Phevamine A, a small molecule that suppresses plant immune responses

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Bacterial plant pathogens cause significant crop damage worldwide. They invade plant cells by producing a variety of virulence factors, including small-molecule toxins and phytotaxonomic mimics. Virulence of the model pathogen Pseudomonas syringae pv. tomato DC3000 (Pto) is regulated in part by the sigma factor HrpL. Our study of the HrpL regulon identified an uncharacterized, three-gene operon in Pto that is controlled by HrpL and related to the Erwinia hsp-associated systemic virulence (hsv) operon. Here, we demonstrate that the hsv operon contributes to the virulence of Pto on Arabidopsis thaliana and suppresses bacteria-induced immune responses. We show that the hsv-encoded enzymes in Pto synthesize a small molecule, phevamine A. This molecule consists of 1-phenylalanine, 1-valine, and a modified spermidine, and is different from known small molecules produced by phytopathogens. We show that phevamine A suppresses a potentiation effect of spermidine and L-arginine on the reactive oxygen species burst generated upon recognition of bacterial flagellin. The hsv operon is found in the genomes of divergent bacterial genera, including ∼37% of P. syringae genomes, suggesting that phevamine A is a widely distributed virulence factor in phytopathogens. Our work identifies a small-molecule virulence factor and reveals a mechanism by which bacterial pathogens overcome plant defense. This work highlights the power of omics approaches in identifying important small molecules in bacteria–host interactions.

Significance

Bacterial pathogens cause plant diseases that threaten the global food supply. To control diseases, it is important to understand how pathogenic bacteria evade plant defense and promote infection. We identify from the phytopathogen Pseudomonas syringae a small-molecule virulence factor—phevamine A. Both the chemical structure and mode of action of phevamine A are different from known bacterial phytotoxins. Phevamine A promotes bacterial growth by suppressing plant immune responses, including both early (the generation of reactive oxygen species) and late (the deposition of cell wall reinforcing callose in leaves and leaf cell death) markers. This work uncovers a widely distributed, small-molecule virulence factor and shows the power of a multidisciplinary approach to identify small molecules important for plant infection.


The authors declare no conflict of interest.

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operon synthesize a small molecule that is important for virulence. By integrating heterologous expression, metabolomics, and in vitro biosynthesis, we identify a bioactive small molecule, phevamine A, a conjugate of L-phenylalanine, L-valine, and a modified spermidine. We further show that phevamine A suppresses the potentiation of MAMP-induced ROS bursts by spermidine and L-arginine. Thus, phevamine A is a small-molecule virulence factor that promotes bacterial growth and virulence, in part by suppressing plant immune responses.

Results and Discussion

The P. syringae hsv Operon Promotes Virulence and Suppresses Defense Responses. To investigate the function of the hsv operon, we first generated independent clean deletion mutants in Pto. The parental strain, PtoΔhsv1, PtoΔhsv2 were used to infect A. thaliana Col-0 seedlings by dip inoculation (SI Appendix, Materials and Methods) and the growth of these bacteria was monitored. We observed that the mutants grew slightly less than the Pto strains (SI Appendix, Fig. S2) in two independent experiments, but this reduced growth trend was not statistically significant. This observation is not surprising, as Pto is an aggressive pathogen on A. thaliana and its large suite of virulence factors can act collectively (17). It is therefore rare to observe significant loss of virulence phenotypes when deleting a single candidate virulence factor from this strain. To circumvent this problem, we used a weakly pathogenic Pto derivative deficient in coronatine production (Pto DC3118, hereafter Pto-Cor−) (17) to generate independent hsv mutants Pto-Cor−Δhsv1, and Pto-Cor−Δhsv2. Three days postinfection, significantly less bacteria for Pto-Cor−Δhsv1 and Pto-Cor−Δhsv2 were recovered from the seedlings than Pto-Cor− (Fig. L1). This result indicates that hsv is required for full virulence in planta.

We next addressed whether the hsv operon had an effect on host callus deposition, a cell wall reinforcing response typically triggered during MTI (18, 19) (SI Appendix, Fig. S1). Several type III secretion effector proteins can inhibit callus deposition (20–24) and thus may obscure phenotypes resulting from hsv deletion. We therefore generated two independent hsv mutants in PtoΔ28E (PtoΔ28EΔhsv1 and PtoΔ28EΔhsv2), a strain lacking 28 different type III secretion effectors (25). PtoΔ28E, PtoΔ28EΔhsv1, and PtoΔ28EΔhsv2 were used to infiltrate 4-wk-old leaves of A. thaliana. Both PtoΔ28EΔhsv alleles induced higher callus deposition than PtoΔ28E (Fig. 1B); complementation of PtoΔ28EΔhsv2 with constitutively expressed hsv reduced the level of callus back to that induced by PtoΔ28E (Fig. 1C). These results demonstrate that hsv suppresses a late marker of plant defense at the cell wall.

