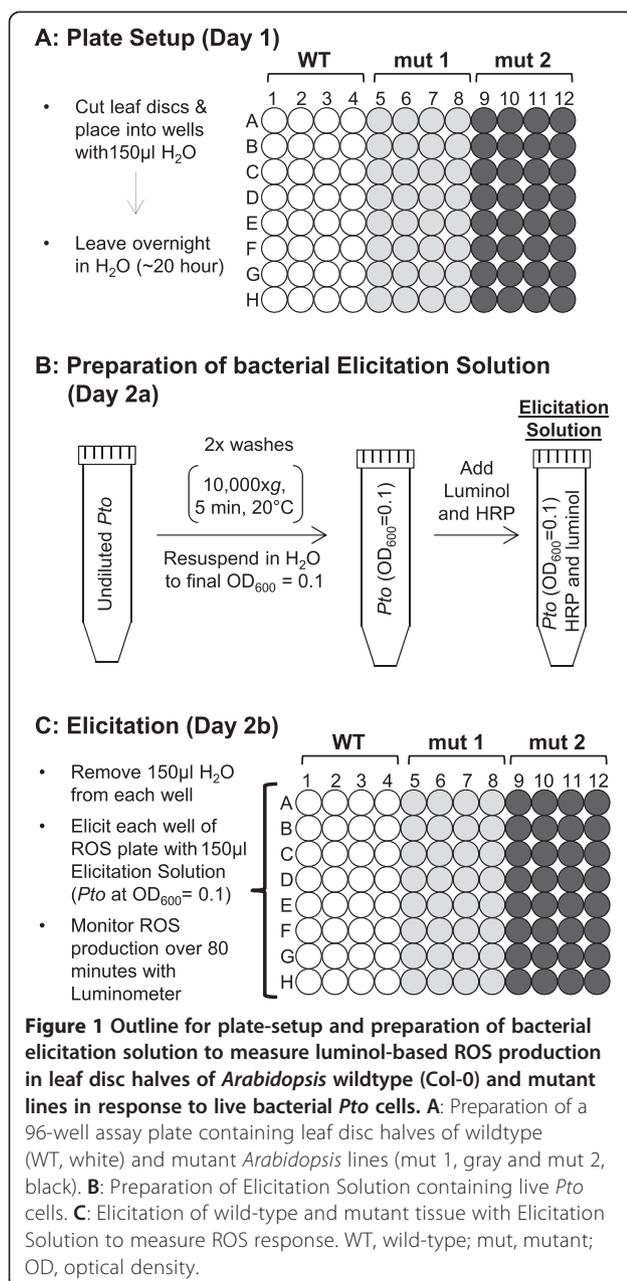
Our goal was to establish a rapid and robust *in planta* method to quantitatively measure early ROS production

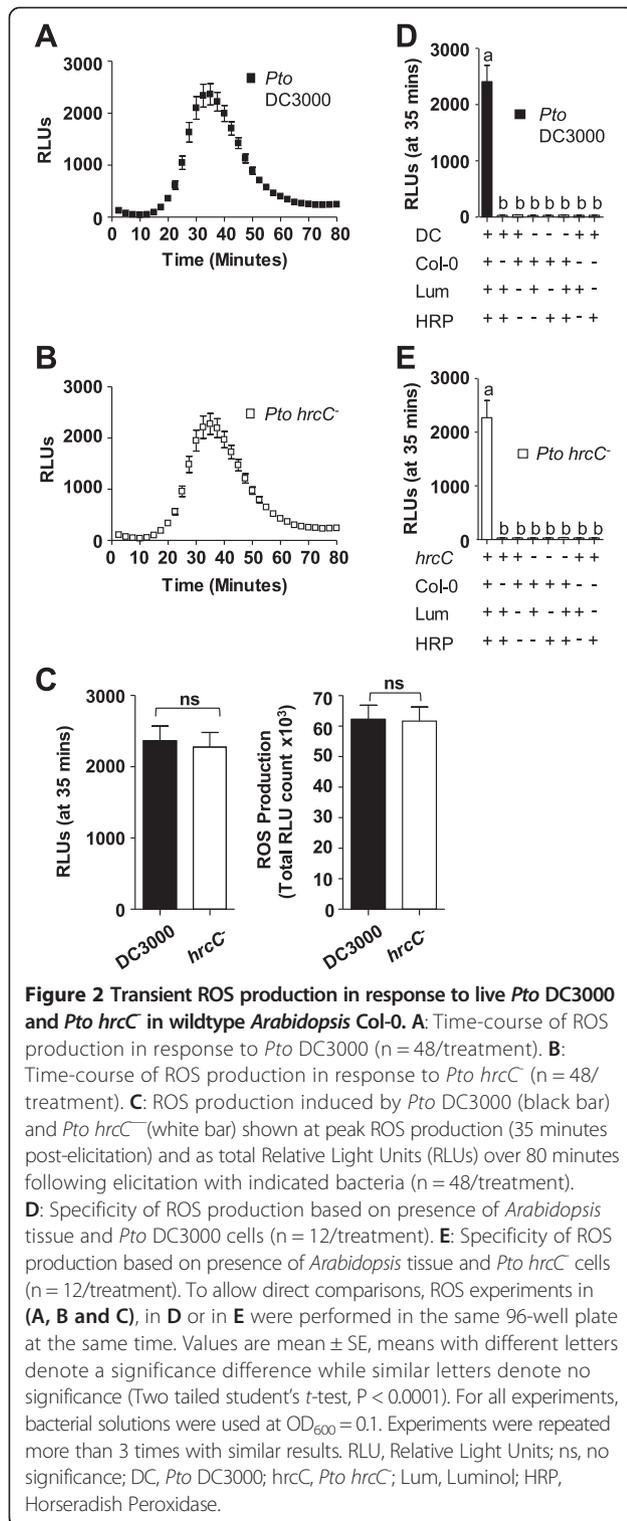
in response to live *Pto* strains in *Arabidopsis* leaves, a tissue commonly used for bacterial infection assays. To this end, we adapted the luminol-based ROS assay used for PAMP-elicitation [18,21,36,37] in that we substituted the synthetic PAMP peptide with bacterial cells of *Pto* strains that were freshly grown for 36-48 hours at 22°C on King's B media (KBM) plates containing appropriate antibiotics [Figure 1; see Methods for details]. In brief, *Arabidopsis* leaf discs obtained from 5 week old plants were cut in half with a sharp razor blade to increase surface area, and each half was placed into a distilled water (dH₂O)-containing well of a 96-well titer plate for at

