Widely Conserved Attenuation of Plant MAMP-Induced Calcium Influx by Bacteria Depends on Multiple Virulence Factors and May Involve Desensitization of Host Pattern Recognition Receptors

Meltem Lammertz,¹ Hannah Kuhn,¹ Sebastian Pfeilmeier,^{2,3} Jacob Malone,^{2,4} Cyril Zipfel,³ Mark Kwaaitaal,¹ Nai-Chun Lin,⁵ Brian H. Kvitko,⁶ and Ralph Panstruga¹

¹ Unit of Plant Molecular Cell Biology, Institute for Biology I, RWTH Aachen University, 52074 Aachen, Germany; ² John Innes Centre, Norwich Research Park, Norwich NR4 7UH, U.K.; ³ The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, U.K.; ⁴ University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.; ⁵ Department of Agricultural Chemistry, National Taiwan University, Taipei 106, Taiwan, Republic of China; and ⁶ Department of Plant Pathology, University of Georgia, Athens, GA 30602, U.S.A.

Accepted 14 November 2018.

Successful pathogens must efficiently defeat or delay host immune responses, including those triggered by release or exposure of microbe-associated molecular patterns (MAMPs). Knowledge of the molecular details leading to this phenomenon in genuine plant–pathogen interactions is still scarce. We took advantage of the well-established *Arabidopsis thaliana*–*Pseudomonas syringae* pv. *tomato* DC3000 pathosystem to explore the molecular prerequisites for the suppression of MAMP-triggered host defense by the bacterial invader. Using a transgenic *Arabidopsis* line expressing the calcium sensor apoaequorin, we discovered that strain DC3000 colonization results in a complete inhibition of MAMP-induced cytosolic

calcium influx, a key event of immediate-early host immune signaling. A range of further plant-associated bacterial species is also able to prevent, either partially or fully, the MAMP-triggered cytosolic calcium pattern. Genetic analysis revealed that this suppressive effect partially relies on the bacterial type III secretion system (T3SS) but cannot be attributed to individual members of the currently known arsenal of strain DC3000 effector proteins. Although the phytotoxin coronatine and bacterial flagellin individually are dispensable for the effective inhibition of MAMP-induced calcium signatures, they contribute to the attenuation of calcium influx in the absence of the T3SS. Our findings suggest that the capacity to interfere with early plant immune responses is a widespread ability among plant-associated bacteria that, at least in strain DC3000, requires the combinatorial effect of multiple virulence determinants. This may also include the desensitization of host pattern recognition receptors by the prolonged exposure to MAMPs during bacterial pathogenesis.

M. Lammertz and H. Kuhn contributed equally to the work.

Current address for S. Pfeilmeier: Institute of Microbiology, Department of Biology, ETH Zurich, 8093 Zurich, Switzerland.

Current address for C. Zipfel: Institute of Plant and Microbial Biology, Zurich-Basel Plant Science Center, University of Zurich, 8008 Zurich, Switzerland.

Corresponding author: R. Panstruga;
E-mail: panstruga@bio1.rwth-aachen.de

Funding: Work in the laboratory of R. Panstruga is supported by RWTH Aachen University core funds. This study was additionally supported by the Excellence Initiative of the German federal and state governments (Seed Fund provided to R. Panstruga by RWTH Aachen University and Diversity Fund provided to M. Lammertz by RWTH Aachen University), which is administrated by the Deutsche Forschungsgemeinschaft. S. Pfeilmeier was funded by a studentship from the Norwich Research Park. Research in the J. Malone and C. Zipfel laboratories is supported by the Biotechnology and Biological Sciences Research Council Institute Strategic Program Grant BB/J004553/1. The C. Zipfel laboratory is further supported by the Gatsby Charitable Foundation.

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table and eight supplementary figures are published online.

The author(s) declare no conflict of interest.

Modified: 21 Jan 2022.

The bacterial phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 is the causal agent of the bacterial speck disease of tomato (*Solanum lycopersicum*) and thale cress (*Arabidopsis thaliana*). First described in 1986 (Cuppels 1986), *P. syringae* pv. *tomato* strain DC3000 rapidly emerged as a model organism to study the molecular principles underlying bacterial virulence and host immunity (Xin and He 2013). The availability of ample genetic resources renders this pathogen exquisitely suited to explore the molecular pathways required for bacterial pathogenesis and plant resistance.

P. syringae pv. *tomato* DC3000 colonizes the intercellular spaces in leaves and other aerial organs. To defeat plant innate immunity, the pathogen injects effector proteins into host cells via the type-III secretion system (T3SS), which often is described as a molecular syringe (“injectisome”) (Erhardt et al. 2010). In strain DC3000, the T3SS is encoded by *hypersensitive response and pathogenicity* (*hrp*) and *hypersensitive response and conserved* (*hrc*) genes, and the effectors are accordingly designated as Hrp outer proteins (Hop) or, when inducing an avirulence reaction in the plant host, avirulence (Avr) proteins (Lindeberg et al. 2005). More than half of the effector genes known to encode fully active protein effectors (at least 28)

are organized in five genomic clusters and a conserved effector locus (CEL), which is considered as the sixth cluster (Cunnac et al. 2009; Kvitko et al. 2009). In addition to T3SS-dependent effector genes grouped in clusters, at least 10 further effector genes are individually dispersed throughout the strain DC3000 genome (Kvitko et al. 2009).

In general, bacterial effector proteins act transcriptionally and posttranscriptionally to suppress plant immunity, including microbe-associated molecular pattern (MAMP)-triggered responses (de Torres et al. 2006; Hauck et al. 2003). Although great progress regarding the elucidation of effector functions has been achieved during the past decade, the biochemical activity of many *P. syringae* pv. *tomato* DC3000 effectors remains elusive (Cunnac et al. 2009). However, for some key effectors, the mode of action has been deciphered. These include, for example, AvrPto and AvrPtoB, which were initially discovered based on their avirulence function in tomato (Kim et al. 2002; Scofield et al. 1996). AvrPto binds to and inhibits the autophosphorylation of plasma membrane-localized pattern recognition receptors (PRRs) that play a key role in the perception of MAMPs and the initiation of immune responses, thereby abolishing their function (Xiang et al. 2008). AvrPtoB has E3 ubiquitin ligase activity and targets several PRRs, including the flagellin receptor FLAGELLIN SENSING2 (FLS2) and the oligosaccharide coreceptor CHITIN ELICITOR RECEPTOR KINASE1 (CERK1), for proteolytic removal via the 26S proteasome (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Janjusevic et al. 2006). In addition, AvrPtoB dissociates the common PRR coreceptor BRASSINOSTEROID INSENSITIVE1-associated receptor kinase 1 (BAK1) from MAMP receptor complexes (Shan et al. 2008). Consistent with their role in suppression of plant immunity, strain DC3000 mutants defective in AvrPto and AvrPtoB show reduced virulence in tomato plants deficient of the *Pto* kinase (Lin and Martin 2005) and in *Arabidopsis* (Macho et al. 2007).

In addition to proteinaceous effectors, which are predominantly delivered via the T3SS, nonproteinaceous molecules contribute to bacterial virulence. One class of such compounds comprises secreted bacterial secondary metabolites that enhance virulence, which are sometimes also referred to as phytotoxins (Bender et al. 1999). One of the best-studied secreted secondary metabolites is the nonhost-specific phytotoxin coronatine, which is produced by several pathovars of *P. syringae*, including DC3000 (Moore et al. 1989). Coronatine is known to reopen closed stomata to promote bacterial entry and to activate the jasmonic acid signaling pathway in *Arabidopsis* and tomato (Bender et al. 1999; Melotto et al. 2006). *P. syringae* pv. *tomato* DC3000 Δcfa and Δcma mutants, which are deficient in the biosynthesis of coronafacic acid and coronamic acid, the two coronatine precursors, show severely attenuated virulence on *Arabidopsis* and tomato leaves (Brooks et al. 2004; Melotto et al. 2008).

Another major class of compounds that may contribute to bacterial virulence are secreted surface molecules such as extracellular polysaccharides (exopolysaccharides [EPSs]). EPSs have been described as virulence factors of bacteria infecting animals or humans (Vuong et al. 2004) and plants (Denny 1995). In the case of strain DC3000, it was demonstrated that these polyanionic molecules (in particular, alginate) suppress MAMP-based immunity by chelating extracellular calcium (Ca^{2+}) ions (Aslam et al. 2008). Influx of Ca^{2+} from the apoplastic space into the cytosol is known as an immediate-early response during MAMP signaling (Blume et al. 2000; Jeworutzki et al. 2010; Lecourieux et al. 2006). Thus, interference with MAMP-induced Ca^{2+} influx by bacterial EPSs is a potentially potent way to block plant innate immunity (Aslam et al. 2008).

Exogenous application of MAMPs to plant organs (leaves) or plant cells triggers a characteristic pattern of Ca^{2+} influx

that can be visualized by suitable Ca^{2+} reporter systems. Transgenic *Arabidopsis* lines expressing the heterologous apoprotein aequorin allow visualization of MAMP-induced Ca^{2+} signatures at the tissue or whole-seedling level (Aslam et al. 2009; Knight et al. 1991). Although defense suppression by phytopathogens is a well-established phenomenon in plant-microbe interactions, many molecular details of this process remain to be explored. Monitoring Ca^{2+} signatures is an exquisite means to study this feature because Ca^{2+} influx is an immediate-early MAMP-triggered immune response that can be readily measured and quantified in a noninvasive manner.

Here, we used a hydroponic culture system to study the effect of defense suppression by *P. syringae* pv. *tomato* DC3000, choosing cytosolic MAMP-induced Ca^{2+} influx as a proxy for early plant immunity. Transgenic, apoaequorin-expressing *Arabidopsis* seedlings were first challenged with strain DC3000, and a MAMP response was triggered subsequently. This experimental setup revealed near-complete attenuation of MAMP-triggered cytosolic Ca^{2+} influx by the bacteria. A comprehensive collection of strain DC3000 mutants was deployed to decipher the molecular basis of this phenomenon, which revealed a partial and cooperative contribution of the T3SS, coronatine, and flagellin to the phenotype.

RESULTS

To study whether phytopathogenic bacteria are able to suppress canonical MAMP-induced Ca^{2+} signatures, we used an experimental setup based on a 96-well liquid culture system. We inoculated 10- to 14-day-old hydroponically grown, apoaequorin-expressing *Arabidopsis* seedlings (a transgenic Col-0 *P35S::apoaequorin* line) (Knight et al. 1991) with virulent *P. syringae* pv. *tomato* DC3000, which essentially resembles infection of soil-grown plants with regard to pathogen proliferation (Supplementary Fig. S1) (Faulkner et al. 2013). At various time points after inoculation (12, 24, 36, and 48 h postinoculation [hpi]), we triggered a MAMP response by adding the flagellin-derived flg22 peptide (1 μM) to the growth medium. Immediately after MAMP addition, we recorded alterations in cytosolic Ca^{2+} levels ($[\text{Ca}^{2+}]_{\text{cyt}}$) by measuring aequorin-derived luminescence over a period of 45 min in a microplate luminometer. This experimental design, comprising inoculation and a subsequent MAMP stimulus, allowed us to unravel whether strain DC3000 bacteria were able to interfere with a canonical MAMP response in the course of the genuine infection process.