Conversely, we also examined whether hsv expression is sufficient to suppress plant callus deposition induced by a nonpathogenic bacterium. The hsv operon was constitutively expressed in Pseudomonas fluorescens P0-1 (P0-1), a strain that lacks both hsv and a T3SS, but still induces callus deposition (Fig. 1D) (26). Expression of hsv had no effect on the level of P0-1-induced callus deposition (Fig. 1D). A P0-1 derivative engineered to contain a complete P. syringae T3SS locus (P0-1+T3SS) (27) induced more callus than wild-type P0-1 (SI Appendix, Fig. S3), suggesting that P0-1 T3SS components are recognized by the plant (28). Expression of hsv in the P0-1+T3SS strain reduced the level of callus back to the level elicited by wild-type P0-1 (Fig. 1D and SI Appendix, Fig. S3). Thus, hsv suppresses the callus deposition induced by P0-1 T3SS components. To extend this observation, we evaluated the activities of these P. fluorescens strains on a second plant species, N. benthamiana. Infiltration of N. benthamiana leaves with P0-1 or P0-1-expressing hsv had no effect; the tissue infiltrated with bacteria was identical to the noninfiltrated tissue (Fig. 1E). In contrast, infiltration with P0-1+T3SS elicited a dose-dependent leaf cell death. This response was suppressed by hsv expression (Fig. 1E). Thus, hsv suppresses the cell death caused by a component of the T3SS or the linked harpin gene transferred from P. syringae pv. syringae to P0-1 (29, 30).

 hsv-Encoded Enzymes Synthesize Three Related Small Molecules. The hsv operon encodes three enzymes, including a putative amidotransferase, HsvA, and two putative ATP-grasp-type enzymes, HsvB and HsvC (Fig. 2A). To test the hypothesis that these hsv-encoded enzymes synthesize small molecules, we conducted hsv heterologous expression and comparative metabolomics experiments. The hsv cluster was overexpressed in Escherichia coli as a heterologous host to enhance small molecule production. Organic extracts of culture supernatants were analyzed using liquid chromatography-coupled high-resolution mass spectrometry (LC-HRMS) to generate metabolomic profiles for comparison. Three species with mass-to-charge ratios (m/z) of 287.255, 434.324, and 581.392 were present at high levels in the hsv-expressing E. coli, but were absent in the control E. coli carrying the empty vector (Fig. 2B and SI Appendix, Figs. S4–S6). The species with the m/z of 287.255 and 434.324 were also detected by LC-HRMS in the culture extract of the wild-type Pto, but not in PtoΔhsv (Fig. 2C and SI Appendix, Fig. S5). The production of the metabolite with the m/z of 434.324 is also observed in Pto overexpressing hrpL−, but is reduced by hrpL− deletion (Fig. 2C), consistent with the regulation of hsv by hrpL− (12). This metabolite was detected at the same level in the supernatants of both P0-1- and P0-1+T3SS-expressing hsv (SI Appendix, Fig. S7), indicating that secretion of the compound is independent of the T3SS.

We analyzed the structures of the identified metabolites from E. coli and P. syringae strains expressing the hsv operon by tandem MS, which revealed that these metabolites share similar MS fragments (SI Appendix, Figs. S8 and S9) and likely possess related structures. The mass differences between 287.255 and 434.324, and between 434.324 and 581.392 are both 147.068, corresponding to the mass of a phenylalanine in an amide linkage. Based on these data, we propose that the structures of the compounds with the m/z of 434.324 and 581.392 contain one and two phenylalanines, respectively. We name the identified metabolites phevamine A (m/z 434.324; PHVA), phevamine B (m/z 581.392; PHVB) and phevamine C (m/z 287.255; PFHV).