least 20 hours to reduce wounding effect [Figure 1A]. Immediately prior to elicitation, bacteria were harvested directly from plates [38] and washed twice in sterile dH₂O. After the second wash step, bacterial cells were resuspended in sterile dH₂O [38], and their cell densities (OD₆₀₀) were adjusted between OD₆₀₀ = 0.001 to OD₆₀₀ = 0.1 [Figure 1B]. The OD₆₀₀ can be used as a rapid means to provide an estimation of bacterial cell density (as colony-forming units (cfu)/mL) [38,39]. After Luminol and Horseradish Peroxidase (HRP) was given to the *Pto*-containing solution, this Elicitation Solution was added to *Arabidopsis* leaf halves to measure luminol-based ROS production between 0 and 80 minutes using a luminometer [Figure 1C].

First, we established that virulent live *Pto* DC3000 cells induced ROS-production in wild-type *Arabidopsis* Col-0 leaves. As shown in Figure 2A, ROS production after elicitation with *Pto* DC3000 (OD₆₀₀ = 0.1) was transient, in that increases in ROS was first detected around 20 minutes, peaking around 35-40 minutes and returning to near-basal levels around 70 minutes post-elicitation. Thus, it was delayed compared to that of PAMP peptide flg22-induced ROS which can be detected within 2-4 minutes, peaks at 10-14 minutes and returns to basal levels around 30-35 minutes [Additional file 1]. An explanation for the delayed ROS response to *Pto* DC3000 cells may be that in contrast to pure and short PAMP peptide(s), the PAMP(s) derived from living bacterial cells may not be readily accessible but may need to be released and/or processed for perception by the plant PRR(s) as suggested for flagellin-FLS2 system [40]. The reduced amplitude in response to *Pto* DC3000 cells may be an indication that living bacteria possibly release lower concentrations of PAMP(s) into the host apoplast compared to the concentration of applied synthesized flg22 peptide.

It is also possible that the reduced *Pto* DC3000-induced ROS amplitude may be due to suppression of ROS by bacterial effectors delivered into host cells. To address this hypothesis, we measured ROS production in response to non-pathogenic *Pto hrcC* cells (OD₆₀₀ = 0.1) that lacked functional T3SS and are defective in effector delivery [Figure 2B]. Importantly, ROS induced by *Pto hrcC* was similar in the time of initiation, amplitude and duration compared to that by virulent *Pto* DC3000 [Figure 2B; also see Figure 2C]. ROS production at their peaks (at 35 minutes) and total ROS productions over the 80 minute time-course did not show any statistically significant difference between these two *Pto* strains [Figure 2C], indicating that this *Pto*-elicited ROS production is independent of a functional T3SS. These results also suggest that compared to PAMP-induced ROS, the lower level ROS amplitude in response to *Pto* cells is unlikely due to interference of ROS by bacterial effectors. Similar ROS results were obtained over 75 min time-course after treatment with *Pto avrRpm1*





and *Pto avrRps4*, two avirulent bacterial strains known to inject the avirulence proteins AvrRpm1 and AvrRps4, respectively, into host cells resulting in ETI-dependent responses [5-8]. Comparing *Pto avrRpm1* and *Pto avrRps4*