Mock-inoculated Col-0 (*P35S::apoaequorin*) control seedlings showed at all time points the characteristic flg22-induced transient increase of $[\text{Ca}^{2+}]_{\text{cyt}}$ with a maximum $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ of approximately 70 to 90 nM (Aslam et al. 2009), while *P. syringae* pv. *tomato* DC3000-inoculated seedlings revealed a gradual attenuation of this response over time. Whereas, at 12 hpi, the $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ of control and strain DC3000-challenged seedlings was undistinguishable, we noted an evident reduction in $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ at 24 hpi and a near-complete absence of any flg22-induced Ca^{2+} transient in Col-0 bacteria-treated seedlings at 36 and 48 hpi (Fig. 1A). We quantified this effect by determining the respective areas under the curve (AUCs) of data from multiple independent experiments, focusing on 24 and 48 hpi as characteristic early and late time points, respectively, after inoculation. In relation to mock-inoculated control seedlings set as 100% AUC, strain DC3000-inoculated seedlings showed a reduction to approximately 40% AUC at 24 hpi and approximately 10% AUC at 48 hpi (Fig. 1B). This effect depends on the bacterial titer used as a start inoculum because, at optical densities at 600 nm (OD_{600}) of 10^{-7} and 10^{-8} , the reduction of $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ was partially or fully relieved,

respectively (Supplementary Fig. S2). Lack of intensive trypan blue staining in both mock-treated and inoculated seedlings at 48 hpi excludes the possibility that an impaired viability of strain DC3000-inoculated seedlings is responsible for the abrogated flg22-triggered Ca^{2+} influx.

To explore whether the observed phenomenon is confined to the bacterial peptide MAMP flg22, we performed the same assay as described above but used either the bacterial ELONGATION FACTOR-THERMO UNSTABLE-derived peptide MAMP elf18 (1 μM) or the fungal polycarbohydrate MAMP chitin (0.1 mg/ml) as inducers. We noted a similar outcome as observed for flg22, with an intermediate reduction of the Ca^{2+} transient at 24 hpi (approximately 40 and 50% AUC for elf18 and chitin, respectively) and a more pronounced decline of the Ca^{2+} transient at 48 hpi (approximately 10 and 30% AUC for elf18 and chitin, respectively) (Fig. 1B). We further broadened our approach and tested two non-MAMPs as triggers of a cytoplasmic Ca^{2+} signature: the defense-associated phytohormone salicylic acid (SA; 1 mM) and the osmotically active polyol,

mannitol (700 mM), which induces an osmotic shock. Similar to the tested MAMPs, these two substances elicited a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, which was, however, more irregular in amplitude (especially for SA) and duration (Supplementary Fig. S3). Notably, neither the SA- nor the mannitol-induced Ca^{2+} response was markedly reduced in the presence of *P. syringae* pv. *tomato* DC3000 at 24 or 48 hpi under our experimental conditions (Fig. 1B). Taken together, these findings suggest that strain DC3000 is able to attenuate strongly and specifically MAMP-induced Ca^{2+} signatures during the course of infection. In the following work, we concentrated on flg22 as a well-characterized and widely used MAMP for further experiments and 48 hpi as a representative time point.

Thus far, all experiments described above were conducted with hydroponically grown *Arabidopsis* seedlings. To find out whether our findings are also of relevance in the context of intact soil-grown plants, we performed an assay similar to that described above with leaf discs derived from 5-week-old transgenic Col-0 (*P35S::apocaequorin*) plants. Leaf discs were placed in 96-well plates and infected with *P. syringae* pv. *tomato* DC3000 bacteria prior to a flg22 stimulus and recording of the Ca^{2+} response. Similar to the hydroponic culture system, leaf discs showed a reduced flg22-triggered Ca^{2+} signature. However, in contrast to the hydroponic system, (nearly) full suppression of the flg22-induced Ca^{2+} transient was already reached at 24 hpi (Fig. 2A and B). Influx of Ca^{2+} from external and internal stores into the cytosol is a well-known immediate-early response of plant cells upon MAMP perception (Blume et al. 2000; Jeworutzki et al. 2010; Lecourieux et al. 2006). To explore whether immune responses downstream of cytosolic Ca^{2+} influx are also affected by the suppressive activity of strain DC3000 bacteria, we analyzed the flg22-triggered transcript accumulation of the prototypical defense marker gene, *FLAGEL-LIN-RESPONSIVE KINASE 1* (*FRK1*). We noted that the formation of flg22-triggered ROS was fully abolished subsequent to strain DC3000 infection, again regardless of the time point (24 or 48 hpi) (Fig. 2C and D). By contrast, *FRK1* mRNA abundance showed a gradual decline, with reduced transcript levels (approximately 50% compared with 0 hpi) at 24 hpi and a near-complete absence of *FRK1* transcripts at 48 hpi (Fig. 2E; Supplementary Fig. S4). The latter findings rule out the possibility that the suppressive effect seen at the level of Ca^{2+} influx is an artifact of the transgenic aequorin reporter system.

To assess whether the ability to inhibit a MAMP-induced Ca^{2+} signature is restricted to *P. syringae* pv. *tomato* DC3000, we next analyzed a broader panel of microorganisms in our assay conditions, which covered pathogenic, nonpathogenic, and beneficial species. This selection included baker's yeast (*Saccharomyces cerevisiae*) as a fungal representative, a gram-positive bacterium (*Bacillus subtilis*), and six gram-negative bacteria (*Agrobacterium tumefaciens*, *Burkholderia plantarii*, *Escherichia coli*, *P. fluorescens*, *P. putida*, and *Xanthomonas campestris*) (Table 1). Of these, most are known to be associated with plants as either pathogens (*A. tumefaciens*, *B. plantarii*, and *X. campestris*), plant growth-promoting bacteria (*Bacillus subtilis*, *P. fluorescens*, and *P. putida*), or a sporadic colonizer of ripe fruits (*S. cerevisiae*) (Goddard and Greig 2015), whereas *E. coli* has no strong established relationship to plant biology. Apart from *A. tumefaciens*, *E. coli*, and *S. cerevisiae*, most of the tested microorganisms were capable of preventing the flg22-triggered Ca^{2+} response to a considerable extent (Fig. 3A). However, with the exception of *Burkholderia plantarii*, the suppressive effect of these microorganisms was either delayed or weaker than that of strain DC3000 because, at 48 hpi, the AUC was higher in the case of *Bacillus subtilis*, *P. fluorescens*, *P. putida*, and *X. campestris* (approximately 30 to 40% of the mock value compared with approximately 10% for strain DC3000).

Fig. 1. *Pseudomonas syringae* pv. *tomato* DC3000 attenuates microbe-associated molecular pattern- but not salicylic acid (SA)- or mannitol-triggered Ca^{2+} transients in a time-dependent manner. Ten- to fourteen-day-old transgenic (*P35S::apocaequorin*) *Arabidopsis* seedlings grown in hydroponic culture were inoculated with *P. syringae* pv. *tomato* (*Pst*) DC3000 at an optical density at 600 nm of 0.001 or were mock treated. **A**, The flg22-triggered Ca^{2+} transients (1 μM) were recorded at 12, 24, 36, and 48 h postinoculation (hpi) for 45 min at 30-s intervals; flg22 was added after 2 min. Data represent means and standard deviations of the means of 12 seedlings/treatment. The experiment was repeated three times with similar results. **B**, The flg22 (1 μM), elf18 (1 μM), chitin (100 $\mu\text{g}/\text{ml}$), SA (1 mM), or osmotic shock (700 mM mannitol)-triggered Ca^{2+} transients were determined at 24 and 48 hpi and total Ca^{2+} influx following elicitor addition was estimated as area under the curve (AUC). Data represent means and standard errors of the means (SEM) of ≥ 3 independent biological replicates with 12 seedlings/replicate and treatment. Lowercase letters indicate statistically significant differences ($P \leq 0.05$; one-way analysis of variance and Tukey's honestly significant difference post hoc test).

Burkholderia plantarii, however, showed a stronger suppression of the Ca^{2+} transient than *P. syringae* pv. *tomato* (Fig. 3A). In summary, numerous other microorganisms in addition to strain DC3000 are competent to interfere with MAMP-induced Ca^{2+} signatures.

We next wondered whether the suppressive activity of these microorganisms requires living entities. To address this question, we assessed the ability of heat-treated (95°C, 5 min) microorganisms to interfere with the flg22-induced Ca^{2+}

response. Notably, none of the microorganisms that had previously shown statistically significant effects (Fig. 3A) retained the capacity to impede the flg22-triggered Ca^{2+} signature upon heat inactivation (Fig. 3B). This finding suggests that the critical factors are either temperature sensitive or depend on a living organism.

According to current understanding, secreted effector proteins are considered to be key tools deployed by phytopathogens for host cell manipulation (Büttner 2016; Toruño et al. 2016). The T3SS is believed to be the main device for the delivery of effector proteins in *P. syringae* pv. *tomato* DC3000 (Guo et al. 2009). To find out whether the observed reduction of $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ was dependent on the T3SS, we performed the above-described assay with strain DC3000 ΔhrcC and ΔhrpA mutants, which are defective in essential components of the T3SS (Roine et al. 1997; Yuan and He 1996). The two independent mutants behaved similarly and showed a reduction of the Ca^{2+} response to approximately 40 and 60% AUC (Fig. 4A), indicating that the suppressive effect is associated, in part, with a functional T3SS. We obtained similar results with the ΔhrpL mutant, which lacks the alternative σ factor HrpL, controlling the expression of more than 70 virulence-associated genes, including most of the T3SS genes (Lam et al. 2014) (Fig. 4A).

Because we found the T3SS to be partially required for the suppression of the MAMP-triggered Ca^{2+} transient by *P. syringae* pv. *tomato* DC3000 bacteria, we wondered whether we could identify one or more known effector proteins that may mediate this suppressive effect. We first focused on deletion mutants of effectors AvrPto and AvrPtoB, which are known to interfere with flg22-induced, FLS2-mediated immune responses in *Arabidopsis* by binding to PRRs, including FLS2 (AvrPto) (Xiang et al. 2008), or by tagging FLS2 with ubiquitin for proteasomal degradation (AvrPtoB) (Göhre et al. 2008). The deletion mutants ΔavrPto and $\Delta\text{avrPtoB}$ as well as the ΔavrPto

Fig. 2. *Pseudomonas syringae* pv. *tomato* DC3000 attenuates flg22-triggered Ca^{2+} transients and reactive oxygen species (ROS) accumulation in soil-grown adult *Arabidopsis* leaves and *FRK1* expression in seedlings. **A** and **B**, The flg22 (1 μM)-triggered Ca^{2+} transients of leaf discs from 5-week-old transgenic (*P35S::apoaquorin*) *Arabidopsis* plants were determined 24 or 48 h after mock treatment or after *P. syringae* pv. *tomato* DC3000 inoculation (optical density at 600 nm [OD_{600}] = 0.001). **A**, Ca^{2+} influx was recorded for 45 min at 30-s intervals; flg22 was added after 2 min. Data represent means and standard deviations of the means (SDM) of ≥ 6 leaf discs/treatment. The experiment was repeated four times with similar results. **B**, Total Ca^{2+} influx after flg22 addition was estimated as area under the curve (AUC). Data represent means and standard error of the mean (SEM) of four independent biological replicates with 6 leaf discs/replicate and treatment. **C** and **D**, The flg22 (1 μM)-triggered ROS accumulation of leaf discs from 6- to 8-week-old *Arabidopsis* plants was recorded as relative light units (RLU) in a luminol-based assay at 24 or 48 h after mock treatment or after strain DC3000 inoculation (OD_{600} = 0.001). **C**, ROS accumulation was recorded for 45 min at 30-s intervals; flg22 was added after 2 min. Data represent means and SDM of ≥ 9 leaf discs/treatment. The experiment was repeated four times with similar results. **D**, Total ROS accumulation after flg22 addition was estimated as AUC of experiments shown in C. Data represent means and SEM of three independent biological replicates with ≥ 9 leaf discs/replicate and treatment. **E**, *FRK1* transcript accumulation of either untreated or flg22 (1 μM)-treated 10-day-old hydroponically grown *Arabidopsis* seedlings prior to (0 h) or after (24 and 48 h postinoculation [hpi]) inoculation with strain DC3000 (OD_{600} = 0.001) was quantified by quantitative reverse-transcription polymerase chain reaction. Seedlings were harvested for RNA extraction at 60 min after addition of flg22. Shown values were normalized to the mRNA abundance of the reference gene *At4g26420* and relativized to the untreated sample at 0 hpi. Data represent means and SEM of two independent biological replicates with 10 seedlings/replicate and treatment. Lowercase letters indicate statistically significant differences ($P \leq 0.05$; one-way analysis of variance and Tukey's honestly significant difference posthoc test).