In Vitro Biosynthesis of Phevamines Reveals Structures. To characterize the structures of phevamines, we reconstituted the biosynthetic enzymes encoded by the hsv operon in vitro. HsvA shares homology with amidotransferases that transfer an amido group from arginine to an amine, suggesting the presence of an amidino group. HsvB and HsvC do not belong to the amidotransferase family, and it is likely that these enzymes, HsvB and HsvC, are involved in the biosynthesis of phevamines with amido groups.


that L-phenylalanine is a structural component of the phevamines (SI Appendix, Fig. S12). To validate this proposal, we incubated prephevamine with HsvB and L-Phe, and the formation of phevamine A and phevamine B was indeed observed (Fig. 3 and SI Appendix, Figs. S10 and S13). This result also indicates that HsvB can catalyze two rounds of condensation with L-Phe.

Tandem MS analysis of phevamine A suggests that the amidino group is connected to the propylamine end of spermidine (Fig. 3B and SI Appendix, Fig. S9).

Having identified that phevamines consist of L-Phe, L-Val, and amidinospermidine, we next determined the connectivity of these components by NMR analysis. Due to high polarity, phevamines were difficult to isolate from bacterial culture extracts. To isolate sufficient materials for NMR, we developed in vitro biosynthetic methods for phevamine A and phevamine B (Methods). From 16 mL of in vitro enzymatic assays, 5.7 mg of phevamine A and 5.5 mg of phevamine B were purified. These compounds were analyzed by $^1$H, ($^1$H, $^1$H)-COSY, ($^1$H, $^{13}$C)-HMBC, and ($^1$H, $^{13}$C)-HSQC NMR experiments (Fig. 3C and SI Appendix, Figs. S14–S21). The NMR data support that the amidino group is linked to the propylamine side of spermidine and that phevamine B contains an additional L-Phe at the N-terminus of phevamine A. Phevamine B was only detected under conditions of heterologous expression and in vitro synthesis, but not in the culture extract of Pto (SI Appendix, Fig. S6), suggesting that phevamine B is unlikely a physiologically relevant molecule. The production of phevamine B may be due to the overexpression of HsvB in E. coli and the high concentration of HsvB and L-Phe added in vitro. Thus, we focused on phevamine A for further structural characterization. To confirm the absolute stereochemistry, we developed a total synthesis method for phevamine A (SI Appendix, Materials and Methods). Synthetic
and in vitro isolated phevamine A exhibit identical $^{1}$H and $^{13}$C NMR signals, and the same LC retention time and MS fragmentation pattern as the species detected in the bacterial culture extract (Fig. 3D and SI Appendix, Figs. S14–S22). These results confirmed the structure assignment for phevamine A and provided materials for biological activity testing of this molecule. In an initial activity test, we examined the ability of phevamine A to bind ferric iron using a chromeazurol S assay and observed no significant binding to ferric iron (SI Appendix, Fig. S23), suggesting phevamine A is unlikely acting as a siderophore.

Comparative Genomics Suggests a Potential Host Target for Phevamines. We investigated the phylogenetic distribution of hsv in bacterial genomes using MultiGeneBlast that identifies homologous gene clusters based on sequence similarity and gene synteny (34). We found that hsv is widely distributed across plant-pathogenic bacterial genera including Pseudomonas, Erwinia, and Pantoea. We compared the distribution of the hsv operon to those biosynthetic gene clusters encoding known P. syringae phytotoxins, focusing on coronatine, mangotoxin, syringolin, phaseolotoxin, and tabtoxin (Fig. 4A and Dataset S1) (1, 35). The hsv operon is present in ~37% of P. syringae (107 out of 292) and is the most widely distributed gene cluster of the six analyzed. The hsv operon rarely cooccurs with any of the aforementioned small-molecule biosynthetic clusters. This anticorrelation is especially prominent between hsv and phaseolotoxin biosynthetic genes in the genomes of otherwise extremely closely related strains of P. syringae pv. actinidiae (36). We previously showed that functionally redundant virulence factors rarely cooccur in the same strain (37). Specifically, the small molecule coronatine and three different type III secretion system protein effectors use four distinct mechanisms to target the same host defense signaling pathway, and only genes responsible for a single mechanism are typically found in any given P. syringae genome (37). Based on the clear anticorrelation of hsv with the phaseolotoxin biosynthetic genes, and the knowledge that phaseolotoxin targets host arginine and polyamine biosynthesis by inhibiting ornithine carbamoyltransferase and ornithine decarboxylase (Fig. 4B) (4, 5, 38), we hypothesized that the action of phevamine A involves polyamides and/or arginine. This hypothesis is consistent with previous implication of polyamine function in plant immune responses (39, 40). Furthermore, our genomic analysis revealed that many P. syringae strains do not contain the biosynthetic gene clusters for phevamine A or the five other phytotoxins described herein (Fig. 4A). These strains could produce different small molecules that might play a role in bacteria-plant interaction.