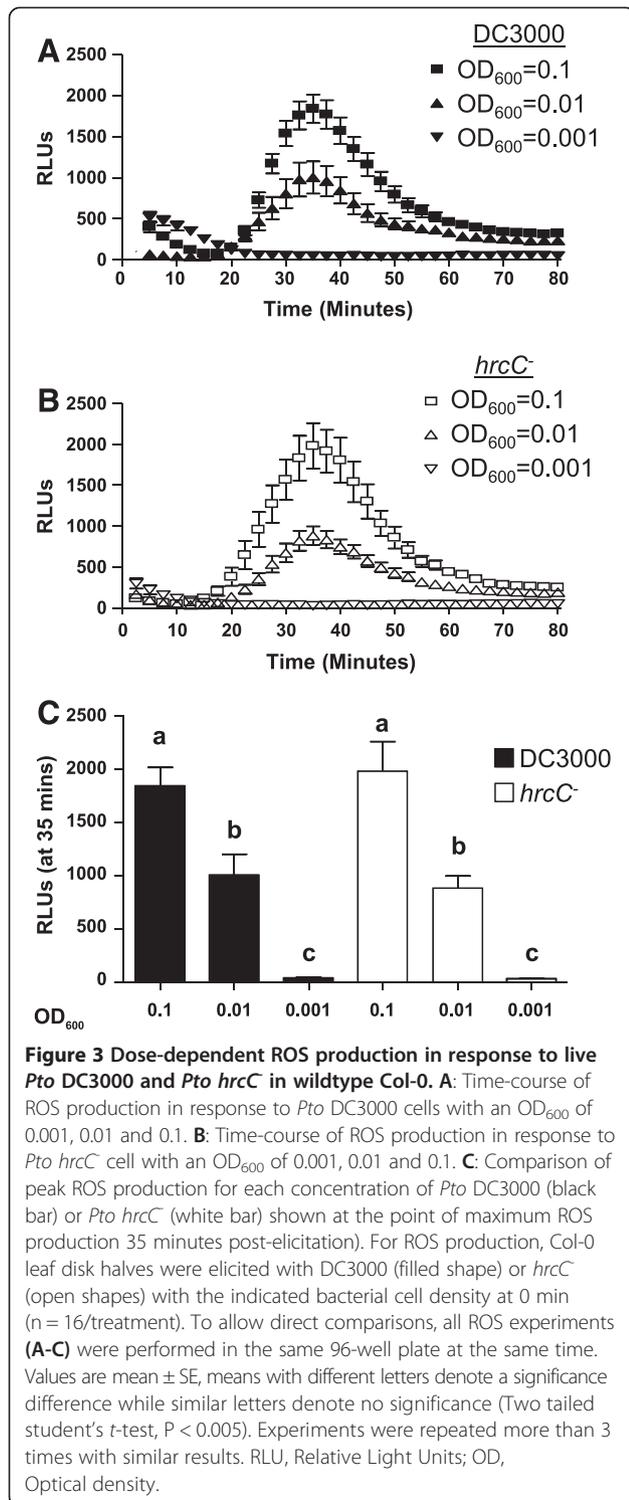
to *Pto* DC3000, no difference in ROS initiation, amplitude and attenuation as well as total ROS production was observed over the 80 min time-course [Additional file 2]. Taken together, our results using virulent, non-pathogenic and avirulent *Pto* strains are in agreement that the observed ROS response was due to PTI-dependent events and are consistent with *Pto* effector delivery into host cells occurring at significantly later times post-infection (> 3 hours) [41] than the ROS response investigated in our study.

In control experiments, ROS production was measured using Elicitation Solutions that contained or lacked *Pto* strain (*Pto*), luminol, HRP and Col-0 leaf discs (Col-0) in different combinations. As evident in Figure 2D and E, ROS was produced only when the *Pto* strain, luminol, HRP and the leaf discs were present. Lack of any of these components did not result in any significant ROS production. No difference in the ROS production was observed in *Pto* strains resuspended in dH₂O or 10 mM MgCl₂ [Additional file 3].

Next, we showed that *Pto*-dependent ROS production was dose-dependent by eliciting Col-0 leaf tissue with different bacterial cell densities between $OD_{600} = 0.001$ and 0.1 [Figure 3]. Either strain, *Pto* DC3000 or *Pto hrcC*, elicited a ROS response in a dose-dependent manner [Figure 3A or B, respectively]. Consistent with Figure 2C, we did not observe any statistically significant difference when comparing ROS production elicited by *Pto* DC3000 and *Pto hrcC* at comparable optical densities [Figure 3C]. Furthermore, *Pto* optical densities of $OD = 0.001$ or below (data not shown) did not elicit any significant detectable ROS. *Pto* cells with an $OD_{600} = 0.1$ yielded the most consistent and robust ROS response, thus we used this bacterial cell density for all subsequent experiments.

To investigate the genetic requirement of the plant host for *Pto*-induced ROS, we made use of previously characterized *Arabidopsis* mutants functioning in or upstream of apoplastic ROS production. A *rbohD* null mutant plant line in Col-0 background [22,25] was used to investigate dependency on *RbohD*, the plasma membrane localized protein previously shown to be the sole NADPH oxidase required for rapid induction of apoplastic ROS after elicitation with PAMP peptides [22,24,26,33]. In response to either live *Pto* DC3000 or *hrcC* cells, no ROS production was detected in *rbohD* compared to wild-type Col-0 for the 80 min time-course [Figure 4A or B, respectively; see also Figure 4C for peak comparison at 35 min post-elicitation]. We conclude that similar to the synthetic flg22 peptide, *Pto* DC3000 or *hrcC* elicited ROS production was fully dependent on *RbohD*. These result also suggested that the ROS measured in our assays was unlikely due to cell death-associated ROS.

Next, we determined whether *Pto*-elicited ROS was dependent on *BAK1*, the receptor-like kinase known to be required very early in initiating signaling after flg22-



elicitation [18,20,42]. Underlining its crucial role in PAMP-signaling and PTI, BAK1 forms a ligand-induced receptor complex with FLS2 within seconds [18,20,42,43]. In response to *Pto* DC3000 or *hrcC*, we observed an increase in ROS production over time in *bak1-4* null

mutant leaf discs; but importantly, the ROS amplitude was significantly reduced compared to Col-0 [Figure 4D or E, respectively]. No statistical significant difference in ROS production was observed in *bak1-4* mutant tissue in response to *Pto* DC3000 or *Pto hrcC* [Figure 4F]. Taken together, these results suggest that *Pto*-elicited ROS was only in part dependent on BAK1. Our studies are consistent with previous reports showing that full signaling responses to the bacterial PAMP peptides flg22 require other proteins in addition to BAK1 [18,20,42].