$\Delta avrPtoB$ double mutant showed a similar reduction of the flg22-induced Ca^{2+} transient as strain DC3000 wild-type (WT) bacteria, excluding a major contribution of these two effectors to the suppression of the MAMP-triggered Ca^{2+} signature (Fig. 4B).

We next broadened our approach and took advantage of a collection of effector cluster deletion mutants that each represent a knockout of multiple effector genes (Kvitko et al. 2009). None of the six tested single- or double-cluster mutants (ΔI , ΔIV , ΔCEL , ΔIX , ΔX , and $\Delta II \Delta X$) or the $\Delta I \Delta II \Delta IV \Delta CEL \Delta IX \Delta X$ multicluster mutant which, altogether, is deficient in 18 effector genes, showed altered suppression of the flg22-triggered Ca^{2+} signature (Fig. 4C). We finally deployed the mutant $\Delta I \Delta II \Delta IV \Delta CEL \Delta IX \Delta X + 10$ which, in addition to the effector genes located in clusters, also lacks 10 additional effector genes located as single-copy genes elsewhere in the bacterial genome. This mutant, which is devoid of the 28 supposedly functional *P. syringae* pv. *tomato* DC3000 effectors (Cunnac et al. 2011), including AvrPto and AvrPtoB, revealed only a slight relief in the suppressive effect (approximately 20% AUC instead of approximately 10% AUC for strain DC3000; difference not statistically significant) (Fig. 4C). In sum, these data suggest that neither individual strain DC3000 effector proteins nor any tested combination thereof conditions the suppression of the flg22-triggered Ca^{2+} influx.

Because only a partial relief of the prohibitory effect was observed with the T3SS-defective *P. syringae* pv. *tomato* DC3000 $\Delta hrcC$, $\Delta hrpA$, and $\Delta hrpL$ mutants (Fig. 4A), we conclude that additional factors must be involved in this phenomenon. In addition to the T3SS, bacteria possess further dedicated systems for the delivery of proteins and possibly other metabolites to the exterior. In total, gram-negative bacteria deploy six major secretion systems (Green and Mecsas 2016). We explored a potential contribution of the twin-arginine translocation (Tat) pathway, which feeds into the type II secretion system (T2SS), by pharmacological inhibition and studied a possible involvement of the type VI secretion system (T6SS) via mutant analysis. Treatment of seedlings with the Tat pathway inhibitors Bay 11-7082 and *N*-phenyl maleimide (Vasil et al. 2012) increased the flg22-induced Ca^{2+} transient markedly in the absence of bacteria (Supplementary Fig. S5). However, in the presence of strain DC3000, even this elevated response was largely suppressed. Similarly, none of the T6SS mutants ($\Delta hcp1$, $\Delta hcp2$, $\Delta hcp1 \Delta hcp2$, $\Delta HSI I$, $\Delta HSI II$, and $\Delta HSI I \Delta HSI II$) (Haapalainen et al. 2012; Sarris et al. 2010) revealed a relief of the strain DC3000-mediated suppression of flg22-induced Ca^{2+} influx.

Apart from the T3SS, other nonproteinaceous molecules have been attributed to the virulence of *P. syringae* pv. *tomato* DC3000. Among these is the phytotoxin coronatine, which has been implicated in multiple facets of bacterial pathogenesis (Geng et al. 2014). Therefore, we included the coronatine-deficient strain DC3000 Δcma mutant in our assay and found that it behaved like strain DC3000 WT bacteria. Additional

nonproteinaceous factors with a presumed role in the defeat of plant defense are EPSs. Alginates, for example, have been shown to be involved in the chelation of extracellular Ca^{2+} for the suppression of innate plant immunity (Aslam et al. 2008). Therefore, we tested a range of mutants defective in the production

Fig. 3. Ability to attenuate flg22-triggered Ca^{2+} transients is widespread among plant-associated microorganisms. Ten- to fourteen-day-old transgenic (*P35S::apoaquorin*) *Arabidopsis* seedlings were grown in hydroponic culture. The flg22 (1 μ M)-triggered Ca^{2+} transients were determined at 48 h postinoculation after mock treatment or inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 wild type at an optical density at 600 nm = 0.001 and **A**, the indicated microorganisms (in alphabetical order) or **B**, heat-inactivated microorganisms that showed statistically significant effects in **A**. Total Ca^{2+} influx after flg22 addition was estimated as area under the curve (AUC). Data represent means and standard error of the mean of ≥ 3 independent biological replicates with 12 seedlings/replicate and treatment. Lowercase letters indicate statistically significant differences ($P \leq 0.05$; one-way analysis of variance and Tukey's honestly significant difference posthoc test).

Table 1. Microorganisms other than *Pseudomonas syringae* pv. *tomato* DC3000 used in this study

Microorganisms	Growth conditions ^a	Reference
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> 85-10	King's B + Rif (50 mg/liter); 28°C	Thieme et al. 2005
<i>P. putida</i> KT2440	LB + glucose; 28°C	Nelson et al. 2002
<i>P. fluorescens</i> DSM 50090 T	LB + glucose; 28°C	DSMZ catalog number 50090
<i>Bacillus subtilis</i> DSM 347	LB + glucose; 28°C	DSMZ catalog number 347
<i>Burkholderia plantarii</i> DSM 9509	LB + glucose; 30°C	DSMZ catalog number 9509
<i>Escherichia coli</i> DH5 α	LB; 37°C	Taylor et al. 1993
<i>Agrobacterium tumefaciens</i> GV3101 pMP90RK	DYT + Rif (100 mg/liter), Km, Gm; 28°C	Koncz and Schell 1986
<i>Saccharomyces cerevisiae</i> EBY100	YEB; 28°C	Thermo Fisher Scientific, Dreieich, Germany

^a Rif = rifampicin at 50 or 100 mg/liter, Km = kanamycin at 50 mg/liter, and Gm = gentamycin at 25 mg/liter. LB = lysogeny broth, DYT = double yeast tryptone medium, and YEB = yeast extract broth.

of various EPS molecules. These included $\Delta algD$ (Aslam et al. 2008) and $\Delta algG/X$ (Jain et al. 2003) mutants, a $\Delta pslD/E$ mutant (Byrd et al. 2009), a $\Delta wssB/C$ mutant (Spiers et al. 2002; Gal et al. 2003), as well as some double mutants and a $\Delta algG/X \Delta pslD/E \Delta wssB/C$ triple mutant. These mutants lack alginates ($\Delta algD$ and $\Delta algG/X$); do not produce a polysaccharide consisting of a repeated pentasaccharide containing D-mannose, D-glucose, and L-rhamnose ($\Delta pslD/E$); or are deficient in acetylated cellulose polymer production ($\Delta wssB/C$). All tested single and double mutants, as well as the $\Delta algG/X \Delta pslD/E \Delta wssB/C$ triple mutant, retained full suppression of the flg22-induced Ca^{2+} response.

An explanation for the absence of any MAMP-triggered Ca^{2+} signature and further downstream immune responses such as ROS formation and defense gene activation would be the pathogen-induced reduction or membrane depletion of host PRRs such as FLS2, which are indispensable to initiate intracellular defense signaling, including Ca^{2+} signatures. To

test whether proliferation of *P. syringae* pv. *tomato* DC3000 correlates with a depletion of the cellular FLS2 pool, we performed an immunoblot analysis in the course of bacterial infection of hydroponically grown seedlings. This experiment revealed no reduction and even possibly a slight increase in total FLS2 levels in Col-0 (*P35S::apoaquorin*) seedlings at 24 and 48 hpi in comparison with either untreated seedlings or seedlings at 0 hpi (Fig. 5A). Confocal imaging further indicated that abundance of membrane-associated FLS2::green fluorescent protein (GFP) fluorescence remained largely unaltered at 48 hpi in either WT strain DC3000 or flagella-deficient strain DC3000 mutants (Supplementary Fig. S6). These findings suggest that degradation or intracellular sequestration of FLS2 is unlikely to be the primary reason for the attenuated MAMP-triggered Ca^{2+} pattern.

Another possible explanation for the suppressed MAMP-induced Ca^{2+} response would be a desensitization of host PRRs by MAMPs inadvertently released by the bacterial pathogen during

Fig. 4. Loss of the type III secretion system (T3SS); however, neither single nor multiple T3SS effectors deletions impair *Pseudomonas syringae* pv. *tomato* DC3000-mediated attenuation of flg22-triggered Ca^{2+} transients. Ten- to fourteen-day-old transgenic (*P35S::apoaquorin*) *Arabidopsis* seedlings were grown in hydroponic culture. The flg22 (1 μ M)-triggered Ca^{2+} transients were determined at 48 h postinoculation after mock treatment or inoculation with an optical density at 600 nm = 0.001 of **A**, strain DC3000 wild type or the T3SS-deficient mutants $\Delta hrcC$, $\Delta hrpA$, and $\Delta hrpL$; **B**, the T3SS effector mutants $\Delta AvrPto$, $\Delta AvrPtoB$, and $\Delta AvrPto \Delta AvrPtoB$; or **C**, deletions of T3SS effector clusters. Total Ca^{2+} influx after flg22 addition was estimated as area under the curve (AUC). Data represent means and standard error of the mean of ≥ 3 independent biological replicates with 12 seedlings/replicate and treatment. Lowercase letters indicate statistically significant differences ($P \leq 0.05$; one-way analysis of variance and Tukey's honestly significant difference posthoc test).

Fig. 5. FLS2 desensitization may contribute to the *Pseudomonas syringae* pv. *tomato* DC3000-mediated attenuation of flg22-triggered Ca^{2+} transients. Ten- to fourteen-day-old transgenic (*P35S::apoaquorin*) *Arabidopsis* wild-type (WT) (Col-0) or *fls2* seedlings were grown in hydroponic culture. **A**, In all, 24 seedlings were sampled immediately (0 h) or at 24 and 48 h postinoculation (hpi) with strain DC3000 (optical density at 600 nm [OD₆₀₀] = 0.001). FLS2 protein abundance was determined by immunoblot analysis with α FLS2 antibodies. Ponceau staining served as a loading control. A second independent replicate showed similar results. **B**, The flg22 (1 μ M)-triggered Ca^{2+} transients were determined at 48 hpi after mock treatment or inoculation (OD₆₀₀ = 0.001) with strain DC3000 WT and flagellin-deficient strain DC3000 $\Delta fliC$. Total Ca^{2+} influx after flg22 addition was estimated as area under the curve (AUC). Data represent means and SEM of seven independent biological replicates with 12 seedlings/replicate and treatment. **C**, The flg22 (1 μ M)-triggered Ca^{2+} transients were determined at 48 h after mock treatment or treatment with 1 μ M flg22, 1 μ M elf18, or chitin at 100 μ g/ml. Total Ca^{2+} influx after flg22 addition was estimated as AUC. Data represent means and SEM of three independent biological replicates with 16 seedlings/replicate and treatment. Lowercase letters indicate statistically significant differences ($P \leq 0.05$; one-way analysis of variance and Tukey's honestly significant difference posthoc test).