**Polyamines and Arginine Potentiate an Early MTI Response.** We investigated the effect of arginine and polyamides on the MTI response induced by the well-studied MAMP flg22, a short peptide derived from the Pseudomonas flagellin (18). We monitored the ROS burst following recognition of flg22 by the pattern recognition receptor FLS2 (SI Appendix, Fig. S1) (41, 42). The polyamines putrescine, spermidine, and spermine potentiated the flg22-induced ROS burst in N. benthamiana, resulting in an earlier and larger amplitude response (Fig. 5 A and B and SI Appendix, Fig. S24A). The potentiation was dose-dependent (SI Appendix, Fig. S24B). Spermidine alone induced only a slight ROS burst (SI Appendix, Fig. S24C) (40), and the increase of the flg22-induced ROS burst in the presence of spermidine was higher than an additive effect (SI Appendix, Fig. S24C). The potentiation by spermidine was also observed in A. thaliana, but was not observed in the flg22 receptor mutant fliC (Fig. S24C and SI Appendix, Fig. S25A and B). Spermidine potentiation was also observed using the elicitor elf18 (SI Appendix, Fig. S25C) (43). Surprisingly, the Arabidopsis Ca$^{2+}$ burst induced by flg22 was reduced in the presence of spermidine (Fig. 5D and SI Appendix, Figs. S1 and S25D). Arginine had a similar effect on ROS burst in A. thaliana and N. benthamiana, while 1-citrulline did not (SI Appendix, Figs. S26 and S27). Thus, spermidine and arginine potentiate MAMP-induced ROS bursts in both plant species, and spermidine modulates the flg22-induced Ca$^{2+}$ burst, at least in A. thaliana. These results are consistent with previously proposed functions of polyamines in plant defense responses (39, 40), including up-regulation of polyamine biosynthesis in plants following challenge with bacterial pathogens (44–47), subsequent polyamine transport to the apoplast (44), and increased resistance toward Pseudomonas viridiflava observed after exogenous addition of spermine (47).

**Phevamine A Suppresses Spermidine and Arginine Potentiation of the flg22-Induced ROS Burst.** The anticorrelation of phevamine A- and phaseolotoxin-encoding genes suggested that phevamines might impair the effect of spermidine and arginine on the early MAMP-dependent ROS burst. We therefore tested whether phevamine A could impair the effect of exogenously supplied spermidine and arginine in the MTI-induced ROS burst potentiation assay. Leaf disks of N. benthamiana were challenged with flg22 in the presence of phevamine A, spermidine, or both (Fig. 5A). We observed
that phevamine A suppressed the spermidine potentiation of the flg22-induced ROS burst in *N. benthamiana*, but did not directly affect the flg22 response (Fig. 5A). We noted that prephevamine also suppressed the spermidine potentiation of the flg22-induced ROS burst, but phevamine B did not (Fig. 5B). This result is consistent with our observation that phevamine B is not naturally produced by *Pto* ([SI Appendix](#supplementary-material), Fig. S6). In contrast, only phevamine A suppressed the spermidine-mediated flg22-induced ROS burst in *A. thaliana* leaf disks in a similar experimental setup (Fig. 5C). This indicates that the molecular mechanism(s) of the spermidine potentiation might differ slightly between *N. benthamiana* and *A. thaliana*.

Spermidine affects both the flg22-induced ROS and Ca\(^{2+}\) bursts (Fig. 5D and [SI Appendix](#supplementary-material), Fig. S24C). We therefore tested the effect of phevamine A on the flg22-induced Ca\(^{2+}\) burst in the presence and absence of spermidine using a transgenic *A. thaliana* line that expresses the aequorin reporter ([48]). As noted above, spermidine suppressed the flg22-induced Ca\(^{2+}\) burst, but the addition of phevamine A did not affect this suppression. Phevamine A also had no direct effect on the Ca\(^{2+}\) burst induced by flg22 (Fig. 5D). These data suggest that phevamine A acts downstream of the Ca\(^{2+}\) burst.