Previous studies have shown that *Arabidopsis* mutants lacking functional FLS2 receptor are unable to perceive flg22 which results in a complete loss of any flg22/FLS2-dependent signaling event including lack of apoplastic ROS production [16,31,36]. In addition, FLS2-dependent responses contribute to restriction of *Pto* DC3000 growth [31]. However when treated with crude boiled *Pto* DC3000 extracts, *fls2* null mutants are still able to elicit signaling responses including ROS production [29,31]. These results are consistent with the idea that in addition to flagellin (flg22), *Pto* DC3000 contains multiple extra- and intracellular PAMPs that are exposed upon boiling of bacterial strains. To determine whether FLS2 contributed to ROS production induced by intact living *Pto* DC3000 or *hrcC*, we utilized previously characterized *fls2* null mutants [18,31,36]. Interestingly, *fls2* null mutants in a Col-0 background did not elicit any ROS in response to either *Pto* strain [Figure 5A-C]. To confirm this observation, we challenged Wassilewskija (Ws-0), an *Arabidopsis* ecotype that is flagellin-insensitive and considered a natural *fls2* mutant due to an early stop mutation in the *FLS2* gene [31], with *Pto* DC3000 or *Pto hrcC*. Similar to the *fls2* null mutant in Col-0, Ws-0 displayed a complete lack of ROS production in response to either *Pto* strains [Additional file 4]. Based on our studies utilizing two different *Arabidopsis* ecotypes lacking functional FLS2, the flagellin/flg22 receptor FLS2 appeared to be the predominant host receptor responsible for initiating early ROS production in response to intact, living *Pto* DC3000 and *hrcC*. Our results are consistent with observations that in response to intact *Pto* DC3000 and DC3118 at 1 to 2 hours post-treatment, stomatal closure of epidermal leaf peels is entirely dependent on the presence of functional FLS2 [33,34]. Thus, these and our present studies suggest that not all potential *Pto* PAMPs appear to be exposed simultaneously during the initial bacterial infection. Our results further underline the advantages of utilizing living intact *Pto* cells in contrast to crude boiled bacterial extract [14,29,31] when studying early signaling responses induced by bacteria *in planta*. Boiling of bacteria likely releases both extra- and intracellular localized PAMPs simultaneously. In contrast, utilizing intact live bacterial cells enables researchers to determine the biologically relevant contribution and/or order of PAMP recognition by specific host PRRs.