Fig. 6. Coronatine, flagellin, and the type III secretion system (T3SS) cooperatively attenuate flg22-triggered Ca^{2+} influx. Ten- to fourteen-day-old transgenic (*P35S::apoaquorin*) *Arabidopsis* seedlings were grown in hydroponic culture. **A**, The flg22 (1 μ M)-triggered Ca^{2+} transients were determined at 48 h postinoculation (hpi) after mock treatment or inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 wild type (WT), strain DC3000 $\Delta cfa \Delta hrcC$ (coronatine-deficient [COR⁻] and T3SS-deficient), $\Delta fliC \Delta cfa$ (flagellin-deficient and COR⁻), $\Delta fliC \Delta hrcC$ (flagellin- and T3SS-deficient), or $\Delta fliC \Delta cfa \Delta hrcC$ (flagellin-deficient, COR⁻, and T3SS-deficient). Total Ca^{2+} influx after flg22 addition was estimated as area under the curve (AUC). Data represent means and standard error of the mean (SEM) of three independent biological replicates with 12 seedlings/replicate and treatment. **B**, Seedlings were inoculated with strain DC3000 WT and indicated mutants (optical density at 600 nm = 0.001). Immediately after inoculation (0 hpi) or after 4, 24, and 48 hpi, seedlings were surface sterilized, weighed, and homogenized and bacterial densities of the extracts were determined by evaluating CFU of serial dilutions. **C**, Culture supernatants of seedlings in B were collected and bacterial densities were determined by evaluating CFU of serial dilutions. Data represent means and SEM of three independent biological replicates with 8 seedlings/replicate and treatment. Lowercase letters indicate statistically significant differences ($P \leq 0.05$; one-way analysis of variance and Tukey's honestly significant difference posthoc test).

proliferation. To address this possibility, we performed an experiment with the *P. syringae* pv. *tomato* DC3000 $\Delta fliC$ mutant, which is defective in the gene encoding flagellin, an essential building block of bacterial flagella and the natural source of flagellum-derived MAMPs during plant colonization. Inoculation with the $\Delta fliC$ mutant did result in a slight yet not statistically significant relief of suppression of flg22-triggered cytosolic Ca^{2+} influx (Fig. 5B). However, pretreatment of seedlings with MAMPs at 48 h prior to a secondary flg22 stimulus led to a severely reduced Ca^{2+} response in the case of flg22 (10% of control) and less severe reductions in the case of elf18 and chitin (65 and 93% of control, respectively) (Fig. 5C). This finding raises the possibility that the prolonged exposure of MAMPs during bacterial colonization may contribute to the prevention of MAMP-triggered Ca^{2+} influx.

Because mutants in individual pathogenicity components did not markedly relieve the *P. syringae* pv. *tomato* DC3000-mediated suppression of the flg22-triggered Ca^{2+} transient (Figs. 4A and 5B), we considered combining individual mutations in higher order mutants. The double mutants deficient in T3SS and coronatine ($\Delta cfa \Delta hrcC$), T3SS and flagellin ($\Delta fliC \Delta hrcC$), and flagellin and coronatine ($\Delta fliC \Delta cfa$) showed only a partial release of suppression (to approximately 36, 53, and 41% AUC, respectively) (Fig. 6A). By contrast, inoculation with a $\Delta fliC \Delta cfa \Delta hrcC$ triple mutant resulted in a fully restored Ca^{2+} response (Fig. 6A). A lack of suppression of the flg22-induced Ca^{2+} transient by the $\Delta fliC \Delta cfa \Delta hrcC$ triple mutant correlates with reduced bacterial titers in the seedlings, in particular at 24 hpi (Fig. 6B), whereas bacterial titers in the culture supernatant (and, thus, overall bacterial fitness) were unaffected by the three mutations (Fig. 6C).

DISCUSSION

In this study, we used a hydroponic culture system in combination with a transgenic *Arabidopsis* Ca^{2+} reporter line (Col-0 *P35S::apoaquorin*) to analyze the molecular prerequisites for effective defense suppression by the phytopathogenic bacterium *P. syringae* pv. *tomato* DC3000 in the course of the genuine infection process. We focused on the MAMP-induced immediate-early Ca^{2+} transient as a convenient proxy for the level of bacterial interference with plant immunity. The hydroponic system allows the cultivation of highly uniform host plants, the largely stress-free application of bacteria and MAMPs, and quantitative assessment of the MAMP-triggered response (Ca^{2+} transient). Strain DC3000 infection of *Arabidopsis* seedlings grown in hydroponic culture resembles bacterial proliferation in soil-grown plants. The aequorin Ca^{2+} reporter system is well established in the context of plant immunity research and enables accurate measurements of cytoplasmic Ca^{2+} levels (Kwaaijtaal et al. 2011; Ranf et al. 2011; Vatsa et al. 2011). In combination with the 96-well microtiter plate format, this experimental setup permits the measurement of a comparatively high number of plant samples per treatment and the analysis of a large number of conditions and bacterial mutant strains. Thus, the format may also be suitable for studying other aspects of plant–bacteria interactions.

We observed that presence of *P. syringae* pv. *tomato* DC3000 caused a suppression of MAMP-induced immune responses within 48 hpi (from 24 hpi onward). This relates to MAMP-triggered Ca^{2+} transients (Fig. 1A and B), the generation of ROS (Fig. 2C and D), and transcript accumulation of defense genes (Fig. 2E). At least with respect to the MAMP-induced Ca^{2+} signature, this effect is dependent on bacterial titers, is specific for MAMPs (Fig. 1B), and relies on one or more heat-sensitive bacterial factors (Fig. 3B). Together, these data demonstrate the surprising and hitherto undescribed capacity of

phytopathogenic bacteria to undermine early plant immune signaling in a near-complete manner. Notably, suppression of Ca^{2+} influx was effective for cell surface immune receptors with external leucine-rich repeat (FLS2 and EFR) and LysM (CERK1) domains (Fig. 1B), which operate either in dependence (FLS2 and EFR) or independently (CERK1) of the BAK1 coreceptor (Chinchilla et al. 2007; Ranf et al. 2011). These findings indicate a broad suppressive activity of strain DC3000, affecting signals transmitted through different types of PRRs and operating via different downstream signaling routes.

At present, there is little published data on the timing of defense suppression in the course of authentic plant–bacteria interactions. Most studies focus on artificial systems such as the heterologous expression or overexpression of individual effectors in plants. However, even under such simplified conditions, it takes a considerable period of time for effects to become detectable. For example, dexamethasone-induced expression of AvrPtoB requires 12 to 24 h to result in a marked reduction of CERK1 levels in transgenic *Arabidopsis* plants (Gimenez-Ibanez et al. 2009). Likewise, transient expression of XopJ in *Nicotiana benthamiana* resulted in a reduction of proteasome activity at 48 hpi (Üstün et al. 2012). It is well conceivable that, in the course of an authentic plant–bacteria interaction, lag times are in a similar order of magnitude. We like to emphasize that we start with a rather low inoculum (OD of 0.001, corresponding to 10^3 to 10^4 CFU/g fresh weight of seedlings) (Fig. 6B) and that bacteria have to enter into plant tissue, assemble the type III secretion apparatus, and deliver effectors that then still need to affect the host machinery, which may require additional time. Given these constraints and the likely asynchronous infection process, we consider it credible that we observe a reduction in MAMP-induced Ca^{2+} signature from 24 h onward but not at 12 hpi (Fig. 1A). The reduction in the Ca^{2+} signature is still partial at 24 hpi—possibly because the response to be complete has to be suppressed in all cells of the seedlings, which probably does not happen fast and synchronously, resulting in an intermediate outcome at 24 hpi. Realistically, although molecular events related to defense suppression are likely to happen locally before, effects are unlikely to be detectable experimentally at earlier time points at the whole-seedling level.

We were unable to pinpoint further the partial loss of suppressive activity in the *P. syringae* pv. *tomato* DC3000 ΔhrcC , ΔhrpA , and ΔhrpL mutants to particular T3SS effector proteins. Individual effector cluster mutants (ΔI , ΔIV , ΔCEL , ΔIX , and ΔX) as well as the multicuster mutants $\Delta\text{II}\Delta\text{X}$ and $\Delta\text{I}\Delta\text{II}\Delta\text{IV}\Delta\text{CEL}\Delta\text{IX}\Delta\text{X}$ or the $\Delta\text{I}\Delta\text{II}\Delta\text{IV}\Delta\text{CEL}\Delta\text{IX}\Delta\text{X}+10$ mutant, which is defective in 28 TSS functional effector proteins, failed to phenocopy the partially relieved suppression phenotype of the ΔhrcC , ΔhrpA , and ΔhrpL mutants (Fig. 4A and C). This finding suggests that likely yet-unidentified proteinaceous effectors or possibly nonproteinaceous effectors such as secondary metabolites exert the suppressive effect via the T3SS. Given that strain DC3000 effectors AvrPto and AvrPtoB were previously reported to target the FLS2 immune receptor (Göhre et al. 2008; Xiang et al. 2008), it was surprising that strain DC3000 mutants defective in *AvrPto* or *AvrPtoB* did not show any relief of the suppression of the flg22-triggered Ca^{2+} transient (Fig. 4B). This result suggests that either additional effectors may target this immune receptor or that the bacterial blockade of Ca^{2+} influx might happen downstream of MAMP perception.

In principle, suppression of MAMP-triggered Ca^{2+} transients could be achieved by obstructing the respective MAMP receptors, interfering with cytoplasmic signaling downstream of MAMP receptors, or by impeding the activity of Ca^{2+} channels.

Given that responses to multiple MAMPs such as flg22, elf18, and chitin are hindered (Fig. 1B), suppression at the level of individual MAMP receptors seems rather unlikely. The molecular events downstream of MAMP receptor activation that lead to the opening of Ca^{2+} channels remain largely obscure. Similarly, the molecular identity of Ca^{2+} channels involved in mediating MAMP-triggered Ca^{2+} influx from the apoplastic space are unknown at present. Results from pharmacological inhibitor experiments point to an involvement of ionotropic glutamate receptor-like channels in this respect (Kwaaitaal et al. 2011; Vatsa et al. 2011). Because Ca^{2+} transients induced by SA or mannitol remain unaltered by strain DC3000 (Fig. 1B), direct interference with Ca^{2+} channels also seems unlikely, unless there were MAMP-specific Ca^{2+} channels that are blocked by bacteria in a very specific manner. In the future, identification of bacterial components that specifically inhibit MAMP-induced Ca^{2+} influx might pave the way toward identification of the responsible host Ca^{2+} channels.

Unexpectedly, suppression of the MAMP-induced Ca^{2+} transient occurred, in part, independently of the T3SS, which is usually considered the key device for bacterial pathogenicity (Deng et al. 2017). Largely apathogenic strain DC3000 ΔhrcC (Hauck et al. 2003), ΔhrpA (Roine et al. 1997), and ΔhrpL (Lam et al. 2014) mutants, which are unable to deliver any T3SS effectors into host cells, were still capable of suppressing the flg22-induced Ca^{2+} transient for the most part (Fig. 4A). Thus, the T3SS is necessary but not sufficient for full defense suppression, suggesting that factors different from the T3SS contribute to this activity. By genetic or pharmacological analysis, we ruled out the individual contribution of EPSs, coronatine, flagellin (Fig. 5B), the Tat translocation pathway, and the T6SS. However, a triple mutant defective in the T3SS and coronatine biosynthesis and lacking flagellin (ΔfliC Δcfa ΔhrcC) was unable to prevent flg22-triggered Ca^{2+} influx (Fig. 6A), indicating that the suppressive activity is likely a combinatorial effect involving multiple bacterial virulence factors. This notion is further corroborated by the fact that each of the tested double mutants (ΔfliC Δcfa , Δcfa ΔhrcC , and ΔfliC ΔhrcC) partially retained the ability to abrogate the flg22-induced Ca^{2+} signature (Fig. 6A). Thus, it seems as if motility or flagellin exposure (*fliC*), effector delivery (*hrcC*), and phytotoxin production (*cfa*) collectively contribute to the suppressive effect. However, the restored MAMP-induced Ca^{2+} levels in the case of the ΔfliC Δcfa ΔhrcC triple mutant correlated with reduced bacterial titers (Fig. 6B). Thus, on the basis of these data, it is impossible to discriminate whether the inability of strain DC3000 to suppress a Ca^{2+} response results in reduced bacterial proliferation or whether reduced bacterial growth causes a reduction in the ability to interfere with Ca^{2+} influx in the host.