Additionally, both phevamine A and prephevamine were tested for their effect on the ROS potentiation mediated by arginine in both *N. benthamiana* and *A. thaliana*. Phevamine A, but not prephevamine, could suppress the arginine-mediated potentiation of the flg22-induced ROS burst in *N. benthamiana* ([SI Appendix](#supplementary-material), Fig. S27). This may imply that the potentiation mediated by spermidine and arginine in *N. benthamiana* involves slightly divergent molecular mechanism(s), supported by the different potentiation phenotypes for prephevamine. Similarly, only phevamine A and not prephevamine could inhibit the arginine potentiation of the flg22-induced ROS burst in *A. thaliana*.

Overall, we observed that phevamine A consistently suppressed both the spermidine- and arginine-mediated potentiation of the flg22-induced ROS burst across two divergent plant species. Thus, phevamine A is likely the most physiologically relevant small molecule produced by the *hsv* operon in *Pto*.

Conclusions

We identify a bacterial small-molecule virulence factor, phevamine A. The biosynthesis of phevamine A is controlled by the HrpL virulence regulator in *P. syringae*. Interestingly, phevamine A shares structural similarity with insect polyamine toxins: argiotoxin from the orb-weaver spider, and philanthotoxin from the Egyptian solitary wasp, both of which contain a polyamine and two amino acids, and are powerful neurotoxins that target
Our study suggests that targeted mining of uncharacterized ATP-grasp enzymes will likely lead to novel small molecules.

Our approach for identifying cryptic small molecules builds on the rich knowledge of pathogen gene expression and integrates comparative genomic analysis, biochemical enzyme reconstitution, and physiological assays. Bacterial genomes harbor many biosynthetic operons of unknown function; therefore, this approach holds potential for identifying many more small molecules essential for bacteria–host interactions.

**Methods**

**Metabolite Extraction for Metabolomics.** *E. coli*, *P. syringae*, and *P. fluorescens* cultures (100 mL) were spun down at 3,500 × g and 4 °C for 10 min. Solvent extractions were performed using an equal volume of supernatant to chloroform-methanol (2:1:1). The upper aqueous layer was separated into a round-bottom flask and concentrated under vacuum. The dried-down material was resuspended and transferred to a small glass vial, concentrated under reduced pressure, and stored at −20 °C until MS analysis.

**MS and MS/MS Analysis.** Culture extracts, in vitro synthesized phevamines, and chemically synthesized phevamine A, were analyzed by Agilent 6520 accurate-mass quadrupole-time of flight (Q-TOF) LC/MS using a Phenomenex Kinetex 5-μm C18 column (100 Å, 150 mm × 4.60 mm). Mobile phases were water and acetonitrile, each containing 0.1% formic acid. The gradient was held at 2% acetonitrile for 2 min before ramping up to 45% acetonitrile over 17 min at a flow rate of 0.4 mL/min. For each sample, m/z 434.324 was selected at a retention time of 8 ± 5 min and analyzed by MS fragmentation. Targeted MS/MS was conducted using 30-V collision energy, 970.9-ms acquisition time, and a 4 m/z isolation width.

**Preparative Scale in Vitro Enzymatic Synthesis of Phevamines.** One milliliter, one-pot enzymatic assays for phevamine A were conducted at room temperature for 2 h. Assays contained 5 mM l-arginine, 5 mM spermidine, 1 mM L-valine, 0.5 mM L-phenylalanine, 100 mM Hepes (pH 7.5), 2 mM MgCl₂, 1 mM ATP, 20 μM HsvA, 10 μM HsvB, 10 μM HsvC, and water. The reaction was then quenched with 1 mL acetonitrile to precipitate protein overnight at −20 °C. The precipitated proteins were removed by centrifugation. Phevamines were purified from the supernatant using one round of preparative HPLC, and two rounds of analytical HPLC using conditions described in SI Appendix, Materials and Methods.

**Plant Material and Bacterial Growth.** *A. thaliana* was grown in walk-in growth rooms maintained at 21 °C (18 °C (day/night) with a 9-h/15-h (day/night) cycle. *N. benthamiana* was grown in a walk-in growth room maintained at...
26 °C/22 °C with a 12-h/12-h (day/night) cycle and a LGM550 professional LED grow light system (LED Grow Master Global). For maintenance and transformation, *Pseudomonas* strains were grown in King’s B media at 28 °C. For infiltration or dipping *in planta*, *Pseudomonas* strains were grown in liquid culture overnight with the appropriate antibiotics, then plated on a Petri dish and incubated overnight before resuspension in 10 mM MgCl$_2$.