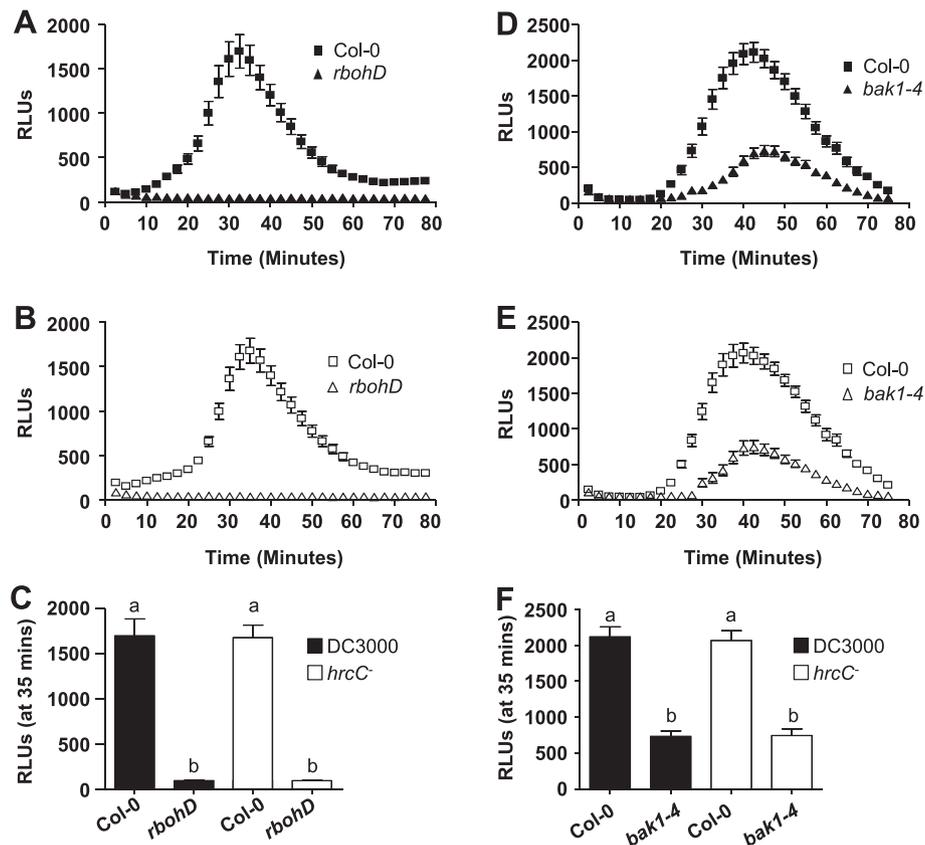


Figure 4 *RbohD* and *BAK1*-dependency of ROS production in response to live *Pto* DC3000 and *Pto hrcC* cells. **A and B:** Time-course of ROS production in response to *Pto* DC3000 (**A**; closed symbols) or *Pto hrcC* (**B**; open symbols) in Col-0 (square) and *rbohD* null mutant (triangle) leaf disc halves ($n = 24/\text{treatment}$). **C:** Comparison of peak ROS production between *Pto* DC3000 or *Pto hrcC* in Col-0 and *rbohD* shown at the point of maximum ROS production (35 minutes post-elicitation from **A** and **B**). **D and E:** Time-course of ROS production in response to *Pto* DC3000 (**D**; closed symbols) or *Pto hrcC* (**E**; open symbols) in Col-0 (square) and *bak1-4* null mutant (triangle) leaf disc halves ($n = 24/\text{treatment}$). **F:** Comparison of peak ROS production between *Pto* DC3000 or *Pto hrcC* in Col-0 and *bak1-4* shown at the point of maximum ROS production (35 minutes post-elicitation from **D** and **E**). To allow for direct comparisons, all ROS experiments shown in (**A**, **B** and **C**) and (**D**, **E** and **F**) were performed in the same 96-well plate at the same time. Values are mean \pm SE, means with different letters denote a significance difference while similar letters denote no significance (Two tailed student's *t*-test, $P < 0.0001$). For all experiments, bacterial solutions were used at $OD_{600} = 0.1$. Experiments were repeated more than 3 times with similar results. RLU, Relative Light Units.

Conclusions

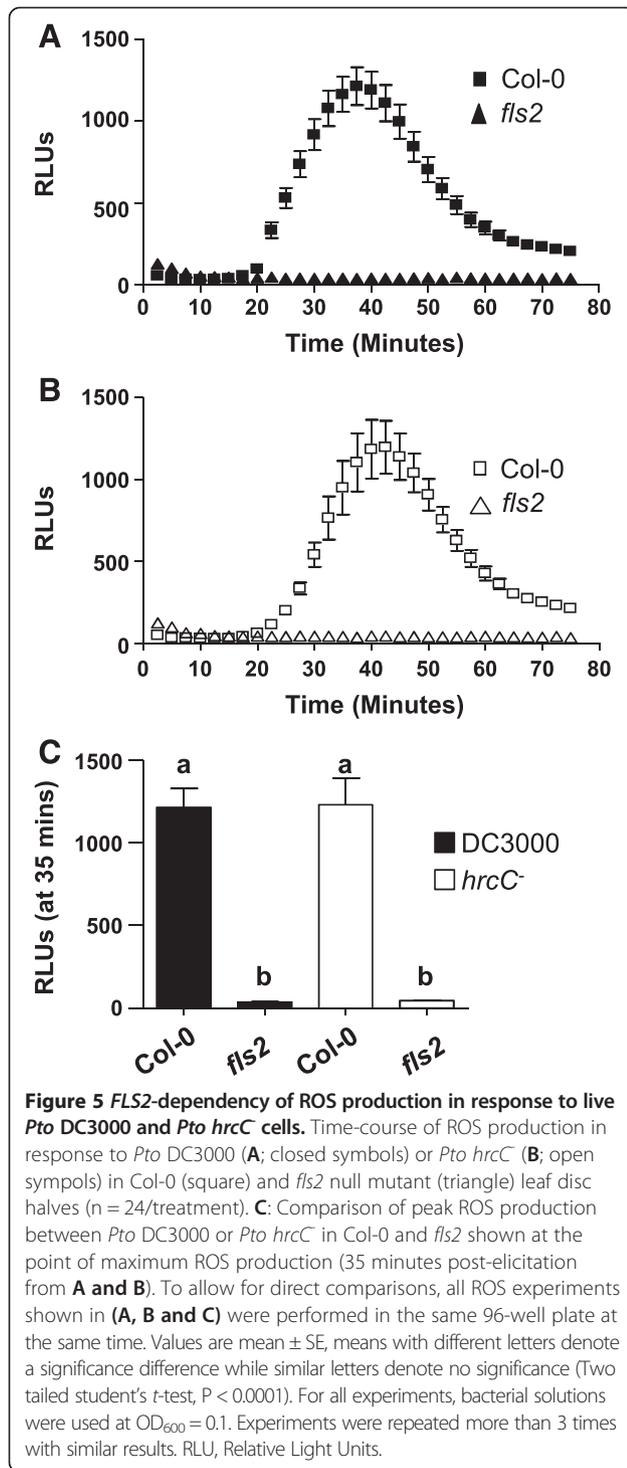
In this study, we report the advancement of a rapid and convenient bioassay allowing the quantitative assessment of ROS production between *Arabidopsis* leaf tissue (the primary site of *Pto* infection) and living *Pto* bacterial strains. Because of the ease in setting up ROS assays in a 96-well plate assay, this quantitative analysis is highly suitable to screen large populations of *Arabidopsis* accession lines in combination with diverse *Pto* mutant strains to define the genetic requirements of host and bacterial pathogen. In future experiments, this ROS bioassay may also allow addressing which PAMP/PRR pair quantitatively contributes to early signaling in response to bacterial pathogens in leaf tissue. Furthermore, the utility of this bioassay can be easily extended beyond *Arabidopsis* and *Pto* to diverse model or crop species and their cognate microbial

pathogen to define components required for early ROS responses.