We discovered that a diverse panel of bacteria has the capacity to exert a level of suppression of flg22-induced Ca^{2+} influx similar to that of *P. syringae* pv. *tomato* DC3000 (Fig. 3A). These comprise gram-positive (*Bacillus subtilis*) and gram-negative (*Burkholderia plantarii*, *P. fluorescens*, *P. putida*, and *X. campestris*) bacteria with different lifestyles, including various phytopathogens and plant endophytes. This observation raises the question of whether the respective underlying molecular mechanism might be conserved across these species. Given that the gram-positive bacterium *Bacillus subtilis* as well as the genome-sequenced strains of *A. tumefaciens* and *P. putida* used in our experiments lack a T3SS (Economou et al. 2006; Nelson et al. 2002; Wood et al. 2001), which partially contributes to the suppression of Ca^{2+} in strain DC3000 (Fig. 4A), this is an unlikely scenario. Among gram-positive and gram-negative bacteria, the general secretion (Sec) and Tat secretion pathways are highly conserved (Green and Meccas 2016) and, thus, could represent common routes for the delivery

of suppressive molecules. In addition, presence of the T1SS, T2SS, T6SS, and possibly the T5SS is a joint feature of the gram-negative bacteria that showed a suppressive effect in our experiments (Supplementary Table S1). Nevertheless, we cannot rule out the possibility that each bacterial species might exert its own version of defense suppression, using species- or isolate-specific molecular components or pathways (such as, for example, coronatine biosynthesis in the case of strain DC3000).

Microorganisms that failed to suppress the flg22-induced Ca^{2+} transient comprise the yeast *S. cerevisiae* as well as the gram-negative bacteria *A. tumefaciens* and *E. coli* (Fig. 3A). These organisms lack flagellae (*S. cerevisiae*), have a flagellin version that cannot be perceived by *Arabidopsis* (*A. tumefaciens*) (Felix et al. 1999; Gómez-Gómez et al. 1999), or, despite the presence of flagellae, are essentially immobile (*E. coli* DH5 α laboratory strain used in this study) (Wood et al. 2006). This commonality may hint at FLS2 desensitization or bacterial motility as a common denominator of the ability to suppress the flg22-triggered Ca^{2+} pattern. However, the *P. syringae* pv. *tomato* DC3000 Δ *flhC* mutant, which lacks flagellae and is rather immobile, largely retained the capacity to interfere with flg22-triggered Ca^{2+} influx (Fig. 5B). Similarly, crude extracts of different *X. campestris* pathovars were not able to trigger FLS2-dependent medium alkalinization (Felix et al. 1999) but the living bacteria have the ability to interfere with flg22-triggered Ca^{2+} influx (Fig. 3A). We also did not measure a significant reduction of the FLS2 pool (Fig. 5A) or depletion of FLS2 from the plasma membrane after treatment with bacterial WT and flagellin-deficient mutant strains but did observe flg22-provoked desensitization of the flg22-induced Ca^{2+} pattern (Fig. 5C). Although the latter finding might support the idea of exposed bacterial MAMPs indirectly attenuating plant immune signaling, we do not know whether the MAMP concentrations used in this experiment reflect physiological levels. It is also noteworthy that we observed little if any cross-desensitization of flg22 perception by unrelated MAMPs such as elf18 or chitin (Fig. 5C). Hence, it is difficult to reconcile why the Δ *flhC* mutant would retain the ability to attenuate MAMP-induced Ca^{2+} influx (Fig. 5B). Thus, although flagellin perception or FLS2 desensitization may contribute to the phenomenon of inhibiting the MAMP-triggered Ca^{2+} signature, these mechanisms are insufficient to explain all findings. Nevertheless, it is conceivable that prolonged MAMP exposition upon plant colonization might support bacterial pathogenesis by desensitizing the host PRR system. Therefore, in summary, we conclude that likely multiple virulence factors (T3SS, coronatine biosynthesis, and presence of flagella), possibly in combination with desensitization of PRRs upon prolonged exposure to bacterial MAMPs, mediate the suppressive effect on the flg22-induced Ca^{2+} pattern.

MATERIALS AND METHODS

Plant materials and plant growth conditions.

Arabidopsis thaliana WT (ecotype Col-0), a transgenic apoaquorin-expressing Col-0 line (*P35S::apoaquorin*) (Knight et al. 1991), and the *fls2c* mutant (Nekrasov et al. 2009; Zipfel et al. 2004) were used in this study. For cultivation of seedlings in hydroponic culture, seeds were surface sterilized with 70% ethanol and then with 100% ethanol (1 min each) and subsequently dried before distribution for cultivation. Seedlings were grown in white 96-well plates (1 seedling/well; Perkin Elmer, Rodgau, Germany) in 160 μ l of sterile 1 $\times$ Murashige-Skoog (MS) medium supplemented with 0.25% sucrose under short-day conditions (10 h light at 22°C and 14 h of darkness at 19°C). At the age of 10 to 14 days, seedlings were inoculated with microorganisms and used for Ca^{2+} measurements, RNA

extraction, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis. For growth in soil, *Arabidopsis* seeds were germinated on wet soil and stratified overnight at 4°C. Plants were maintained under short-day conditions (10 h of light at 22°C and 4 h of darkness at 19°C) for 5 weeks until usage in the respective experiments.

Phytopathogens and other microorganisms.

All *P. syringae* pv. *tomato* DC3000 strains used in this study (Table 2) were grown at 28°C in King's B medium supplemented with suitable antibiotics. Indicated mutations were confirmed by PCR with gene-specific oligonucleotide primers. Instead of genomic DNA, a colony suspension of the strain DC3000 strains prepared in sterile deionized water was used as a template for PCR-based genotyping. Examination of the previously molecularly uncharacterized Δ *algD* mutant revealed a partial duplication of the *algD* gene, resulting in *algD* dysfunction (see Supplementary Fig. S7 for further details). All microorganisms other than strain DC3000 strains used in this study and the respective growth conditions are given in Table 1.

Trypan blue staining of dead tissue.

Mock-inoculated or *P. syringae* pv. *tomato* DC3000-treated seedlings were stained with trypan blue at 1 mg/ml in 1:1:1:1 lactic acid-phenol-glycerol-distilled H_2O for 1 h and destained in 80% ethanol, as described previously (Fernández-Bautista et al. 2016).

Generation of *P. syringae* pv. *tomato* DC3000 deletion strains.

The *P. syringae* pv. *tomato* DC3000 EPS deletion strains (Table 2) were generated via an adaptation of the protocol described elsewhere (Hmelo et al. 2015). Briefly, 500-bp homologous flanking regions to the target genes *wssB/C*, *pslD/E*, and *algG/X* were PCR amplified and ligated individually into the suicide vector *pTSl* (Scott et al. 2017). Gene deletion was done by allelic exchange in a two-step homologous recombination process. Strain DC3000 was transformed with the resulting gene deletion vectors by electroporation and cells with successful homologous recombination, leading to chromosomal integration of the plasmid selected on plates containing tetracycline at 12.5 μ g/ml. Counter selection on 5% sucrose plates was used to identify cells with a second homologous recombination event resulting in excision of the plasmid and in-frame deletion of the respective genes, leaving 6 to 15 bp between the intact start and stop codons. In all cases, deletion strains were confirmed by PCR and sequencing. Strain DC3000 *flhC* was deleted by a similar approach using the pK18*mob-sacB*-derivative allelic exchange vector pCPP5615 (Kvitko et al. 2009). Allelic replacements of *flhC* were screened for loss of motility on 0.2% agar swim plates.

Time-resolved quantification of Ca^{2+} levels in seedlings.

Col-0 (*P35S::apoaquorin*) seedlings were grown for 10 to 14 days under short-day conditions (see above) in white 96-well plates (1 seed/well; Perkin Elmer) in 1 $\times$ MS medium supplemented with 0.25% sucrose (with vitamins and morpholineethanesulfonic acid [MES]). Then, 100 μ l of *P. syringae* pv. *tomato* DC3000 bacteria, other microorganisms (if indicated the microorganisms were heat killed at 95°C for 5 min prior to addition to the seedlings), or indicated MAMPs (1 μ M flg22, 1 mM elf18, or chitin at 0.1 mg/ml) in 0.5 $\times$ MS medium without sucrose were added to the seedlings. The microorganism suspensions were prepared by dilution of an overnight culture to a final theoretical OD₆₀₀ of 0.001. Until 1 day prior to the measurement (performed at 24 or 48 hpi), the seedlings were kept under short-day conditions and then the aequorin substrate coelenterazine (5 mM

stock; Biosynth, Staad, Switzerland) dissolved in methanol was added to yield a final concentration of 10 μ M. The seedlings were incubated overnight at room temperature in the dark (due to the photosensitivity of the coelenterazine). Recordings of MAMP-induced Ca^{2+} signatures were performed as follows. Measurements were accomplished with a Centro XS³ LB 960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) based on at least 12 WT and 24 mutant seedlings per genotype. Baseline luminescence was measured for four cycles and, subsequently, 25 μ l of the MAMP solution was injected into 100 μ l of medium by the luminometer during the fifth cycle,

resulting in a final concentration of 1 μ M flg22, 1 μ M elf18, or chitin at 0.1 mg/ml (all elicitors were solved in 0.25 $\times$ MS medium and injected as 5 $\times$ stock solutions). Luminescence originating from one seedling was repeatedly recorded for 0.25 s at 30-s intervals over a total time of 45 min. Data are given as relative light units (RLUs).

Thereafter, in an independent cycling program, a measurement to determine the total aequorin content was performed. For this purpose 100 μ l of 2 M CaCl_2 in 40% ethanol was injected during the fifth cycle. In this program, RLUs of each seedling were measured every 65 s for 0.25 s over a total time of

Table 2. *Pseudomonas syringae* pv. *tomato* DC3000 wild-type and mutant strains used in this study^a