**Bacterial Growth Assay.** *Pto*, *PtoΔhsv*, *Pto-Cor−*, and *Pto-Cor−Δhsv* cells were resuspended in 10 mM MgCl$_2$ to a final concentration of 2 × 10$^6$ cfu/mL. Twenty-day-old *A. thaliana* Col-0 seedlings were dipped in bacterial solutions supplemented with Silwet L-77 (Momentive), and growth was assessed at day 0 and 3-d postinfection as described by Tornero and Dangl (57). Eight samples containing three seedlings were collected for each treatment.

**Callose Deposition Measurement.** Three- to 5-wk-old *Arabidopsis* plants were infiltrated with *Pseudomonas* strains at an OD$_{600}$ of 0.2 in 10 mM MgCl$_2$ and collected after about 20 h. To visualize callose deposition, leaves were stained with aniline blue (58). The tissue was cleared and dehydrated with 96% ethanol overnight at 37 °C. Cleared leaves were washed with distilled water and then stained in 0.01% aniline blue in 150 mM K$_2$HPO$_4$ (pH 9.5) for 4 h at room temperature. Stained samples were washed and mounted in distilled water and examined by epifluorescence (LEICA M205 FA) with 100× magnification. Images were taken at the region below the infiltrated zone of each leaf. Counting of accumulated callose foci was carried out using ImageJ (NIH). For each treatment, 10–20 leaves were processed.

**Phylogenetic Analysis.** The phylogenetic history of *P. syringae* was inferred from Yang et al. (37). Protein sequences of toxin biosynthetic genes listed in Dataset S1 were used as basic local alignment search tool (BLAST) queries to search for homologous protein sequences in the 292 *P. syringae* genomes [available as of January 2017, Pathosystems Resource Integration Center (PATRIC)]. A hit with over 80% protein sequence identity was considered positive. The tree was visualized using interactive tree of life (iTOL) (59).

**ROS Burst Measurement.** Leaf disks from 4-wk-old *A. thaliana* Col-0, or *N. benthamiana* were placed into a 96-well plate with 100 μL of water in each well. Twelve leaf disks were used per treatment. After overnight incubation for *Arabidopsis* leaf disks, or 20 h for *N. benthamiana* leaf disks, each sample was treated with 100 μL of reaction mix, including 17 mg/mL of luminol (Sigma), 10 mg/mL of horseradish peroxidase (Sigma), distilled water, flg22 at 4 h at room temperature. Stained samples were washed and mounted in distilled water and examined by epifluorescence (LEICA M205 FA) with 100× magnification. Images were taken at the region below the infiltrated zone of each leaf. Counting of accumulated callose foci was carried out using ImageJ (NIH). For each treatment, 10–20 leaves were processed.

**Fig. 5.** Effect of the phevamines and prephevamine on the spermidine potentiation of the ROS burst and suppression of the calcium burst. (A) Phevamine A suppresses the spermidine potentiation of the flg22-induced ROS burst in *N. benthamiana*, but does not affect the flg22-induced ROS burst directly. Leaf disks were treated with 50 nM flg22, with or without spermidine at 400 μM, and with or without phevamine A at 400 μM. (B) Phevamine A and prephevamine suppress the spermidine potentiation in *N. benthamiana*, but phevamine B does not. Leaf disks were treated with flg22 at 10 nM, spermidine, phevamines, and prephevamine were used at 300 μM. (C) In *Arabidopsis*, phevamine A suppresses the spermidine potentiation, while prephevamine does not. Leaf disks were treated with flg22 at 10 nM, spermidine and phevamines were used at 300 μM. (D) Phevamine A does not affect the flg22-induced Ca$^{2+}$ burst, or the reduction of this burst by spermidine. Leaf disks were treated with flg22 at 20 nM, spermidine and phevamine A were used at 300 μM. Error bars represent SEs. PHVA, phevamine A; PHVB, phevamine B; pPHV, prephevamine; RLU, relative luminescence units; Spd, spermidine. All experiments presented here were repeated at least three times.
concentrations ranging from 5 to 100 nM, l-arginine, and polyamines at concentrations typically ranging from 200 to 800 μM. Pheomazines and pre-pheomazine were used at the same concentration as L-arginine (Sigma) or spermidine. Luminescence was measured immediately with 0.5-s integration and 1-min interval over 45 min using a SpectraMax L ( Molecular Devices). For each experiment, 8-12 leaf-disk per condition were monitored. The flg22 peptide ( QRSLGSTSRCADKDAGLQIA ) was synthesized by Genscript (41).

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55. Blasiak LC, Clardy J (2010) Discovery of 3-formyl-tyrosine metabolites from Pseu-