Methods

Plant material and growth conditions

Arabidopsis seeds were sterilized with 10% bleach + 1% Triton X-100 for 1 hour, rinsed with water and plated aseptically on 0.5% agar containing 2.14 g L^{-1} Murashige and Skoog (MS) salts (Sigma Chemical Company, St. Louis, MO, USA, <http://www.sigmaaldrich.com/>) + 1% sucrose, pH 5.7. Following stratification for 2 days at 4°C , seedlings were germinated in Percival CU-36 L4 growth chambers (Percival, Perry, IA) under continuous light at 22°C [18]. Seven day-old seedlings were transplanted in soil and grown in an 8-h light/16-h dark photoperiod at $82 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Fully expanded rosette leaves were



used from 4-5 week old plants for all ROS experiments. The *Ws-0*, *fls2Δ* (Col-0), *bak1-4* (Col-0), and *rbohD* (Col-0) mutants have been previously described [18,22,25].

Chemicals

Synthetic flg22 peptide [21] was made by GenScript (Scotch Plains, NJ) and used at indicated concentrations.

Horseradish Peroxidase (HRP; Sigma, catalog # P6782) was prepared as a 500x HRP stock solution by dissolving 10 mg/mL in sterile H₂O. Aliquots (10-30 μ L) of the 500x HRP stock solution were stored at -20°C and used at a final concentration of 20 μ g/mL. For the 500x Luminol stock solution, 17 mg Luminol (\geq 97% purity-HPLC; Sigma; catalog # A8511) was completely dissolved in 1 ml of 200 mM KOH and used at a final concentration of 0.2 μ M. For example, 10 μ l of each of the 500x stocks of luminol and HRP were added to 5 ml of resuspended bacterial solution. Because Luminol is light-sensitive, all solutions containing Luminol must be protected from light by wrapping tubes in aluminum foil. The 500x Luminol stock solution is made fresh prior to use and discarded daily.

Bacterial preparation

Two days prior to ROS experiments, *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pto* DC3000) or *Pto hrcC* was streaked from glycerol stocks (stored at -80°C) onto King's B medium (KBM) agar plates containing 50 μ g ml⁻¹ kanamycin and 30 μ g ml⁻¹ rifampicin (*Pto* DC3000) or 30 μ g ml⁻¹ rifampicin (*Pto hrcC*) [38]. KMB plates containing bacteria were incubated for 36 to 48 hours at room temperature. Prior to elicitation, bacteria were scraped from plates and washed twice in sterile dH₂O by repeated centrifugation at 10,000 \times g for 5 minutes. After the second wash step, a 1:10 dilution of the bacterial solution was made and its optical density (OD_{600}) was measured using a spectrophotometer as a means to provide an approximate quantification of bacterial cell density [38,39]. The final bacterial elicitation solution was adjusted to an OD_{600} between 0.001 and 0.1, which under our conditions equated to 1×10^6 to 10^8 colony forming units (cfu)/mL based on serial dilution plating. The OD_{600} of the final bacterial elicitation solution was measured again to ensure accuracy of the dilution. To accurately compare the dose-dependency of the ROS response [Figure 3], bacteria were serially diluted from a stock solution.

Measurement of apoplastic ROS production

One day before the ROS assay, leaf disks (1.1 cm²) from 4-5 week old plants were cut into two equal halves with a sharp razor blade to increase the cellular surface area exposed to elicitation solution, an important step for obtaining reproducible responses with less variability within and between experiment. Each leaf disc half was floated adaxial side up in an individual well of a 96-well microtiter plate (Costar; Fisher Scientific, catalog # 3912) containing 150 μ l dH₂O and then incubated overnight at 22°C in continuous light for 20 to 24 hours to reduce the wounding response. Prior to elicitation, the Elicitation Solution was prepared containing bacteria, Luminol and

HRP. For a 10 ml Elicitation Solution, 20 μ l of 500x HRP stock solution and 20 μ l of the 500x Luminol stock solution is added to 10 ml of bacterial cells that have been already diluted to the desired concentration. For flg22-induced ROS production, flg22 peptide was used instead of bacteria in the Elicitation Solution. All Elicitation Solutions were kept at room temperature. Immediately prior to the elicitation, the incubating dH₂O solution was carefully removed from each well avoiding any tissue damage or desiccation. Then using a multichannel pipetman, 100 μ l of the Elicitation Solution was quickly added to each well containing leaf disc half. For Luminol-based ROS production, the plate was placed without delay into a GloMax[®] 96-well microplate luminometer (Promega, Madison, USA) to measure *Pto*-induced ROS production between 0 and 80 minutes.

Statistical analysis

Each experiment was done at least 3 independent times with similar results. Statistical significances based on unpaired two sample *t*-test were determined with Graph Pad Prism4 software (La Jolla, CA).

Additional files

Additional file 1: Dose-dependent ROS production in response to synthetic flg22 peptide. A: Time-course of ROS production in response to 1, 10 and 100 nM flg22 in wild-type Col-0 leaf disc halves (n = 16/treatment). B: Bar graph representation of peak ROS production for each flg22 concentration from experiment shown in A. To allow direct comparisons, all ROS experiments were performed in the same 96-well plate at the same time. Values are mean \pm SE, means with different letters denote a significance difference (Two tailed student's *t*-test, $P < 0.0001$). Experiment was repeated more than 3 times with similar results. RLU, Relative Light Units.