Designation	Relevant characteristics	Additional information	Antibiotics	Reference or source
Wild-type	...	...	Rif	Buell et al. 2003
$\Delta hrcC$	T3SS deficient	HrcC forms the T3SS outer ring	Rif	Yuan and He 1996; Peñaloza-Vázquez et al. 2000
$\Delta hrpA$	T3SS deficient	HrpA is the major component of the T3SS pilus	Rif, Km	Roine et al. 1997
$\Delta hrpL$	T3SS deficient	HrpL is an alternative σ factor regulating T3SS genes	Rif	Zwiesler-Vollick et al. 2002
$\Delta fliC$	Flagella deficient	Flagellin (<i>fliC</i>) is the flagella filament structural protein	Rif	Kvitko et al. 2009
Δcfa ($\Delta cfl-cfa9$)	COR deficient	The <i>cfa</i> operon codes for biosynthesis of CFA, one of the two precursors of COR		Worley et al. 2012
$\Delta cfa \Delta hrcC$	COR deficient; T3SS deficient	See above	Rif, Sp	Worley et al. 2012
$\Delta fliC \Delta cfa$	Flagella deficient; COR deficient	See above	Rif, Gm	This study
$\Delta fliC \Delta hrcC$	Flagella deficient; T3SS deficient	See above	Rif, Gm	This study
$\Delta fliC \Delta cfa \Delta hrcC$	Flagella deficient; COR deficient; T3SS deficient	See above	Rif, Sp, Gm	This study
$\Delta avrPto$	T3SS effector deficient	AvrPto inhibits the kinase activity of FLS2 and EFR	Rif, Sp	Ronald et al. 1992
$\Delta avrPtoB$	T3SS effector deficient	AvrPtoB is an E3 ubiquitin ligase that degrades PRRs, including FLS2	Rif, Km	Abramovitch et al. 2003
$\Delta avrPto \Delta avrPtoB$	T3SS effector deficient	AvrPto and AvrPtoB block the function of PRR complexes	Rif, Km, Sp	Lin and Martin 2005
$\Delta cmaA$ ($\Delta cmaD-U$)	COR deficient	The <i>cma</i> operon codes for biosynthesis of CMA	Rif, Km, Sp	Worley et al. 2012
$\Delta algD$	Alginate (EPS) deficient	Biosynthesis of EPS alginate	Rif, Km	Aslam et al. 2008
$\Delta wssB/C$	Deficient in an EPS (acetylated form of cellulose)	<i>wssB/C</i> encode the cellulose synthase of the <i>wss</i> operon	Rif	This study
$\Delta pslD/E$	Psl (EPS) deficient	Biosynthesis of Psl	Rif	This study
$\Delta algG/X$	Alginate deficient	Biosynthesis of alginate	Rif	This study
$\Delta wssB/C \Delta pslD/E$	Wss and Psl deficient	Biosynthesis of alginate	Rif	This study
$\Delta wssB/C \Delta algG/X$	Wss and alginate deficient	Biosynthesis of alginate	Rif	This study
$\Delta pslD/E \Delta algG/X$	Psl and alginate deficient	Biosynthesis of alginate	Rif	This study
$\Delta wssB/C \Delta pslD/E \Delta algG/X$	Wss, Psl and alginate deficient	Biosynthesis of alginate	Rif	This study
ΔI	$\Delta hopU1 \Delta hopF2$	T3SS effector cluster deletion	Rif	A. Collmer, unpublished
ΔIV	$\Delta hopD1 \Delta hopR1 \Delta hopQ1-1$	T3SS effector cluster deletion	Rif	Wei et al. 2007
ΔCEL (VI)	$\Delta hopN1 \Delta hopAA1-1 \Delta hopM1 \Delta avrE$	T3SS effector cluster deletion	Rif, Sp	Alfano et al. 2000
ΔIX	$\Delta hopAA1-2 \Delta hopV1 \Delta hopAO1 \Delta hopG1$	T3SS effector cluster deletion	Rif	Wei et al. 2007
ΔX	$\Delta hopAM1-2 \Delta hopX1 \Delta hopO1-1 \Delta hopT1-1$	T3SS effector cluster deletion	Rif	Cunnac et al. 2011
$\Delta \Pi \Delta X$	See above + $\Delta hopH1 \Delta hopC1$	T3SS effector cluster deletion	Rif	Kvitko et al. 2009
$\Delta I \Delta II \Delta IV \Delta CEL \Delta IX \Delta X$	See above	T3SS effector cluster deletion	Rif	Kvitko et al. 2009
$\Delta I \Delta II \Delta IV \Delta CEL \Delta IX \Delta X$ + 10 (DC3000D28E)	See above + $\Delta hopI1 \Delta hopAM1-1 \Delta hopAF1, \Delta avrPtoB \Delta avrPto \Delta hopK1 \Delta hopB1 \Delta hopE1 \Delta hopA1 \Delta hopY1$	T3SS effector cluster deletion + 10 individual effector deletions	Rif	Cunnac et al. 2011
$\Delta hcp1$	T6SS-dependent secreted protein deficient	<i>hcp1</i> gene in the HSI-I T6SS gene cluster	Rif	Haapalainen et al. 2012
$\Delta hcp2$	T6SS-dependent secreted protein deficient	Hcp2 is involved in intermicrobial competition	Rif	Haapalainen et al. 2012
$\Delta hcp1 \Delta hcp2$	See above	See above	Rif	Haapalainen et al. 2012
$\Delta HSI-I$	T6SS deficient	T6SS chromosomal gene cluster; <i>Hcp secretion island-I</i>	Rif	This study
$\Delta HSI-II$	T6SS deficient	T6SS chromosomal gene cluster; <i>Hcp secretion island-II</i>	Rif	This study
$\Delta HSI-I \Delta HSI-II$	See above	See above	Rif	This study

^a Strains were grown in King's B medium or NYG at 28°C with respective antibiotics. Km = kanamycin, Sp = spectinomycin, and Gm = gentamycin. Concentration of all antibiotics was 50 mg/liter. Abbreviations: T3SS = type III secretion system, COR = coronatine, CFA = coronafacic acid, PRR = pattern recognition receptor, CMA = coronamic acid, EPS = exopolysaccharide, and T6SS = type VI secretion system.

30 min. The CaCl_2 /ethanol solution permeated the cell membrane and saturated the aequorin/coelenterazine complexes. The data obtained from this measurement were used to calculate the $[\text{Ca}^{2+}]$ and as well to normalize for variation due to plant condition and size differences.

Calculation of the $\Delta[\text{Ca}^{2+}]_{\text{cyt}}$ and the corresponding relative AUC.

Ca^{2+} concentrations were calculated based on RLUs according to the method of Rentel and Knight (2004). To plot $\Delta[\text{Ca}^{2+}]$, the average $[\text{Ca}^{2+}]$ before the injection was subtracted from the $[\text{Ca}^{2+}]$ at each time point. For the assessment of quantitative differences, the integrals (AUCs) of the curves after the injection peak were calculated (based on at least 12 seedlings/treatment). The integral of the averaged curve after the injection peak was approximated by summing up the products of the means of two consecutive measurements and the respective measurement intervals (30 s) (Supplementary Fig. S8).

Time-resolved quantification of Ca^{2+} levels in leaf discs.

At minimum, six leaf discs (5 mm in diameter) per sample, measured from 5-week-old transgenic (*P35S::apoeaquorin*) *Arabidopsis* plants grown in soil under short-day conditions, were pretreated for 24 or 48 h with 100 μl of strain DC3000 (diluted to $\text{OD}_{600} = 0.001$ in 0.5 $\times$ MS medium without sucrose) in a white 96-well plate (Perkin Elmer). The leaf discs were kept under short-day conditions and, 1 day prior to the measurement, coelenterazine (5 mM stock; Biosynth) dissolved in methanol was added to yield a final concentration of 10 μM . The leaf discs were incubated in the dark at room temperature overnight. The Ca^{2+} measurements and calculations were performed as described above for seedlings.

RNA extraction and cDNA synthesis.

Ten 10-day-old hydroponically grown Col-0 seedlings were treated with 1 μM flg22 (in 0.5 $\times$ MS medium) or mock treated (0.5 $\times$ MS medium) prior to (at 0 h) or after (at 24 and 48 h) inoculation with *P. syringae* pv. *tomato* DC3000 (diluted to $\text{OD}_{600} = 0.001$ in 0.5 $\times$ MS medium without sucrose). All samples were taken at 60 min after MAMP addition and immediately transferred to liquid nitrogen. Total RNA was isolated using the NucleoSpin RNA plus Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's manual. The RNA samples were stored at -80°C until cDNA synthesis. cDNA was synthesized using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's manual. The cDNA samples were stored at -20°C until qRT-PCR analysis. *FRK1* (*At2g19190*) transcript accumulation was quantified with primers 5'-CGGTCAGATTTCAACAGTTGTC-3' (forward primer) and 5'-AATAGCAGGTTGGCCTGTAATC-3' (reverse primer; amplicon size = 142 bp) using the Bio Rad CFX Connect Real-Time System (Bio-Rad, Munich, Germany). The *FRK1* expression level was normalized to the reference gene *At4g26410* (forward primer 5'-GAGCTGAAGTGGCTTCCATGAC-3'; reverse primer 5'-GGTCCGACATACCCATGATCC-3'; amplicon size = 81 bp), which was previously described to be stably expressed under various biotic stress conditions and encodes an uncharacterized conserved protein (Czechowski et al. 2005). Transcript abundance was calculated according to the comparative cycle threshold method (Pfaffl 2001) and values normalized to time point 0 h of Col-0, set to 1. Three technical replicates were performed per sample and experiment.

Protein extraction and immunoblot analysis.

In total, 24 10- to 14-day-old transgenic (*P35S::apoeaquorin*) Col-0 or *fls2c* (negative control) seedlings were sampled immediately (at 0 h) or at 24 and 48 hpi with strain

DC3000 (diluted to $\text{OD}_{600} = 0.001$ in 0.5 $\times$ MS medium without sucrose) and frozen in liquid nitrogen. Extraction buffer (150 μl per 24 seedlings; 250 mM sucrose, 50 mM HEPES-KOH [pH 7.5], 5% glycerol [vol/vol], 0.5% Triton X-100 [vol/vol], 50 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM Na_2MoO_4 , 25 mM NaF, 2 mM dithiothreitol, and Sigma plant protease inhibitor cocktail [EDTA-free]) was added to the frozen plant material and samples were homogenized with an electrical tissue grinder (Heidolph, Schwabach, Germany). The samples were centrifuged at $21,000 \times g$ for 20 min at 4°C . The supernatant (100 μl) was transferred to a new reaction tube and the protein concentration was determined using the Bradford reagent Roti-Quant (Roth, Karlsruhe, Germany). Following sodium dodecyl sulfate electrophoresis, proteins were transferred to a BioTrace pure nitrocellulose membrane (PALL Corporation, Dreieich, Germany), which was subsequently probed with a commercial polyclonal anti-FLS2 antiserum (1:5000; AgriSera, Vännäs, Sweden). The membrane was washed and then probed with a polyclonal horseradish-coupled goat antirabbit immunoglobulin G secondary antibody (1:2000; Cell Signaling Technology, Danvers, MA, U.S.A.). The chemiluminescence detection was carried out using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Dreieich, Germany) according to the provided instructions in a ChemiDoc imaging system of Bio-Rad. For visualization of equal loading of the protein samples, the membrane was stained with Ponceau S solution from AppliChem (Darmstadt, Germany).

Time-resolved quantification of ROS.

Leaf discs (5 mm in diameter) from 6- to 8-week-old Col-0 plants grown in soil under short-day conditions were cut (at least nine leaf discs per sample per measurement) and pretreated for 24 or 48 h with 100 μl of strain DC3000 suspension (diluted to $\text{OD}_{600} = 0.001$ in 0.5 $\times$ MS medium without sucrose) in a white 96-well plate (Perkin Elmer). Until 1 day prior to the measurement, the leaf discs were kept under short-day conditions and then transferred for one night into the dark at room temperature. The next day, the bacterial solution was replaced by 50 μl of sterile deionized water and incubated for 30 min on the bench. To each well, 50 μl of 400 μM luminol dissolved in dimethyl sulfoxide, peroxidase at 20 $\mu\text{g}/\text{ml}$, and 2 μM flg22 were added with a repeating pipette (HandyStep; BrandTech Scientific, Essex, UK). The measurement was started 1 min after addition of the solution in a Centro XS³ LB 960 Microplate Luminometer (Berthold Technologies). The luminescence (RLU) was repeatedly measured for 2 min/leaf disc at 60-s intervals over a total time of 60 min.

Determination of bacterial titer in hydroponically grown seedlings and culture supernatants.

Stably transgenic (*P35S::apoeaquorin*) *Arabidopsis* seedlings were grown in 1 $\times$ MS medium with 0.25% sucrose, vitamins, and MES under short-day conditions for 10 to 14 days in a 96-well plate. *P. syringae* pv. *tomato* DC3000 were grown overnight at 28°C in liquid NYG medium (tryptone or peptone at 5 g/liter, yeast extract at 3 g/liter, and glycerol at 20 ml/liter; pH 7) supplemented with rifampicin at 50 mg/liter. Bacteria were diluted in 1 $\times$ MS medium without sugar but with vitamins and MES to an OD_{600} of 0.001. Then, 75 μl of the bacterial suspension of one genotype was added to single seedlings in each of eight wells after removal of the initial medium. Samples of seedlings and culture supernatant were taken immediately (approximately 30 min) and 4, 24, and 48 h after the inoculation. Seedlings of eight wells were sampled and combined, surface sterilized in 70% ethanol for 30 s, and washed twice in sterile water. The weight of the seedlings was determined

before they were ground in 100 µl of 10 mM MgCl₂ with a plastic pestle. Supernatants of each of the eight wells (40 µl each) were collected and pooled. The ground seedling suspension or the supernatants (10 µl each) and 10 µl of 10⁻⁰ to 10⁻³ (0 and 4 hpi), 10⁻³ to 10⁻⁶ (24 hpi), or 10⁻⁵ to 10⁻⁸ (48 hpi) dilutions were plated in three technical replicates on NYG agar supplemented with rifampicin at 50 mg/liter. Bacteria were grown at 28°C until colonies appeared. CFU were determined relative to the weight of the seedlings or volume of the culture supernatant.

Detection and quantification of membrane-associated FLS2-GFP fluorescence.