Additional file 2: Transient ROS production in response to live *Pto* DC3000, *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRps4* in Col-0. A: Time-course of ROS production between *Pto* DC3000 and *Pto* DC3000 *avrRpm1* (n = 24/treatment). B: Timecourse of ROS production between *Pto* DC3000 and *Pto* DC3000 *avrRps4* (n = 24/treatment). C: ROS production induced by *Pto* DC3000 (black bar), *Pto* DC3000 *avrRpm1* (white bar) and *Pto* DC3000 *avrRps4* (gray bar) shown at peak ROS production (35 minutes post-elicitation) and as total Relative Light Units (RLUs) over 75 minutes following elicitation with indicated bacteria (n = 24/treatment). To allow direct comparisons, ROS experiments in (A, B and C) were performed in the same 96-well plate at the same time. Values are mean \pm SE, means with different letters denote a significance difference while similar letters denote no significance (Two tailed student's *t*-test, $P \geq 0.4084$). For all experiments, bacterial solutions were used at OD600 = 0.1. Experiments were repeated more than 3 times with similar results. RLU, Relative Light Units.

Additional file 3: ROS production in response to live *Pto* DC3000 cells resuspended in dH₂O or 10 mM MgCl₂. A: Time-course of ROS production in response to *Pto* DC3000 resuspended in sterile dH₂O (closed symbols) or 10 mM MgCl₂ (open symbols) in Col-0 leaf disc halves (n = 24/treatment). B: Comparison of peak ROS production between *Pto* DC3000 resuspended in sterile dH₂O or 10 mM MgCl₂ at the point of maximum ROS production (35 minutes post-elicitation from A). To allow for direct comparisons, all ROS experiments shown in (A and B) were performed in the same 96-well plate at the same time. Values are mean \pm SE, means with different letters denote a significance difference while similar letters denote no significance (Two tailed student's *t*-test, $P = 0.59$). For all experiments, bacterial solutions were used at OD600 = 0.1. Experiment was repeated more than 3 times with similar results. RLU, Relative Light Units.

Additional file 4: Lack of ROS production in *fls2* mutant of different *Arabidopsis* ecotypes in response to live *Pto* DC3000 and *Pto* *hrcC* cells. A and B: Time-course of ROS production in response to *Pto* DC3000 (A; closed symbols) or *Pto* *hrcC* (B; open symbols) in leaf disc halves of Col-0 (square), *fls2* (Col-0) null mutant (triangle) and *Ws-0* (upside down triangle) (n = 16/treatment). C: Comparison of peak ROS production between *Pto* DC3000 or *Pto* *hrcC* in Col-0, *fls2* (Col-0) and *Ws-0* shown at the point of maximum ROS production (35 minutes post-elicitation from A and B). To allow for direct comparisons, all ROS experiments shown in (A, B and C) were performed in the same 96-well plate at the same time. Values are mean \pm SE, means with different letters denote a significance difference while similar letters denote no significance (Two tailed student's *t*-test, $P < 0.0001$). For all experiments, bacterial solutions were used at OD600 = 0.1. Experiments were repeated more than 3 times with similar results. RLU, Relative Light Units.

Competing interests

The authors declare that they do not have any financial or other competing interests.

Authors' contributions

JMS performed experiments and made figures. JMS and AH designed experiments, analyzed and interpreted data, and wrote the manuscript. Both authors read and approved the final manuscript.

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References

1. Dodds PN, Rathjen JP: Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat Rev Genet* 2010, **11**:539-548.
2. Boller T, Felix G: A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol* 2009, **60**:379-406.
3. Monaghan J, Zipfel C: Plant pattern recognition receptor complexes at the plasma membrane. *Curr Opin Plant Biol* 2012, **15**:349-357.
4. Boller T, He SY: Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 2009, **324**:742-744.
5. Lindeberg M, Cunnac S, Collmer A: *Pseudomonas syringae* type III effector repertoires: last words in endless arguments. *Trends Microbiol* 2012, **20**:199-208.
6. Xin X-F, He SY: *Pseudomonas syringae* pv. Tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. *Annu Rev Phytopathol* 2013, **51**:473-498.
7. Munkvold KR, Martin GB: Advances in experimental methods for the elucidation of *Pseudomonas syringae* effector function with a focus on *AvrPtoB*. *Mol Plant Pathol* 2009, **10**:777-793.
8. Mansfield JW: From bacterial avirulence genes to effector functions via the hrp delivery system: an overview of 25 years of progress in our understanding of plant innate immunity. *Mol Plant Pathol* 2009, **10**:721-734.
9. Newman M-A, Sundelin T, Nielsen JT, Erbs G: MAMP (Microbe-Associated Molecular Pattern) triggered immunity in plants. *Front Plant Sci* 2013, **4**:139.
10. Gust AA, Willmann R, Desaki Y, Grabherr HM, Nürnberger T: Plant LysM proteins: modules mediating symbiosis and immunity. *Trends Plant Sci* 2012, **17**:495-502.

11. Robatzek S, Wirthmueller L: Mapping FLS2 function to structure: LRRs, kinase and its working bits. *Protoplasma* 2012, **250**:671–681.
12. Bardy SL, Ng SYM, Jarrell KF: Prokaryotic motility structures. *Microbiology* 2003, **149**:295–304.
13. Clarke CR, Chinchilla D, Hind SR, Taguchi F, Miki R, Ichinose Y, Martin GB, Leman S, Felix G, Vinatzer BA: Allelic variation in two distinct *Pseudomonas syringae* flagellin epitopes modulates the strength of plant immune responses but not bacterial motility. *New Phytol* 2013, **200**:847–860.
14. Felix G, Duran JD, Volko S, Boller T: Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* 1999, **18**:265–276.
15. Sun W, Cao Y, Jansen Labby K, Bittel P, Boller T, Bent AF: Probing the Arabidopsis flagellin receptor: FLS2-FLS2 association and the contributions of specific domains to signaling function. *Plant Cell Online* 2012, **24**:1096–1113.
16. Gómez-Gómez L, Boller T: FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in arabidopsis. *Mol Cell* 2000, **5**:1003–1011.
17. Chinchilla D, Bauer Z, Regenass M, Boller T, Felix G: The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 2006, **18**:465–476.
18. Heese A, Hann DR, Gimenez-Ibanez S, Jones AM, He K, Li J, Schroeder JI, Peck SC, Rathjen JP: The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc Natl Acad Sci U S A* 2007, **104**:12217–12222.
19. Boutrot F, Segonzac C, Chang KN, Qiao H, Ecker JR, Zipfel C, Rathjen JP: Direct transcriptional control of the Arabidopsis immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. *Proc Natl Acad Sci* 2010, **107**:14502–14507.
20. Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nurnberger T, Jones JD, Felix G, Boller T: A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 2007, **448**:497–500.
21. Korasick DA, McMichael C, Walker KA, Anderson JC, Bednarek SY, Heese A: Novel functions of Stomatal Cytokinesis-Defective 1 (SCD1) in innate immune responses against bacteria. *J Biol Chem* 2010, **285**:23342–23350.
22. Nühse TS, Bottrill AR, Jones AME, Peck SC: Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *Plant J* 2007, **51**:931–940.
23. Ranf S, Eschen-Lippold L, Pecher P, Lee J, Scheel D: Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J* 2011, **68**:100–113.
24. Zhang J, Shao F, Li Y, Cui H, Chen L, Li H, Zou Y, Long C, Lan L, Chai J, Zhang Y, Shao F, Li Y, Cui H, Chen L, Li H, Zou Y, Long C, Lan L, Chai J, Chen S, Tang X, Zhou JM: A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell Host Microbe* 2007, **1**:175–185.
25. Torres MA, Dangl JL, Jones JDG: Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc Natl Acad Sci U S A* 2002, **99**:517–522.
26. Torres MA: ROS in biotic interactions. *Physiol Plant* 2010, **138**:414–429.
27. Lamb C, Dixon RA: The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 1997, **48**:251–275.
28. Nicaise V, Roux M, Zipfel C: Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol* 2009, **150**:1638–1647.
29. Gimenez-Ibanez S, Ntoukakis V, Rathjen JP: The LysM receptor kinase CERK1 mediates bacterial perception in Arabidopsis. *Plant Signal Behav* 2009, **4**:539–541.
30. 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. *Plant Cell* 2004, **16**:3496–3507.
31. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G, Boller T: Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 2004, **428**:764–767.
32. de la Torre F, Gutiérrez-Beltrán E, Pareja-Jaime Y, Chakravarthy S, Martin GB, del Pozo O: The tomato calcium sensor Cbl10 and its interacting protein kinase Cipl6 define a signaling pathway in plant immunity. *Plant Cell Online* 2013, **25**:2748–2764.
33. Mersmann S, Bourdais G, Rietz S, Robatzek S: Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. *Plant Physiol* 2010, **154**:391–400.
34. Zeng W, He SY: A prominent role of the flagellin receptor FLAGELLIN-SENSING2 in mediating stomatal response to *Pseudomonas syringae* pv tomato DC3000 in Arabidopsis. *Plant Physiol* 2010, **153**:1188–1198.
35. Zhang J, Li W, Xiang T, Liu Z, Laluk K, Ding X, Zou Y, Gao M, Zhang X, Chen S, Mengiste T, Zhang Y, Zhou JM: Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe* 2010, **7**:290–301.
36. Gómez-Gómez L, Felix G, Boller T: A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *Plant J* 1999, **18**:277–284.
37. Smith JM, Salamango DJ, Leslie ME, Collins CA, Heese A: Sensitivity to Flg22 is modulated by ligand-induced degradation and de novo synthesis of the endogenous flagellin-receptor FLAGELLIN-SENSING2. *Plant Physiol* 2014, **164**:440–454.
38. Katagiri F, Thilmony R, He SY: The Arabidopsis thaliana-*Pseudomonas syringae* interaction. In *The Arabidopsis Book*. doi/10.1199/tab.0039.
39. Ishiga Y, Ishiga T, Uppalapati S, Mysore K: Arabidopsis seedling flood-inoculation technique: a rapid and reliable assay for studying plant-bacterial interactions. *Plant Methods* 2011, **7**:32.
40. Albert M, Jehle AK, Mueller K, Eisele C, Lipschis M, Felix G: Arabidopsis thaliana pattern recognition receptors for bacterial elongation factor Tu and flagellin can be combined to form functional chimeric receptors. *J Biol Chem* 2010, **285**:19035–19042.
41. Schechter LM, Roberts KA, Jamir Y, Alfano JR, Collmer A: *Pseudomonas syringae* type III secretion system targeting signals and novel effectors studied with a *Cya* translocation reporter. *J Bacteriol* 2004, **186**:543–555.
42. Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton N, Malinovsky FG, Tör M, de Vries S, Zipfel C: The arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell Online* 2011, **23**:2440–2455.
43. Schulze B, Mentzel T, Jehle A, Mueller K, Beeler S, Boller T, Felix G, Chinchilla D: Rapid heteromerization and phosphorylation of ligand-activated plant transmembrane receptors and their associated kinase BAK1. *J Biol Chem* 2010, **285**:9444–9451.

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