Fourteen-day-old WT and transgenic (*PFLS2::FLS2-3xmyc-GFP*) (Beck et al. 2012) *Arabidopsis* seedlings were grown in hydroponic culture and mock treated or inoculated with 75 µl of a strain DC3000 WT, Δ *flhC*, or Δ *flhC* Δ *cfa* Δ *hrcC* suspension (diluted to OD₆₀₀ = 0.001), as described above. At 48 hpi, fluorescence (500 to 520 nm) of seedlings was recorded with a photo multiplier detector of a Leica SP8 confocal microscope with a 63× objective. For each genotype and treatment, single z-layer micrographs of two areas of both cotyledons of eight individual seedlings were taken. Autofluorescence was recorded in WT seedlings that did not express FLS2-GFP. Quantification of the images was performed with CellProfiler (Lamprecht et al. 2007). The membrane-associated fluorescence was determined by subtraction of chloroplastic from total fluorescence and exclusion of background and vesicle-associated fluorescence. Autofluorescence-subtracted FLS2-GFP membrane-associated fluorescence was subsequently determined by subtraction of average Col-0 membrane-associated fluorescence from average membrane-associated FLS2-GFP fluorescence.

Statistical analysis.

Quantitative experimental data were tested by Levene's test to confirm homogeneity of variance followed by one-way analysis of variance and Tukey's honestly significant difference posthoc test using R/Bioconductor (Gentleman et al. 2004).

ACKNOWLEDGMENTS

We thank S. Robatzek (The Sainsbury Laboratory, Norwich, U.K.), R. Wilson Jackson (University of Reading, U.K.), A. Collmer (Cornell University, U.S.A.), and J. Dangel (Chapel Hill, NC, U.S.A.) for providing *P. syringae* pv. *tomato* DC3000 mutants strains; T. Lahaye (Tübingen University, Germany) and L. Blank (RWTH Aachen University, Germany) for providing additional microorganisms; T. Scott (John Innes Centre, Norwich, UK) for providing the *pTSL1* vector; and S. Robatzek (The Sainsbury Lab, Norwich, U.K.) for providing the transgenic *Arabidopsis PFLS2::FLS2-GFP* line.

LITERATURE CITED

Abramovitch, R. B., Kim, Y. J., Chen, S., Dickman, M. B., and Martin, G. B. 2003. *Pseudomonas* type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J.* 22:60-69.

Alfano, J. R., Charkowski, A. O., Deng, W. L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97:4856-4861.

Aslam, S. N., Erbs, G., Morrissey, K. L., Newman, M. A., Chinchilla, D., Boller, T., Molinaro, A., Jackson, R. W., and Cooper, R. M. 2009. Microbe-associated molecular pattern (MAMP) signatures, synergy, size and charge: Influences on perception or mobility and host defence responses. *Mol. Plant Pathol.* 10:375-387.

Aslam, S. N., Newman, M. A., Erbs, G., Morrissey, K. L., Chinchilla, D., Boller, T., Jensen, T. T., De Castro, C., Ierano, T., Molinaro, A., Jackson, R. W., Knight, M. R., and Cooper, R. M. 2008. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Curr. Biol.* 18:1078-1083.

Beck, M., Zhou, J., Faulkner, C., MacLean, D., and Robatzek, S. 2012. Spatio-temporal cellular dynamics of the *Arabidopsis* flagellin receptor reveal activation status-dependent endosomal sorting. *Plant Cell* 24: 4205-4219.

Bender, C. L., Alarcón-Chaidez, F., and Gross, D. C. 1999. *Pseudomonas syringae* phytotoxins: Mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiol. Mol. Biol. Rev.* 63:266-292.

Blume, B., Nürnberger, T., Nass, N., and Scheel, D. 2000. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. *Plant Cell* 12:1425-1440.

Brooks, D. M., Hernández-Guzmán, G., Kloeck, A. P., Alarcón-Chaidez, F., Sreedharan, A., Rangaswamy, V., Peñaloza-Vázquez, A., Bender, C. L., and Kunkel, B. N. 2004. Identification and characterization of a well-defined series of coronatine biosynthetic mutants of *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant-Microbe Interact.* 17:162-174.

Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L., Dodson, R. J., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Daugherty, S., Brinkac, L., Beanan, M. J., Haft, D. H., Nelson, W. C., Davidsen, T., Zafar, N., Zhou, L., Liu, J., Yuan, Q., Khouri, H., Fedorova, N., Tran, B., Russell, D., Berry, K., Utterback, T., Van Aken, S. E., Feldblyum, T. V., D'Ascenzo, M., Deng, W. L., Ramos, A. R., Alfano, J. R., Cartinhour, S., Chatterjee, A. K., Delaney, T. P., Lazarowitz, S. G., Martin, G. B., Schneider, D. J., Tang, X., Bender, C. L., White, O., Fraser, C. M., and Collmer, A. 2003. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. U.S.A.* 100:10181-10186.

Büttner, D. 2016. Behind the lines-actions of bacterial type III effector proteins in plant cells. *FEMS Microbiol. Rev.* 40:894-937.

Byrd, M. S., Sadovskaya, I., Vinogradov, E., Lu, H., Sprinkle, A. B., Richardson, S. H., Ma, L., Ralston, B., Parsek, M. R., Anderson, E. M., Lam, J. S., and Wozniak, D. J. 2009. Genetic and biochemical analyses of the *Pseudomonas aeruginosa* Psl exopolysaccharide reveal overlapping roles for polysaccharide synthesis enzymes in Psl and LPS production. *Mol. Microbiol.* 73:622-638.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D. G., Felix, G., and Boller, T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448:497-500.

Cunnac, S., Chakravarthy, S., Kvitko, B. H., Russell, A. B., Martin, G. B., and Collmer, A. 2011. Genetic disassembly and combinatorial reassembly identify a minimal functional repertoire of type III effectors in *Pseudomonas syringae*. *Proc. Natl. Acad. Sci. U.S.A.* 108:2975-2980.

Cunnac, S., Lindeberg, M., and Collmer, A. 2009. *Pseudomonas syringae* type III secretion system effectors: Repertoires in search of functions. *Curr. Opin. Microbiol.* 12:53-60. doi.org/10.1016/j.mib.2008.12.003

Cuppels, D. A. 1986. Generation and Characterization of *Tn5* Insertion Mutations in *Pseudomonas syringae* pv. *tomato*. *Appl. Environ. Microbiol.* 51:323-327.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., and Scheible, W. R. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* 139:5-17.

Deng, W., Marshall, N. C., Rowland, J. L., McCoy, J. M., Worrall, L. J., Santos, A. S., Strynadka, N. C. J., and Finlay, B. B. 2017. Assembly, structure, function and regulation of type III secretion systems. *Nat. Rev. Microbiol.* 15:323-337.

Denny, T. P. 1995. Involvement of bacterial polysaccharides in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:173-197.

de Torres, M., Mansfield, J. W., Grabov, N., Brown, I. R., Ammounieh, H., Tsiamis, G., Forsyth, A., Robatzek, S., Grant, M., and Boch, J. 2006. *Pseudomonas syringae* effector AvrPtoB suppresses basal defence in *Arabidopsis*. *Plant J.* 47:368-382.

Economou, A., Christie, P. J., Fernandez, R. C., Palmer, T., Plano, G. V., and Pugsley, A. P. 2006. Secretion by numbers: Protein traffic in prokaryotes. *Mol. Microbiol.* 62:308-319.

Erhardt, M., Namba, K., and Hughes, K. T. 2010. Bacterial nanomachines: The flagellum and type III injectisome. *Cold Spring Harb. Perspect. Biol.* 2:a000299.

Faulkner, C., Petutschnig, E., Benitez-Alfonso, Y., Beck, M., Robatzek, S., Lipka, V., and Maule, A. J. 2013. LYM2-dependent chitin perception limits molecular flux via plasmodesmata. *Proc. Natl. Acad. Sci. U.S.A.* 110:9166-9170.

- Felix, G., Duran, J. D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* 18:265-276.
- Fernández-Bautista, N., Domínguez-Núñez, J., Moreno, M. M., and Berrocal-Lobo, M. 2016. Plant tissue trypan blue staining during phytopathogen infection. *Bio Protoc.* 6:e2078.
- Gal, M., Preston, G. M., Massey, R. C., Spiers, A. J., and Rainey, P. B. 2003. Genes encoding a cellulosic polymer contribute toward the ecological success of *Pseudomonas fluorescens* SBW25 on plant surfaces. *Mol. Ecol.* 12:3109-3121.
- Geng, X., Jin, L., Shimada, M., Kim, M. G., and Mackey, D. 2014. The phytotoxin coronatine is a multifunctional component of the virulence armament of *Pseudomonas syringae*. *Planta* 240:1149-1165.
- Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y. H., and Zhang, J. 2004. Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol.* 5:R80.
- Gimenez-Ibanez, S., Hann, D. R., Ntoukakis, V., Petutschnig, E., Lipka, V., and Rathjen, J. P. 2009. AvrPtoB targets the LysM receptor kinase CERK1 to promote bacterial virulence on plants. *Curr. Biol.* 19:423-429.
- Goddard, M. R., and Greig, D. 2015. *Saccharomyces cerevisiae*: A nomadic yeast with no niche? *FEMS Yeast Res.* 15:fov009.
- Göhre, V., Spallek, T., Häweker, L., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J. W., and Robatzek, S. 2008. Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* 18:1824-1832.
- Gómez-Gómez, L., Felix, G., and Boller, T. 1999. A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* 18:277-284.
- Green, E. R., and Mecsas, J. 2016. Bacterial secretion systems: An overview. Pages 215-239 in: *Virulence Mechanisms of Bacterial Pathogens*, Fifth ed. I. Kudva, N. Cornick, P. Plummer, Q. Zhang, T. Nicholson, J. Bannantine, and B. Bellaire, eds. American Society for Microbiology Press, Washington, DC.
- Guo, M., Tian, F., Wamboldt, Y., and Alfano, J. R. 2009. The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. *Mol. Plant-Microbe Interact.* 22:1069-1080.
- Haapalainen, M., Mosorin, H., Dorati, F., Wu, R.-F., Roine, E., Taira, S., Nissinen, R., Mattinen, L., Jackson, R., Pirhonen, M., and Lin, N.-C. 2012. Hcp2, a secreted protein of the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000, is required for fitness for competition against bacteria and yeasts. *J. Bacteriol.* 194:4810-4822.
- Hauck, P., Thilmony, R., and He, S. Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. U.S.A.* 100:8577-8582.
- Hmel, L. R., Borlee, B. R., Almlad, H., Love, M. E., Randall, T. E., Tseng, B. S., Lin, C., Irie, Y., Storek, K. M., Yang, J. J., Siehnell, R. J., Howell, P. L., Singh, P. K., Tolker-Nielsen, T., Parsek, M. R., Schweizer, H. P., and Harrison, J. J. 2015. Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. *Nat. Protoc.* 10:1820-1841.
- Jain, S., Franklin, M. J., Ertesvåg, H., Valla, S., and Ohman, D. E. 2003. The dual roles of AlgG in C-5-epimerization and secretion of alginate polymers in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 47:1123-1133.
- Janjusevic, R., Abramovitch, R. B., Martin, G. B., and Stebbins, C. E. 2006. A bacterial inhibitor of host programmed cell death defenses is an E3 ubiquitin ligase. *Science* 311:222-226.
- Jeworutzki, E., Roelfsema, M. R. G., Anschutz, U., Krol, E., Elzenga, J. T. M., Felix, G., Boller, T., Hedrich, R., and Becker, D. 2010. Early signaling through the *Arabidopsis* pattern recognition receptors FLS2 and EFR involves Ca^{2+} -associated opening of plasma membrane anion channels. *Plant J.* 62:367-378.
- Kim, Y. J., Lin, N. C., and Martin, G. B. 2002. Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. *Cell* 109:589-598.
- Knight, M. R., Campbell, A. K., Smith, S. M., and Trewavas, A. J. 1991. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352:524-526.
- Koncz, C., and Schell, J. 1986. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* 204:383-396.
- Kvitko, B. H., Park, D. H., Velásquez, A. C., Wei, C.-F., Russell, A. B., Martin, G. B., Schneider, D. J., and Collmer, A. 2009. Deletions in the repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion effector genes reveal functional overlap among effectors. *PLoS Pathog.* 5:e1000388.
- Kwaaitaal, M., Huisman, R., Maintz, J., Reinstädler, A., and Panstruga, R. 2011. Ionotropic glutamate receptor (iGluR)-like channels mediate MAMP-induced calcium influx in *Arabidopsis thaliana*. *Biochem. J.* 440:355-373.
- Lam, H. N., Chakravarthy, S., Wei, H.-L., BuiNguyen, H., Stodghill, P. V., Collmer, A., Swingle, B. M., and Cartinhour, S. W. 2014. Global analysis of the HrpL regulon in the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 reveals new regulon members with diverse functions. *PLoS One* 9:e106115.
- Lamprecht, M. R., Sabatini, D. M., and Carpenter, A. E. 2007. CellProfiler: Free, versatile software for automated biological image analysis. *Biotechniques* 42:71-75.
- Lecourieux, D., Ranjeva, R., and Pugin, A. 2006. Calcium in plant defence-signalling pathways. *New Phytol.* 171:249-269.
- Lin, N.-C., and Martin, G. B. 2005. An *avrPto* mutant of *Pseudomonas syringae* pv. *tomato* DC3000 does not elicit Pto-mediated resistance and is less virulent on tomato. *Mol. Plant-Microbe Interact.* 18:43-51.
- Lindeberg, M., Stavrinides, J., Chang, J. H., Alfano, J. R., Collmer, A., Dangel, J. L., Greenberg, J. T., Mansfield, J. W., and Guttman, D. S. 2005. Proposed guidelines for a unified nomenclature and phylogenetic analysis of type III Hop effector proteins in the plant pathogen *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 18:275-282.
- Macho, A. P., Zumaquero, A., Ortiz-Martín, I., and Beuzón, C. R. 2007. Competitive index in mixed infections: A sensitive and accurate assay for the genetic analysis of *Pseudomonas syringae*-plant interactions. *Mol. Plant Pathol.* 8:437-450.
- Melotto, M., Underwood, W., and He, S. Y. 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu. Rev. Phytopathol.* 46:101-122.
- Melotto, M., Underwood, W., Koczan, J., Nomura, K., and He, S. Y. 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell* 126:969-980.
- Moore, R. A., Starratt, A. N., Ma, S. W., Morris, V. L., and Cuppels, D. A. 1989. Identification of a chromosomal region required for biosynthesis of the phytotoxin coronatine by *Pseudomonas syringae* pv. *tomato*. *Can. J. Microbiol.* 35:910-917.
- Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z.-H., Lacombe, S., Rougon, A., Bittel, P., Kiss-Papp, M., Chinchilla, D., van Esse, H. P., Jorda, L., Schwesinger, B., Nicaise, V., Thomma, B. P. H. J., Molina, A., Jones, J. D. G., and Zipfel, C. 2009. Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J.* 28:3428-3438.
- Nelson, K. E., Weinl, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A. P., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Chris Lee, P., Holtzapple, E., Scanlan, D., Tran, K., Moazzes, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J. A., Timmis, K. N., Dusterhöft, A., Tümmler, B., and Fraser, C. M. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* 4:799-808.
- Peñaloza-Vázquez, A., Preston, G. M., Collmer, A., and Bender, C. L. 2000. Regulatory interactions between the Hrp type III protein secretion system and coronatine biosynthesis in *Pseudomonas syringae* pv. *tomato* DC3000. *Microbiology* 146:2447-2456.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.
- Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J., and Scheel, D. 2011. Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J.* 68:100-113.
- Rentel, M. C., and Knight, M. R. 2004. Oxidative stress-induced calcium signaling in *Arabidopsis*. *Plant Physiol.* 135:1471-1479.
- Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.-L., Kalkkinen, N., Romantschuk, M., and He, S. Y. 1997. Hrp pilus: An Hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. U.S.A.* 94:3459-3464.
- Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskiewicz, B. J. 1992. The cloned avirulence gene *avrPto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. *J. Bacteriol.* 174:1604-1611.
- Sarris, P. F., Skandalis, N., Kokkinidis, M., and Panopoulos, N. J. 2010. *In silico* analysis reveals multiple putative type VI secretion systems and effector proteins in *Pseudomonas syringae* pathovars. *Mol. Plant Pathol.* 11:795-804.
- Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavelle, D. T., Michelsmore, R. W., and Staskiewicz, B. J. 1996. Molecular basis

- of gene-for-gene specificity in bacterial speck disease of tomato. *Science* 274:2063-2065.
- Scott, T. A., Heine, D., Qin, Z., and Wilkinson, B. 2017. An L-threonine transaldolase is required for L-threo- β -hydroxy- α -amino acid assembly during obafluorin biosynthesis. *Nat. Commun.* 8: Article 15935.
- Shan, L., He, P., Li, J., Heese, A., Peck, S. C., Nürnberger, T., Martin, G. B., and Sheen, J. 2008. Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell Host Microbe* 4:17-27.
- Spiers, A. J., Kahn, S. G., Bohannon, J., Travisano, M., and Rainey, P. B. 2002. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. I. Genetic and phenotypic bases of wrinkly spreader fitness. *Genetics* 161:33-46.
- Taylor, R. G., Walker, D. C., and McInnes, R. R. 1993. *E. coli* host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. *Nucleic Acids Res.* 21:1677-1678.
- Thieme, F., Koebnik, R., Bekel, T., Berger, C., Boch, J., Büttner, D., Caldana, C., Gaigalat, L., Goesmann, A., Kay, S., Kirchner, O., Lanz, C., Linke, B., McHardy, A. C., Meyer, F., Mittenhuber, G., Nies, D. H., Niesbach-Klösgen, U., Patschkowski, T., Rückert, C., Rupp, O., Schneiker, S., Schuster, S. C., Vorhölter, F.-J., Weber, E., Pühler, A., Bonas, U., Bartels, D., and Kaiser, O. 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. *J. Bacteriol.* 187:7254-7266.
- Toruño, T. Y., Stergiopoulos, I., and Coaker, G. 2016. Plant-pathogen effectors: Cellular probes interfering with plant defenses in spatial and temporal manners. *Annu. Rev. Phytopathol.* 54:419-441.
- Üstün, S., Müller, P., Palmisano, R., Hensel, M., and Börnke, F. 2012. SseF, a type III effector protein from the mammalian pathogen *Salmonella enterica*, requires resistance-gene-mediated signalling to activate cell death in the model plant *Nicotiana benthamiana*. *New Phytol.* 194:1046-1060.
- Vasil, M. L., Tomaras, A. P., and Pritchard, A. E. 2012. Identification and evaluation of twin-arginine translocase inhibitors. *Antimicrob. Agents Chemother.* 56:6223-6234.
- Vatsa, P., Chiltz, A., Luini, E., Vandelle, E., Pugin, A., and Roblin, G. 2011. Cytosolic calcium rises and related events in ergosterol-treated *Nicotiana* cells. *Plant Physiol. Biochem.* 49:764-773.
- Vuong, C., Kocianova, S., Voyich, J. M., Yao, Y., Fischer, E. R., DeLeo, F. R., and Otto, M. 2004. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J. Biol. Chem.* 279:54881-54886.
- Wei, C.-F., Kvitko, B. H., Shimizu, R., Crabill, E., Alfano, J. R., Lin, N.-C., Martin, G. B., Huang, H.-C., and Collmer, A. 2007. A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. *Plant J.* 51:32-46.
- Wood, D. W., Setubal, J. C., Kaul, R., Monks, D. E., Kitajima, J. P., Okura, V. K., Zhou, Y., Chen, L., Wood, G. E., Almeida, N. F., Jr., Woo, L., Chen, Y., Paulsen, I. T., Eisen, J. A., Karp, P. D., Bovee, D., Sr., Chapman, P., Clendenning, J., Deatherage, G., Gillet, W., Grant, C., Kutuyavin, T., Levy, R., Li, M. J., McClelland, E., Palmieri, A., Raymond, C., Rouse, G., Saenphimmachak, C., Wu, Z., Romero, P., Gordon, D., Zhang, S., Yoo, H., Tao, Y., Biddle, P., Jung, M., Krespan, W., Perry, M., Gordon-Kamm, B., Liao, L., Kim, S., Hendrick, C., Zhao, Z. Y., Dolan, M., Chumley, F., Tingey, S. V., Tomb, J. F., Gordon, M. P., Olson, M. V., and Nester, E. W. 2001. The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* 294:2317-2323.
- Wood, T. K., González Barrios, A. F., Herzberg, M., and Lee, J. 2006. Motility influences biofilm architecture in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 72:361-367.
- Worley, J. N., Russell, A. B., Wexler, A. G., Bronstein, P. A., Kvitko, B. H., Krasnoff, S. B., Munkvold, K. R., Swingle, B., Gibson, D. M., and Collmer, A. 2012. *Pseudomonas syringae* pv. *tomato* DC3000 CmaL (PSPT04723), a DUF1330 family member, is needed to produce L-*allo*-isoleucine, a precursor for the phytotoxin coronatine. *J. Bacteriol.* 195:287-296.
- Xiang, T., Zong, N., Zou, Y., Wu, Y., Zhang, J., Xing, W., Li, Y., Tang, X., Zhu, L., Chai, J., and Zhou, J. M. 2008. *Pseudomonas syringae* effector AvrPto blocks innate immunity by targeting receptor kinases. *Curr. Biol.* 18:74-80.
- Xin, X.-F., and He, S. Y. 2013. *Pseudomonas syringae* pv. *tomato* DC3000: A model pathogen for probing disease susceptibility and hormone signaling in plants. *Annu. Rev. Phytopathol.* 51:473-498.
- Yuan, J., and He, S. Y. 1996. The *Pseudomonas syringae* Hrp regulation and secretion system controls the production and secretion of multiple extracellular proteins. *J. Bacteriol.* 178:6399-6402.
- Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D. G., Felix, G., and Boller, T. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428:764-767.
- Zwiesler-Vollick, J., Plovianich-Jones, A. E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B. N., and He, S. Y. 2002. Identification of novel hrp-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. *Mol. Microbiol.* 45:1207-1218.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Biotechnology and Biological Sciences Research Council:
<https://bbsrc.ukri.org>
 Deutsche Forschungsgemeinschaft: <http://www.dfg.de>
 DSMZ catalog number 50090:
<https://www.dsmz.de/catalogues/details/culture/dsm-50090.html>
 DSMZ catalog number 347:
<https://www.dsmz.de/catalogues/details/culture/dsm-347.html>
 DSMZ catalog number 9509:
<https://www.dsmz.de/catalogues/details/culture/DSM-9509.html>
 Excellence Initiative of the German federal and state governments:
http://www.dfg.de/en/research_funding/programmes/excellence_initiative
 Gatsby Charitable Foundation: <http://www.gatsby.org.uk>
 Norwich Research Park: <https://www.norwichresearchpark.com>
 RWTH Aachen University: <https://www.rwth-aachen.de>

This article was modified on 21 Jan 2022.

ERRATUM / Volume 32, No. 5, 2019 / [MPMI-10-18-0291-R](#)

In the article “Widely Conserved Attenuation of Plant MAMP-Induced Calcium Influx by Bacteria Depends on Multiple Virulence Factors and May Involve Desensitization of Host Pattern Recognition Receptors” by Meltem Lammertz, Hannah Kuhn, Sebastian Pfeilmeier, Jacob Malone, Cyril Zipfel, Mark Kwaaitaal, Nai-Chun Lin, Brian H. Kvitko, and Ralph Panstruga, the Materials and Methods section erroneously stated that the *fls2-17* mutant was used for our experiments. In fact, we used the *fls2c* mutant (harboring the *P35S::apoaquorin* transgene) in our study (Fig. 5A).

While the *fls2-17* mutant is in the genetic background of the *Arabidopsis thaliana* ecotype Landsberg *erecta* (La-*er*) and harbors a point mutation in the kinase domain (G0164R) of the FLS2 protein, the *fls2c* mutant is a T-DNA insertion null mutant (SAIL_691C4) in the genetic background of the *A. thaliana* ecotype Col-0. This T-DNA mutant was first described by Zipfel et al. (2004) and later termed *fls2c* due to its genetic background (Col-0) (Nekrasov et al. 2009). We apologize for any potential confusion caused.